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Supplementary Materials for
**GABA_A Receptor Availability Is Not Altered In Adults With Autism Spectrum
Disorder (ASD) Or In ASD Mouse Models**

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Supplementary Methods & Results

[¹¹C]flumazenil human PET data acquisition and processing

PET examination with [¹¹C]flumazenil was performed with a Siemens ECAT Exact HR 47 (CTI/Siemens, Knoxville, TN) run in 3D mode with Dual Energy Windows scatter correction. Spatial resolution ranges from 3.6 mm full width half maximum (FWHM) at the centre of the field to 4.5 mm tangentially and 7.4 mm radially at 20 cm from the centre. Prior to each PET examination, an individually fitted plaster helmet was constructed to minimize head movement. A 10 minute transmission scan with three rotating GE rods was performed before emission to correct for attenuation and scatter. The radioligand was prepared as previously described(59) and injected in the antecubital vein as a 10 second bolus dose.

[¹¹C]flumazenil PET data was collected in a dynamic scan for 63 minutes and binned into 26 time frames of increasing length (4×15 s, 4×30 s, 6×60 s, 6 × 180 s, 6 × 360 s). PET images were reconstructed using filtered backprojection and a 2mm FWHM Hann filter to produce 47 horizontal sections with a voxel size of 2.02 × 2.02 × 3.125 mm. PET images were corrected for head motion using a frame to first frames approach.(61) T1 weighted MR images (3T GE Signa) were coregistered to PET using SPM5 (Department of Cognitive Neurology, University College London) run in Matlab 7.5 for Windows. (Natick, Massachusetts, The Mathworks Inc.) MRIs were segmented and regions of interest (ROIs) were delineated using Freesurfer 5.0, (<http://surfer.nmr.mgh.harvard.edu>) according to the atlas of Desikan from which larger cortical ROIs were formed.(60) Segmentation and ROI delineation were quality controlled and manually edited to ensure optimal fit. Pons was manually delineated on the individual MRIs using in house developed software. Regional binding potential values (BP_{ND}) were calculated using the Simplified Reference Tissue Model (SRTM) with pons acting as reference region.(62) Comparisons of mean BP_{ND} between groups for each ROI were evaluated using independent samples t-test run in SPSS 22.

Sensitivity analyses of [¹¹C]flumazenil PET data

In the Stockholm [¹¹C]flumazenil study, 4/15 ASD cases and 4/15 controls were female while in the London [¹¹C]Ro15,4513 study, all participants were male. The Stockholm study followed local ethical guidelines and Swedish radiation protection regulations, according to which both male and female volunteers are allowed in research PET studies, therefore we did not apply any selection by sex. We intended to apply the same policy to the UK sample, but British regulations do not allow exposure of females to radiation for research purposes. Therefore, to permit direct comparison between the two PET studies, we repeated the [¹¹C]flumazenil group comparisons including only the male participants. Consistent with the results from the full sample, there were no group differences in [¹¹C]flumazenil binding in whole brain ($t(20)= 1.589$, $p= 0.132$), grey matter ($t(20)= 1.619$, $p= 0.126$) or any brain region (all $p>0.102$).

To verify that potential group differences in brain structure were not affecting the [¹¹C]flumazenil PET data, secondary analyses with partial volume effects correction (PVEC) were carried out as described by Meltzer(63) for subcortical ROIs and as described by Müller-Gärtner(64) for cortical ROIs. The addition of PVEC did not change the results of the statistical analysis.

To further confirm the results from the primary analysis, BP_{ND} was estimated on voxel level in order to detect any brain region-specific group differences which went undetected by the primary ROI-based analysis. Voxel-wise PET data were preprocessed using stationary wavelet aided parametric imaging (S-WAPI) to create parametric PET images. By averaging BP_{ND} of all voxels contained in a ROI, regional binding potentials were calculated. These showed very high correlation with BP_{ND} obtained with SRTM (mean R = 0.996, range = 0.973 – 0.999). The individual parametric PET images were then normalized to a MNI space using a non-linear method (FNIRT in FSL 5.0, FMRIB, Oxford, UK). The resulting warping parameters were applied to the parametric PET images which were then smoothed using a 5mm Gaussian kernel. Voxel-wise analysis was then carried out using SPM12, with a primary cluster-defining threshold of p<0.001 and a minimum cluster size of 20 voxels.

No clusters with a statistically significant group difference in BP_{ND} were found, confirming our finding of no significant group differences in any of the predefined ROIs.

Behavioural phenotype assessment in the Karolinska (¹¹Cflumazenil) sample

A battery of behavioral tests was administered to all subjects in the Karolinska sample to explore executive function, central coherence and social cognition. Executive functioning was measured with Delis-Kaplan Executive Function System (D-KEFS) Verbal fluency and Tower of Hanoi and Conner's Continuous Performance Test (CPT). Central coherence was evaluated with Embedded Figure Test (EFT)(41) and Fragmented Picture Test (FPT)(65). Social cognition was assessed with Reading the Mind in the Eyes Test(42), Movie for Assessment of Social Cognition (MASC)(66) and Faux Pas test (43). Wechsler Adult Intelligence Scale, fourth version (WAIS-IV), subtests Vocabulary and Matrix reasoning were used to assess the general intellectual ability of the two groups.

The performance of the ASD group was significantly lower than the control group on letter fluency ($t=-3.27$; $p=0.006$) and alterations between categories ($t=-3.96$; $p=0.001$). In social cognition tests, the ASD group performed significantly lower in all tasks: Reading the mind in the eye ($t=-3.96$; $p=0.001$), identifying the number of situations in the MASC test ($t=-4.74$; $p<0.001$) and on the Faux Pas test ($t=-3.89$; $p=0.002$). No significant group difference were found in the central coherence tasks.

There were no correlations between GABA_A receptor binding and performance in any ASD related behavioral phenotypes. However, uncorrected for multiple comparisons there were significant positive correlations between number of errors in CPT and GABA_A receptor binding in both the ASD group and the control group.

[¹¹C]Ro15,4513 Human PET data acquisition and processing

[¹¹C]Ro15,4513(29, 30) is a benzodiazepine inverse agonist with approximately 10 fold higher affinity for the $\alpha 5$ subtype of the GABA_A receptor compared with the other receptor subtypes. [¹¹C]Ro15,4513 was synthesized(29) by *N*-methylation of the corresponding *N*-desmethyl derivative with [¹¹C]iodomethane. The product was purified by reverse phase HPLC.

A bolus of [¹¹C]Ro15,4513 was injected into the dominant vein of the antecubital fossa via a cannula, over approximately 15 seconds, and PET emission data was collected in 3D mode for 90 min after injection. During the PET acquisition, arterial blood data were sampled via the radial artery using a combined automatic-manual approach. A continuous sampling system (ABSS Allogg, Mariefred, Sweden) was employed to measure whole blood activity for first 15 minutes of each scan at the rate of one sample per second. Discrete blood samples were manually withdrawn at 2, 5, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90 minutes, centrifuged and used to determine the plasma over blood activity ratio (POB). Samples at 2, 5 and 10 minutes were used to calibrate the two sampling modalities. Samples taken at 2, 5, 10, 20, 30, 50, 70 and 90 minutes were also analyzed using HPLC to calculate the plasma fraction of tracer free of metabolites (PPf). Both POB and PPf were fitted with an extended Hill model(67) as the one leading to the best data description. Whole blood TACs were fitted using a multi-exponential model.(68) For each scan, a time delay was fitted and applied to the input functions (both parent and whole blood TACs) to account for temporal delay between blood sample measurement and the target tissue data.

Dynamic PET data were corrected for inter-frame motion and aligned with each individual's structural T1 MR image by minimizing a mutual information cost function (SPM8, Wellcome Trust Center for Neuroimaging, <http://www.fil.ion.ucl.ac.uk/spm>). A neuroanatomical atlas was also coregistered on each subject's image space (MIAKAT, <http://www.imanova.co.uk>). Among the 506 regions of interest (ROIs) available, a subset of regions including amygdala, hippocampus and nucleus accumbens, were considered. The selection of these ROIs was motivated by their relevance to ASD and the signal quality obtained.

Two fully-quantitative kinetic methods with metabolite-corrected plasma input functions were applied for the PET data analysis, in agreement with previous [¹¹C]Ro15,4513 brain PET studies (23, 34): firstly, the two-tissue compartmental model (2TCM) solved with non-linear least squares was used to compute the total distribution volume (V_t) of the tracer in the tissues using the Logan method; secondly, a spectral-based quantification (non-linear spectral analysis,

NLSA)(69) was implemented to separate GABA non- $\alpha 5$ and $\alpha 5$ -related components (V_1 and V_5 , respectively) from the total tracer uptake.(34) Model fitting and parameter estimation were performed using in house developed software (Matlab 2012b, The MathWorks Inc.)

Rodent autoradiography: protocol

Frozen mouse brains were placed on a microtome platform and cut into 15 μm coronal sections at different brain levels in a cryostat (#Slee, MNT) cooled to $-17 \pm 2^\circ\text{C}$. The sections were mounted on positively charged slides (VWR#631-0446) and kept frozen at -80°C until the day of the experiment.

The brain anatomical levels were determined according to the Paxinos and Franklin's mouse brain atlas(70) and were assigned as follows: Frontal cortex (Bregma 2.22 mm); striatum (Bregma 0.98 mm); dorsal hippocampus (Bregma -1.94 mm); amygdala (Bregma -1.94mm) and cerebellum (Bregma -6.64 mm). The experimental methodology was performed in a manner similar to that described previously(71) with some modifications as described briefly below.

On the day of the experiment the slides were warmed to room temperature before the start of the experiment. Sections were incubated for 20 minutes in 50 mM Tris buffer (pH 7.4) at room temperature. They were then incubated with 1 nM of the tracer, either [^3H]Ro15,4513 (Perkin Elmer #NET925250UC) or [^3H]flumazenil (Perkin Elmer #NET757001MC), in Tris buffer at 4°C for 60 minutes. The non-specific binding was determined using 10 μM flunitrazepam and 10 μM bretazenil for [^3H]flumazenil and [^3H]Ro15,4513, respectively. After incubation with the tracer, the slides were washed twice (2 x 2 minutes) with buffer at 4°C , followed by a dip in distilled water, and then left to dry overnight. Once dry, they were exposed to [^3H]-sensitive Amersham Hyperfilm (VWR# 28-9068-50) along with [^3H] microscaler (American Radiolabelled Chemicals, #ART0124B). Slides were exposed for four weeks in the case of [^3H]flumazenil and for six weeks in the case of [^3H]Ro-15,4513, before development.

For each mouse, the total binding was determined from the two best sections per slide. The right and left hemispheres were combined, therefore 4 optical density measures were obtained. There was no observable NSB, so a background optical density measure was taken and subtracted from the total binding measure. Data were analyzed using ImageJ software (<http://imagej.nih.gov/ij/>) and the radioactivity was quantified in nCi/mg for the selected brain regions based on the optical density of the [^3H]-microscaler. Non-specific binding was subtracted from the total binding to give the specific binding values. Graphs were drawn from normalised data of all regions with respect to the wild type (WT) for each model, the WT average value being defined as 100%. Unpaired t-tests were carried out and corrected for multiple comparisons using Sidak correction. Data was graphed and analysed in GraphPad Prism version 6.0h (GraphPad Software, La Jolla California, USA).

Rodent autoradiography: left amygdala and right amygdala

For our analyses, we considered each brain region as a bilateral unit (average of left and right hemisphere.) However, in the case of the amygdala region, we here present the results of separate analyses of the left amygdala and right amygdala, in order to permit comparisons with Bertelsen et al. (2017) who found a lateralized increase in Ro15,4513 binding in valproate-exposed rats, a rat model of ASD.(55)

We found no significant differences in either [³H]flumazenil or [³H]Ro15,4513 binding in any of the three ASD mouse models. However, in the *SHANK3* model, a trend of increased binding of both [³H]flumazenil and [³H]Ro15,4513 was observed (adjusted p=0.09) in the left amygdala only. Bertelsen et al. found an increase in Ro15,4513 binding in the left amygdala of valproate-exposed rats (not corrected for multiple comparisons). In *Cntnap2*, there was a trend for an increase in binding of [³H]Ro15,4513 in both the left and right amygdala (adjusted p=0.06 and p=0.1 respectively). No significant differences or trends were observed in the case of the 16p11.2 deletion model. All statistical analysis were carried out using a two way ANOVA with a Sidak correction for multiplicity.

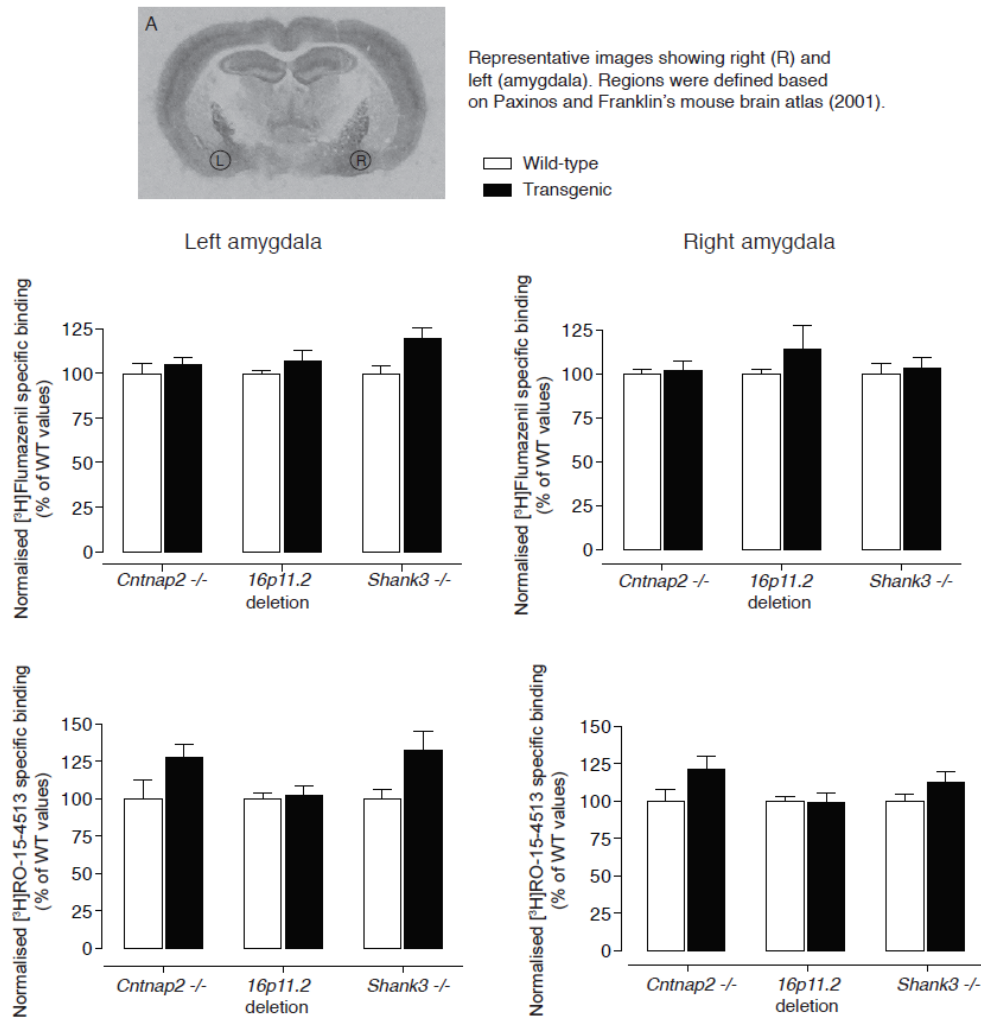


Figure S1. Left and right amygdala autoradiography results.

	Stockholm [¹¹C]Flumazenil PET study			London [¹¹C]Ro15-4513 PET study		
	Control	ASD	P-value (t-test)	Control	ASD	P-value (t-test)
weight (kg)	73.7 ± 12.4	75.9 ± 17.0	0.682	74.4 ± 8.77	75.8 ± 17.3	0.773
injected activity (MBq)	332 ± 69.7	327 ± 67.0	0.855	347 ± 63.2	378 ± 66.8	0.220
activity/weight (MBq/kg)	4.51 ± 0.68	4.33 ± 0.30	0.361	4.68 ± 0.79	5.28 ± 1.75	0.290
specific radioactivity (GBq/μg)	356 ± 155	285 ± 131	0.189	48.2 ± 17.3	40.4 ± 16.0	0.232
injected mass (μg)	0.389 ± 0.375	0.623 ± 1.02	0.409	2.63 ± 1.12	3.60 ± 1.79	0.118
AUC SUV (pons)	3.07 ± 0.65	3.18 ± 0.50	0.612	n/a	n/a	n/a
<p>Values are shown as Mean ± Standard Deviation. P-values are the outcome of independent sample t-tests testing for a difference in group means between typically developing controls and autism spectrum disorder (ASD) adult males, within each study. AUC SUV (pons) is not applicable for [¹¹C]Ro15-4513 PET as an arterial input function was used.</p>						

Table S1. PET tracer dosing in the two human studies.

	STOCKHOLM (n=15 ASD)	LONDON (n=12 ASD)
Employment	<p>Unemployed: 3</p> <p>Student: 5</p> <p>Protected employment (social care): 2</p> <p>Unqualified work (no university education required): 3</p> <p>Qualified work (university education required): 1</p> <p>Other (not employed): 1</p>	<p>Unemployed: 4</p> <p>Student: 3</p> <p>Working from home: 2</p> <p>Unqualified work (no university education required): 2</p> <p>Qualified work (university education required): 1</p>
<p>Psychiatric and Neuroactive Medication History *</p> <p>(Note: all participants were free of psychiatric and neuroactive medications at the time of PET. In the control participants, all reported being medication naïve.)</p>	<p>Medication naïve: 6</p> <p>Previous use of SSRIs ending >six months prior to PET: 6</p> <p>Previous use of mirtazapine ending >six months prior to PET: 1</p> <p>Previous use of bupropion ending >six months prior to PET : 1</p> <p>Occasional use of cannabis >ten years prior to examination: 1</p>	<p>Medication naïve: 6</p> <p>Previous use of SSRIs ending >four months prior to PET: 2</p> <p>Previous use of benzodiazepines ending >five months prior to PET examination: 1</p> <p>Previous use of benzodiazepines and SSRIs, ending >three months prior to PET: 1</p> <p>Previous use of methylphenidate briefly in childhood: 1</p> <p>Use of phenytoin briefly following a childhood seizure, but not diagnosed epilepsy: 1</p>

Table S2. Further information on functional outcome (employment status) and medication history in the ASD participants in the two PET studies.

