

GABA deficiency in NF1: a multimodal [¹¹C]-Flumazenil and spectroscopy study

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Conflict of Interest

The authors declare no conflict of interest.

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Abstract

Objective: To provide a comprehensive investigation of the GABA system in patients with Neurofibromatosis type 1 (NF1) that allows understanding the nature of the GABA imbalance in humans at pre- and post-synaptic levels.

Methods: In this cross-sectional study, we employed multimodal imaging and spectroscopy measures to investigate GABA_A receptor binding, using [¹¹C]-Flumazenil positron emission tomography (PET), and GABA concentration, using magnetic resonance spectroscopy (MRS). 14 adult patients with NF1 and 13 matched controls were included in the study. MRS was performed in the occipital cortex and in a frontal region centered in the functionally localized frontal-eye fields. PET and MRS acquisitions were performed in the same day.

Results: Patients with NF1 have reduced concentration of GABA+ in the occipital cortex ($P = 0.004$) and frontal-eye fields ($P = 0.026$). PET results showed decreased binding of GABA_A receptors in patients in the parieto-occipital cortex, midbrain and thalamus, which are not explained by decreased grey matter levels.

Conclusions: Abnormalities in the GABA system in NF1 involve both GABA concentration and GABA_A receptor density suggestive of neurodevelopmental synaptopathy with both pre- and post-synaptic involvement.

Introduction

Neurofibromatosis type 1 (NF1) is a neurodevelopmental monogenetic disorder, characterized by multisystemic symptoms, including increased incidence of cognitive deficits (50-70%)¹ and predisposition for tumor development².

Cognitive impairments occur even in the absence of visible brain alterations, suggesting the existence of pathophysiological abnormalities at a finer scale.

One hypothesis to explain the cognitive and behavioral profile is based on neurochemical imbalance in the GABA system. Evidence for it includes: 1) cellular and molecular studies in the *Nf1*^{+/-} model, which established a connection between RAS regulation by the *NF1* gene, GABAergic signaling and learning deficits^{3,4}; 2) *in vivo* magnetic resonance spectroscopy (MRS) studies in children with NF1 showing reduced GABA levels^{5,6}.

Moreover, it is possible that GABAergic alterations influence the distribution or binding affinity of GABA receptors, consistent with the 'GABA shift'⁷. This model postulates that increased GABA levels enhance the affinity of GABA_A receptors for benzodiazepine ligands. The directionality of changes in disease is however difficult to predict. On one hand, in homeostatic systems the natural prediction is that, to maintain efficient neurotransmission, low GABA levels lead to increasing numbers of GABA receptors. On the other, reduced GABA concentration in patients could reflect a compensatory mechanism to counterbalance pre- and post-synaptically the effects of increased GABA release caused by reduced neurofibromin expression⁴. GABA metabolism can be downregulated as a response to increased GABAergic neurotransmission, thus limiting GABA availability for packaging and release⁸. If this were the case, we expect observing low GABA levels accompanied by a reduction in the

number of GABA receptors. To further characterize the GABA system in adults with NF1 we studied the distribution of GABA_A receptors using [¹¹C]-Flumazenil positron emission tomography (PET), and GABA concentration using MRS.

Materials and Methods

Participants

We performed a cross-sectional study in 14 adult patients with NF1, diagnosed by the NIH criteria⁹, and 14 healthy controls, matched for age, gender and educational level. All participants were right-handed.

Exclusion criteria were: psychiatric disorder, neurological illness affecting brain function other than NF1, IQ<75, epilepsy, traumatic brain injury, or a clinically significant intracranial abnormality detected on MRI. T2-hyperintensities (commonly found in NF1) were not considered exclusion criteria for patients. However, if present within the voxels prescribed for spectroscopy, the correspondent spectrum was not included. Our final cohort was composed of 14 patients with NF1 (mean age 34.7 ± 7.1 , 4 males) and 13 matched controls (mean age 36 ± 8.9 , 4 males), Table 1.

None of the participants were taking medication. Three patients reported taking antidepressants in the past (for periods ranging between 6 months to 3 years), however none were medicated in the year before the study. One control was medicated with antidepressants and stopped the medication a month before taking part in the study. None of the participants were ever medicated with anticonvulsants. Participants were instructed to withhold from drinking alcohol

24h before testing and from smoking or drinking coffee in the day of testing. All participants scored below the cut-off (<8) defined for hazardous and harmful alcohol use in the Alcohol Use Disorders Identification Test (AUDIT)^{10,11}.

Groups did not significantly differ in their scores (NF1: 2.07 ± 1.69 ; controls: 2.85 ± 2.54 ; $t_{(25)}=0.939$, $P=0.36$).

Neuropsychological testing

Intelligent quotient (IQ) was assessed using the Portuguese version of the Wechsler Intelligence Scale for Adults III (WAIS-III)¹². Patients with NF1 had IQ values in the normal range, however significantly lower than controls (independent-samples t-tests; Total IQ, $P=0.002$; Performance IQ $P<0.001$; Verbal IQ $P=0.030$).

Imaging

Participants underwent all imaging acquisitions in the same day. PET and MRI order was counterbalanced. MRS was performed in the occipital cortex, to investigate whether GABA deficits persist into adulthood, and the frontal eye fields (FEF). The later was chosen because of its numerous cortical inhibitory circuits and because its location is easily identified using a functional localizer.

MRI and MRS acquisitions

Scanning was performed in a 3T TimTrio (Siemens, Germany), using a 12-channel head coil. Acquisitions: (i) T1-weighted MP-RAGE for voxel placement and volumetric measurements; (ii) T2-weighted FLAIR, to identify T2-

hyperintensities, (iii) functional localizer for the FEF using single-shot EPI. Participants performed an oculomotor task as described in the Supplemental Material. Individual brain activation was examined using Neuro3D (Siemens, Germany) to allow immediate placement of the MRS voxel. (iv) 2xGABA-edited spectra, MEGA-PRESS method¹³. The occipital voxel was positioned within the occipital cortex with its lower face aligned with the cerebellar tentorium (Figure 1A and Figure e-1 for position across all participants). The FEF voxel was positioned based on the BOLD activation elicited by the functional localizer (Figure 1B and Figure e-1 for position across all participants). In two NF1 participants the voxel was placed according to anatomical landmarks (junction of precentral sulcus and superior frontal sulcus), due to insufficient BOLD activation. All voxels were placed on the right FEF with the exception of two participants that showed a lateralization of the FEF activation to the left hemisphere (two patients with NF1). (v) ¹H-PRESS in the occipital and FEF positions. Detailed scan parameters can be found in the Supplemental Material.

MRI volumetric analyses

T1-weighted images were segmented using the VBM8 toolbox (<http://dbm.neuro.uni-jena.de/vbm/>) in SPM8 (<http://www.fil.ion.ucl.ac.uk/spm>) to determine the relative proportions of grey matter (GM), white matter (WM) and CSF. This was used to determine tissue proportions in the spectroscopic voxels using Matlab7 (The MathWorks Inc) and to perform voxel based morphometry (Supplemental Material). The later was employed to investigate the potential confound that group differences in [¹¹C]-Flumazenil binding could partially arise from counterpart differences in GM volume¹⁴.

fMRI analyses

Data processing and analysis were performed in BrainVoyager QX2.3 (Brain Innovation, The Netherlands). Pre-processing included slice time correction, linear trend removal, temporal high-pass filtering (3-cycles per run), motion correction and spatial smoothing (FWHM 5 mm Gaussian kernel). No participant had within-run movements >3 mm. Predictors for the general linear model were built by convolving a boxcar time course with a two-gamma function. BOLD signal peak amplitude (% signal change) for the pro- and anti-saccade contrasts, at the peak voxel, were extracted for each participant in the FEF and occipital regions.

MRS analyses

MEGA-PRESS – The signal detected at 3.02 ppm is known to contain contributions from both macromolecules and homocarnosine¹⁵, and is therefore referred to as GABA+. Quantification of GABA+ (Figure 1B) was performed using the Gannet 2.0 toolkit¹⁶ to yield a GABA+/H₂O ratio (i.u.). Only spectra with relative fit error <15% were included. Based on this criterion, the FEF spectra from two controls and four NF1s were excluded. For one control the FEF spectrum was not acquired due to technical difficulties. Therefore, we included data from the FEFs of 10 controls and 9 patients. For the occipital voxel we included data from 12 controls and 14 patients as one control was identified as an outlier (upper fence/hinge) and excluded from the analysis.

To account for differences in voxel tissue composition, the voxel fraction of white matter (f_{WM}) and grey matter (f_{GM}) was used to normalize the GABA+ concentration according to: $GABA+_{corr} = GABA+/(f_{WM} + f_{GM})$.

PRESS – LCModel software (Version 6.3) was used for metabolite quantification applying the internal water reference method, accounting for different water content in GM, WM and CSF¹⁷. Only metabolites with Cramér–Rao bounds <20% were considered. Concentrations of N-acetyl-aspartate (NAA) plus N-acetylaspartylglutamine (NAAG), creatine (Cre) plus phosphocreatine (PCr), glycerophosphocholine (GPC), myo-Inositol (ml), glutamate (Glu) and glutamine (Gln) were included for analysis. Concentrations in mmol units were calculated for all metabolites and results are presented in institutional units (i.u.).

[¹¹C]-Flumazenil PET

A Philips Gemini GXL scanner was used to acquire a CT scan and 26 sequential 3D PET frames of the entire brain (90 slices, 2 mm slice sampling), over a period of 60 min, after the bolus injection of 584.6 ± 25.9 MBq [¹¹C]-Flumazenil. PET data was reconstructed using a LOR-RAMLA algorithm. Voxel-by-voxel BP was calculated from dynamic PET images using the basis function implementation of the Simplified Reference Tissue Model, with the pons as reference^{18,19}. Several studies showed that using the pons activity as a reference is reliable and highly correlated with the BP values estimated by arterial sampling method²⁰. Moreover, in order to ensure that systematic differences in the pons time-activity curves did not affect the results we

compared standard uptake values (SUV) in the pons between the 2 groups (independent-samples, $t_{(25)}=0.370$, $P=0.714$).

PET data were normalized to MNI space after rigidly co-registering the individual PET scan to the participant anatomical T1 scan in SPM8. The latter were segmented using the unified segmentation method²¹ in order to extract the transformation map into MNI space. This was then used to non-linearly warp the PET scans. The resulting normalized images were smoothed using an isotropic Gaussian kernel (12 mm FWHM).

We performed exploratory whole-brain and region of interest (ROI) based analyses. Whole-brain group differences were evaluated in SPM8 using independent-samples t-test. [¹¹C]-Flumazenil BP differences were considered statistically significant at peak height threshold of uncorrected $P<0.005$ with cluster size greater than 230 contiguous voxels, which corresponds to a threshold of $P<0.05$ corrected by cluster level for multiple comparisons as estimated by 10,000 Monte Carlo simulations in 3dClustSim (AFNI, <http://afni.nimh.nih.gov/afni/>). ROI-based analyses were performed in regions used for spectroscopy. The mean BP at these voxels was determined with the objective of investigating the relation between GABA concentration and GABA_A BP.

Statistical analyses

Data were transferred to SPSS v.21 for analysis. All dependent variables were tested for normality of distribution using the Shapiro-Wilk test and for outliers using the Tukey's method. All variables were normally distributed.

Group differences were evaluated using independent-samples t-tests, and corrected for multiple comparisons using the Holm-Sidak method ($\alpha=0.05$). ROI analyses were performed using independent-samples t-test and Pearson correlations (robustness estimated using bootstrapping, 10,000 iterations, to provide 95% confidence intervals). Effect sizes were calculated using Cohen's d . Variance was similar between the groups as tested using the Levene's test of homogeneity of variances.

Standard Protocol Approvals, Registrations, and Patient Consents

Study procedures were reviewed and approved by the Ethics Commission of the Faculty of Medicine of the University of Coimbra. Participants provided written informed consent.

Results

MRS results

Patients with NF1 displayed decreased GABA+ levels, of on average 11.5%, in the occipital cortex ($t_{(24)}=3.172$, $P=0.004$, $d=1.14$) and 22% in the FEF ($t_{(17)}=2.443$, $P=0.026$, $d=1.27$), Figure 2. Additionally, patients showed lower levels of NAA+NAAG ($t_{(25)}=3.361$, $P=0.002$, $d=1.31$; 12% reduction) and glutamate ($t_{(25)}=2.787$, $P=0.01$, $d=1.08$; 10% reduction) in the occipital cortex, Figure 2. A full summary of the spectroscopy results can be found in Supplemental Table e-1.

PET results

Whole brain voxel-based analyses indicated that NF1 patients have decreased [^{11}C]-Flumazenil BP in a left parieto-occipital region that encompasses the precuneus (MNI coordinates ($_{\text{Peak voxel}}$): [-22 -60 30]; z score = 3.65, k = 381, P < 0.05 corrected, d =1.89) and in a region that includes the left midbrain and thalamus (MNI coordinates ($_{\text{Peak voxel}}$): [-2 -34 -10]; z score = 2.95, k = 447, P < 0.05 corrected, d =1.64), whereas no brain regions displayed increased BP in patients, Figure 3. Moreover, VBM showed that decreased [^{11}C]-Flumazenil BP in patients is not explained by decreased GM content, as decrease GM and decrease BP in patients do not co-localize (Figure e-2). Moreover, to ensure that PET results were not hindered by concomitant abnormalities in GM we performed correlation analyses between GM density and BP in the regions showing decreased PET BP. No correlation was observed for patients or controls (Supplementary Material), confirming that decreased BP in patients is not explained by GM alterations.

ROI based analysis for the occipital and FEF voxels showed no statistically significant group differences in BP (occipital: $t_{(25)}=0.519$, $P=0.608$, $d=0.20$; FEF: $t_{(24)}=0.936$, $P=0.359$, $d=0.37$).

Cross modality correlations

The relationship between the density of GABA_A receptors and the concentration of GABA was examined in occipital and FEF voxels. In patients the concentration of GABA+ was negatively correlated with the density of GABA_A receptors in the FEF ($r=-0.842$, $P=0.004$, $N=9$, 95% CI=-1.000 to -0.368), but not in the occipital cortex ($r=-0.211$, $P=0.469$, $N=14$), Figure 5. In controls, we

did not find any significant correlation, neither in the FEF ($r=-0.342$, $P=0.334$, $N=10$) nor in the occipital cortex ($r=0.013$, $P=0.969$, $N=12$). Yet, given the correlation values in the FEF and the low sample size it is possible that the absence of significance is due to a Type II error.

Finally, we examined whether individual peak BOLD amplitudes elicited by the oculomotor task were related with GABA concentration or GABA_A BP. There was no significant correlation between either GABA_A BP or GABA+ in neither the FEF nor occipital cortex for any of the groups.

Discussion

We report a comprehensive study of the GABA system in patients with NF1 that investigated cortical GABA concentration using MR spectroscopy and GABA_A receptor density using PET imaging. Our results indicate that patients with NF1 have decreased GABA+ levels and decreased GABA_A receptors, corroborating our hypothesis that alterations in the GABA system extend beyond reduced GABA concentration.

Spectroscopy results showed that reduced GABA levels in the occipital cortex persist into adulthood, confirming our initial findings in NF1 children^{5,6}, and that this pattern is additionally observed in a frontal region centered in the FEF.

Altogether, the observation of reduced GABA levels in several cortical locations (medial frontal cortex⁵, occipital and FEF) suggests a ubiquitous cortical dysfunction. Moreover, in occipital cortex, GABA reductions were accompanied by lower glutamate and NAA+NAAG concentrations. This reduction in NAA is in

agreement with earlier reports in subcortical structures^{22,23} and white matter²⁴.

The fact that both glutamate and NAA are altered might be explained by the tight link between these metabolites; they are interconnected through a series of biochemical reactions, mainly the tricarboxylic acid cycle (TCA) and glutamate–glutamine cycle²⁵. It is therefore likely that reduced levels of GABA, glutamate and NAA are linked and contributing to maintaining low levels of GABA available for neurotransmission. Furthermore, our results are in agreement with recent findings in the *Nf1*^{+/-} model showing GABAergic and glutamatergic alterations²⁶.

GABA_A receptor density was also found to be reduced in patients in discrete locations: parieto-occipital cortex, midbrain and thalamus. Alterations in the parieto-occipital cortex are in agreement with functional^{27,28} and anatomical^{29,30} abnormalities reported in NF1. Likewise, thalamic metabolic³¹⁻³³ and volumetric alterations³⁰ have been previously reported. Both the thalamus and midbrain are important relay stations of sensory inputs to the cortex. GABAergic neurons in the thalamus are involved in the generation of synchronized activity in thalamocortical networks³⁴, a critical process for cognitive functions. In the midbrain, GABAergic neurons are involved in controlling the firing pattern of dopaminergic neurons³⁵.

Importantly, PET results were not hindered by concomitant abnormalities in GM. It is advisable that studies investigating differences in GABA_A density employing flumazenil perform GM comparisons independently, as the relationship between flumazenil BP and GM volume³⁶ or cortical thickness¹⁴ is not homogeneous across brain regions. VBM results showed that decreased

BP and decreased GM density do not co-localize and are not correlated, indicating that decreased GM does not explain decreased density of GABA_A receptors. Interestingly, we found that increased GM volume and decreased [¹¹C]-Flumazenil BP in patients co-localizes, in a region including left midbrain and left thalamus. Here, GM volume is enlarged in the absence of a parallel increase in inhibitory neurons. This could either be an indication of increased populations of other neuronal types, namely dopaminergic and glutamatergic, or that the population of inhibitory neurons preferentially expresses other types of GABA receptors. Studies with PET ligands to other receptors or *post mortem* studies could disentangle this hypothesis.

Our findings revealed a complex pattern of alterations in the GABA system in NF1, with both GABA⁺ and GABA_A reductions, yet in different locations. Additionally, a negative correlation between GABA⁺ and the density of GABA_A receptors was observed in the FEF of patients and not in the occipital cortex. The fact that this relationship was not observed in controls is an indication that natural variation is not tightly linked for these two variables.

Importantly, MRS and PET are complementary techniques measuring different components of the GABA system and it is possible that different brain regions give primacy to different regulatory mechanisms. Studies measuring GABA⁺ in regions showing altered GABA_A binding could help disentangle the hypothesis that a reduction in both GABA levels and GABA_A receptors can occur as part of a regulatory mechanism to deal with the detrimental effects of increased GABAergic neurotransmission.

Finally, it is noteworthy that both GABA MRS and [^{11}C]-Flumazenil PET are subject to limitations. Particularly important to this study is the fact that MRS is not capable of distinguishing between metabolic and synaptic pools, and [^{11}C]-Flumazenil PET is not specific for the synaptic subtypes of the GABA_A benzodiazepine receptor, as it shows affinity to both synaptic and extrasynaptic receptor subtypes³⁷.

Overall, we now have evidence of alterations occurring at multiple levels of the GABA pathway. Studies in animal models have shown pre-synaptic alterations that lead to increase GABA release^{4,26}, while our studies in humans show that the overall concentration of GABA is reduced as well as the expression of GABA_A receptors. Moreover, we showed that deficits in GABA levels do not ameliorate with age. Importantly, abnormalities in the GABA system in NF1 are involved in relevant behavioral and cognitive domains including social learning²⁶, memory²⁸ and inhibitory control⁵.

In sum, our results point to abnormalities in the GABA system suggestive of synapthopathy, with both pre- and post-synaptic involvement.

However, given the discrete distribution of GABA_A receptor abnormalities it is difficult to support that treatments with GABA_A antagonists, as suggested from the animal literature⁴, would be useful to treat cognitive deficits in humans.

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Table 1 Demographics and neuropsychological testing performance

	NF1	Controls	<i>P</i> value
Demographics			
Number of Participants	14	13	
Age (mean $\pm$ SD)	34.7 $\pm$ 7.1	36 $\pm$ 8.9	0.697
Gender (Female / Male)	10 / 4	9 / 4	0.901
Years of Education	11.4 $\pm$ 4.5	13.7 $\pm$ 3.5	0.160
Handedness (R / L)	14 / 0	13 / 0	
Neuropsychological			
WAIS-III			
Total IQ	99.5 $\pm$ 13.7	116.15 $\pm$ 11.8	0.002*
Performance IQ	99.1 $\pm$ 13.5	119.3 $\pm$ 12.2	<0.001*
Verbal IQ	100.2 $\pm$ 12.5	111.3 $\pm$ 12.6	0.030

Performance data are presented as means $\pm$ standard deviations.

WAIS-III = Wechsler Intelligence Scale for Adults III; IQ = Intelligent quotient; R = right; L = left; SD = standard deviation.

* Significant results after correcting for multiple comparisons using the Holm-Sidak method ($\alpha = 0.05$).

Figure Legends

Figure 1. Magnetic resonance spectroscopy measurements.

Localization of the magnetic resonance spectroscopy voxel (yellow square) in the visual cortex (**A**) and frontal eye-field (FEF) as localized with a functional localizer (**B**). (**C**) Edited magnetic resonance spectroscopy spectrum from a representative participant showing clearly resolved peaks for GABA+ and glutamine + glutamate (Glx). The insert on the right shows the fit output for the GABA+ signal. MEGA-PRESS spectra were processed using the “Gannet 2.0” toolkit, the green line shows the raw GABA data, the blue line the post-phase and frequency aligned GABA data, and the black line is the residual difference between the experimental data and the curve-fit. (**D**) Single-voxel-localized PRESS spectrum (blue line) from a representative participant with spectral fits (red line) and the residual difference between the experimental data and the curve-fit (black line) determined using LCModel. Cr = creatine, Gln = glutamine, Glu = glutamate, GPC = glycerophosphocholine, ml = myo-Inositol, PCr = phosphocreatine, tNAA = total N-acetyl-aspartate (NAA (N-acetyl-aspartate) + NAAG (N-acetylaspartylglutamine)).

Figure 2. MRS alterations in patients with NF1.

Metabolites showing differences between patients with NF1 (green) and controls (grey) in the occipital cortex and the frontal-eye field. The triangle shape indicates a patient with NF1 where GABA+ was measured in the left

hemisphere and the white-filled circle represents a patient where the FEF location was determined using anatomical landmarks. Graphs depict individual values, mean and standard deviation.

Figure 3. [^{11}C]-Flumazenil PET binding differences between patients with NF1 and controls.

Areas showing decreased [^{11}C]-Flumazenil binding in patients (contrast Control > NF1, $P < 0.05$ corrected)

Figure 4. Cross modality correlations between MRS and PET.

Left panel shows results for the occipital voxel and right panel for the frontal-eye field voxel, for patients with NF1 (green) and controls (grey). The triangle shape indicates a patient with NF1 where measurements were performed in the left hemisphere and the white-filled circle represents a patient where the FEF location was determined using anatomical landmarks. In patients with NF1, the concentration of GABA+ was negatively correlated with the density of GABA_A receptors in the FEF ($r = -0.842$, $p = 0.004$, $N = 9$, 95% CI = -1.000 to -0.368 calculated from 10,000 bootstrap samples).

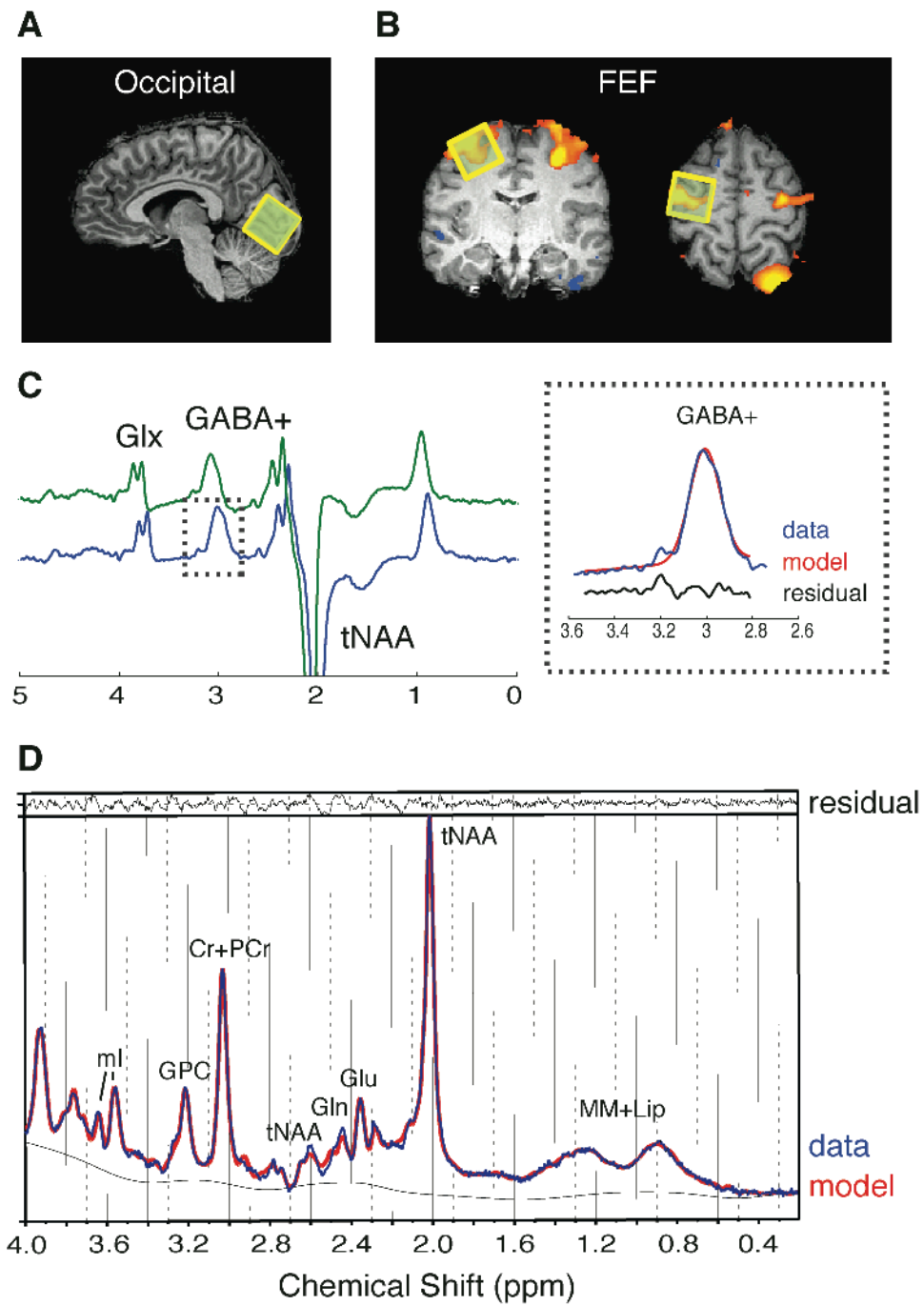


Figure 1

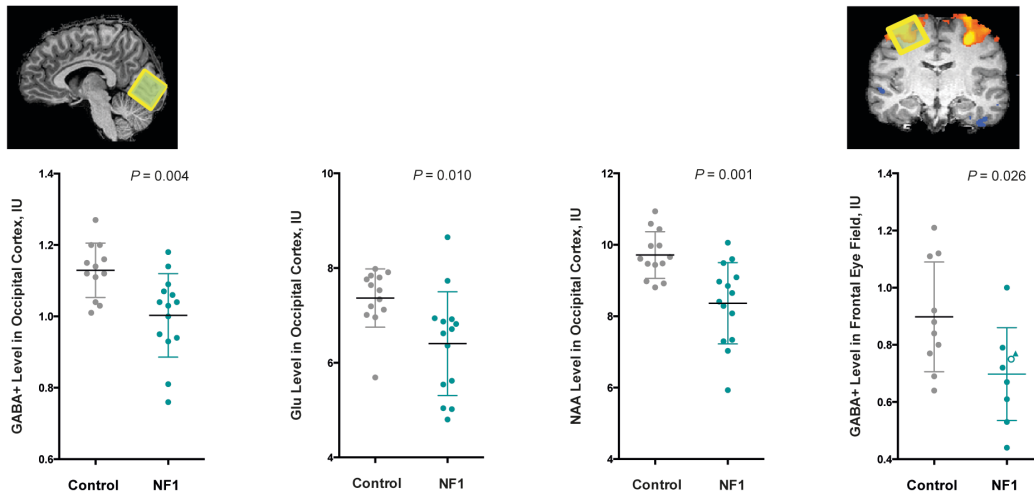


Figure 2

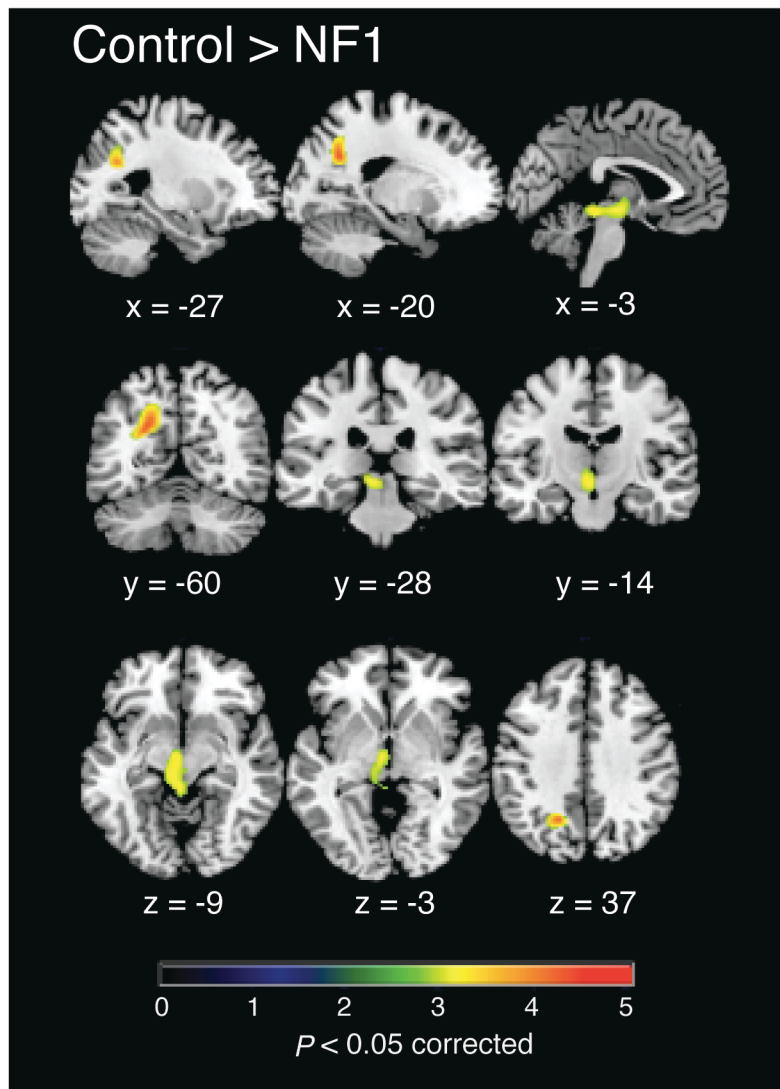


Figure 3

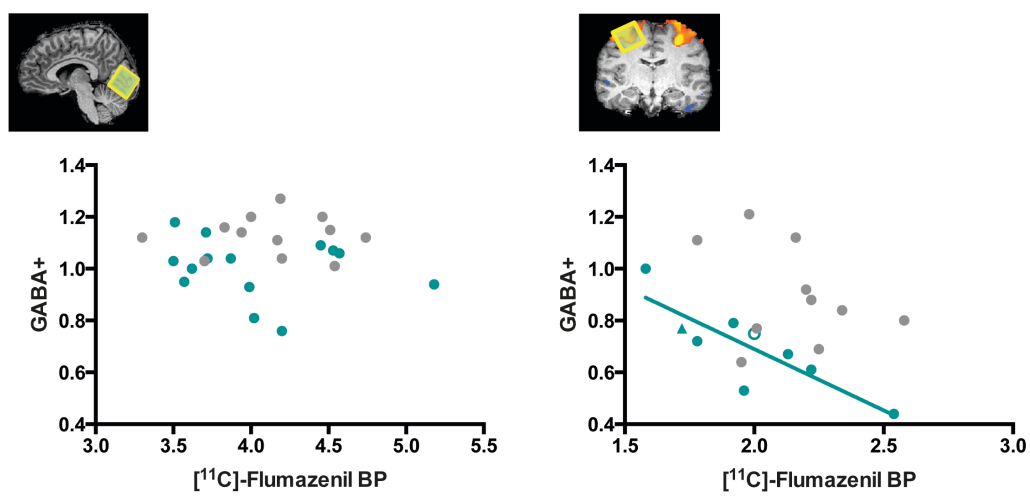


Figure 4

Supplemental Material

GABA deficiency in NF1: a multimodal [^{11}C]-Flumazenil and spectroscopy study

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Supplementary Methods

MRI and MRS acquisitions

Scanning was performed on a 3T Siemens TimTrio (Siemens), using a 12-channel head coil. Acquisitions: (i) T1-weighted MP-RAGE, 1 mm³ isotropic voxel, repetition time (TR) 2530 ms, echo time (TE) 3.42 ms, inversion time (TI) 1100 ms, flip angle (FA) 7°, field of view (FOV) 256 x 256 mm, 256 x 256 matrix, 176 slices, GRAPPA = 2; (ii) T2-weighted FLAIR to identify T2-hyperintensities, 1 mm³ isotropic voxel, TR 5 s, TE 388 ms, TI 1.8 s, FOV 250x250 mm, 256x256 matrix, 160 slices, GRAPPA = 2; (iii) functional localizer for the frontal eye fields (FEF) using single-shot EPI, 3.6 mm³ isotropic voxel, TR 1.5 s, TE 30 ms, FA 76°, FOV 230x230 mm, 64 x 64 matrix, 28 slices, GRAPPA = 2; (iv) 2xGABA-edited spectra using the MEGA-PRESS method^{1,2}, 3 cm³ isotropic voxel, TE 68 ms, TR 1.5 s, 196 averages (occipital) and 264 averages (FEF), 1024 data points. The occipital voxel was positioned within the occipital cortex with its lower face aligned with the cerebellar tentorium (Figure e-1A). The FEF voxel was positioned based on the BOLD activation elicited by the functional localizer. In two NF1 participants the voxel was placed according to anatomical landmarks (junction of precentral sulcus and superior frontal sulcus), due to insufficient BOLD activation. All voxels were placed on the right FEF with the exception of two participants that showed a lateralization of the FEF activation to the left hemisphere (two patients with NF1), (Figure e-1B).

(v) ^1H -PRESS in the occipital and FEF positions, 3 cm³ isotropic voxel, TE 35 ms, TR 2 s, 46 averages, 1024 data points.

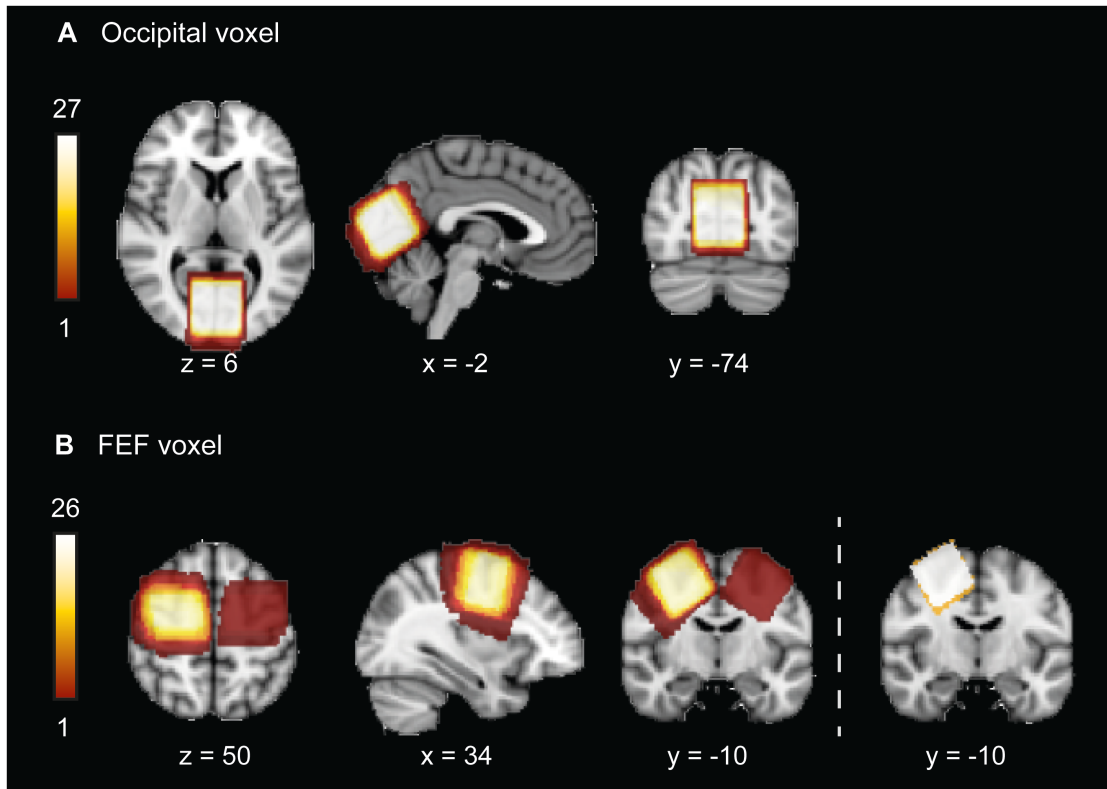


Figure e-1. MRS voxel positions.

A. Overlay of the occipital cortex voxel in all subjects (The color bar indicates the number of subjects). **B.** Left panel: overlay of the FEF voxel in all subjects (color bar indicates the number of subjects). Right panel: overlay of the two MRS voxels positioned according to anatomical landmarks.

Images are in MNI space and radiological orientation.

Occulomotor task to localize the frontal eye fields (FEF)

Trials began with fixation on a central white dot (2° of visual angle) for 1500 ms, followed by presentation of a coloured dot for 1300 ms, which cued for a pro-(green dot) or anti-saccade (red dot). A subsequent gap of 200 ms occurred prior to target presentation for 100 ms (white square 4° of visual angle) at 6 or 7° to the left or right of fixation. Subjects had 1400 ms to perform a saccade to the location of the

target on a pro-saccade trial, or to its mirror location on an anti-saccade trial. A total of 20 pro- and 20 anti-saccade trials were presented in a block design (18 s per block, 5 blocks per task) interleaved with 12 s rest blocks (fixation). We monitored eye movements using a camera placed on the mirror system (Avotec Real Eye 5721).

Voxel-based morphometry

It is important that studies investigating differences in GABA_A density employing flumazenil perform grey matter comparisons independently as the relationship between flumazenil BP and grey matter volume³ or cortical thickness⁴ is not simple as their correlation is not homogeneous across brain regions.

To investigate the potential confound that group differences in binding potential could partially arise from counterpart differences in grey matter (GM) volume, we performed voxel based morphometry, applying standard procedures implemented in the VBM8 toolbox (<http://dbm.neuro.uni-jena.de/vbm/>) in SPM8 (<http://www.fil.ion.ucl.ac.uk/spm>). This included, correction for bias-field inhomogeneity, registration using linear and nonlinear transformations and segmentation into GM, white matter (WM) and CSF images. Images were smoothed with a FWHM of 8 mm.

Group comparisons were performed using voxel-wise independent-samples t-tests in SPM and corrected for multiple comparisons using a peak height threshold of uncorrected $P < 0.005$ with cluster size greater than 1352 contiguous voxels as estimated by 10,000 Monte Carlo simulations in 3dClustSim (AFNI, <http://afni.nimh.nih.gov/afni/>). This corresponds to a threshold of $P < 0.05$ corrected for multiple comparisons.

Supplementary Results

Spectroscopy results

Supplemental Table e-1 Spectroscopy results

Metabolite	Metabolite Level, Mean $\pm$ SD, IU									
	Occipital Cortex					Frontal Eye Field				
	NF1	N	Controls	N	<i>P</i>	NF1	N	Controls	N	<i>P</i>
GABA+	1.00 $\pm$ 0.12	14	1.13 $\pm$ 0.08	12	0.001*	0.70 $\pm$ 0.16	9	0.90 $\pm$ 0.19	10	0.026*
Gln	3.58 $\pm$ 0.53	12	3.86 $\pm$ 0.82	13	0.323	2.53 $\pm$ 0.42	9	2.72 $\pm$ 0.44	10	0.369
Glu	6.40 $\pm$ 1.1	14	7.37 $\pm$ 0.62	13	0.010*	5.11 $\pm$ 0.80	14	5.45 $\pm$ 0.86	12	0.296
GPC	1.02 $\pm$ 0.13	11	1.02 $\pm$ 0.10	12	0.882	1.31 $\pm$ 0.22	12	1.18 $\pm$ 0.19	10	0.169
ml	4.47 $\pm$ 0.50	14	4.52 $\pm$ 0.43	13	0.748	3.63 $\pm$ 0.57	14	3.47 $\pm$ 0.37	12	0.408
PCr+Cr	6.06 $\pm$ 0.78	14	6.37 $\pm$ 0.32	13	0.207	4.94 $\pm$ 0.40	14	4.82 $\pm$ 0.23	12	0.345
tNAA	9.44 $\pm$ 1.17	14	10.69 $\pm$ 0.67	13	0.002*	8.27 $\pm$ 0.83	14	8.63 $\pm$ 1.02	12	0.361

Cr = creatine, Gln = glutamine, Glu = glutamate, GPC = glycerophosphocholine, ml = myo-Inositol, PCr = phosphocreatine, tNAA = total N-acetyl-aspartate (NAA (N-acetyl-aspartate) + NAAG (N-acetylaspartylglutamine)).

* Significant results after correcting for multiple comparisons using the Holm-Sidak method ($\alpha = 0.05$).

The N reflects the number of participants included for the statistical analysis. Only metabolites with Cramér–Rao bounds below 20% were considered.

Voxel-based morphometry results

VBM analyses showed that decreased [^{11}C]-Flumazenil binding potential (BP) in patients do not co-localize with the regions in which patients have decreased GM,

Figure 2 (PET contrast Control > NF1 (magenta) and VBM contrast Control > NF1 (red)). Therefore, decreased density of GABA_A receptors is not explained by decreased GM density.

In addition, VBM analyses identified significant decrease in GM volume in patients with NF1 in the precuneus and extending to the left paracentral lobule (MNI coordinates: [-6 -46 52]; t score 5.91, k = 1842, $P < 0.05$ corrected), Figure 2. The reverse contrast, NF1 > Control, showed increased GM volume in patients in a region encompassing the thalami and the vermis (MNI coordinates: [0 11 1]; t score 5.55, k = 1830, $P < 0.05$ corrected) and in another region that extends from the caudate to the subcallosal cortex and subgenual anterior cingulate cortex (MNI coordinates: [18 -24 -3]; t score 5.32, k = 1953, $P < 0.05$ corrected), Figure e-2.

Interestingly, increased GM volume and decreased flumazenil BP in patients co-localizes in a region including the left midbrain and left thalamus. In this region, patients have increased GM volume in the absence of a parallel increase in inhibitory neurons.

To ensure that PET results were not hindered by concomitant abnormalities in GM we performed correlation analyses between GM density and BP in the regions showing decreased PET BP. No correlation was observed for patients or controls in neither the thalamic/midbrain region [NF1: $r = 0.185$, $P = 0.527$, N=14; Controls: $r = -0.153$, $P = 0.619$, N=13] nor the parietal-occipital region [NF1: $r = 0.479$, $P = 0.083$, N=14; Controls: $r = 0.500$, $P = 0.081$, N=13], confirming that decreased BP in patients is not explained by GM alterations.

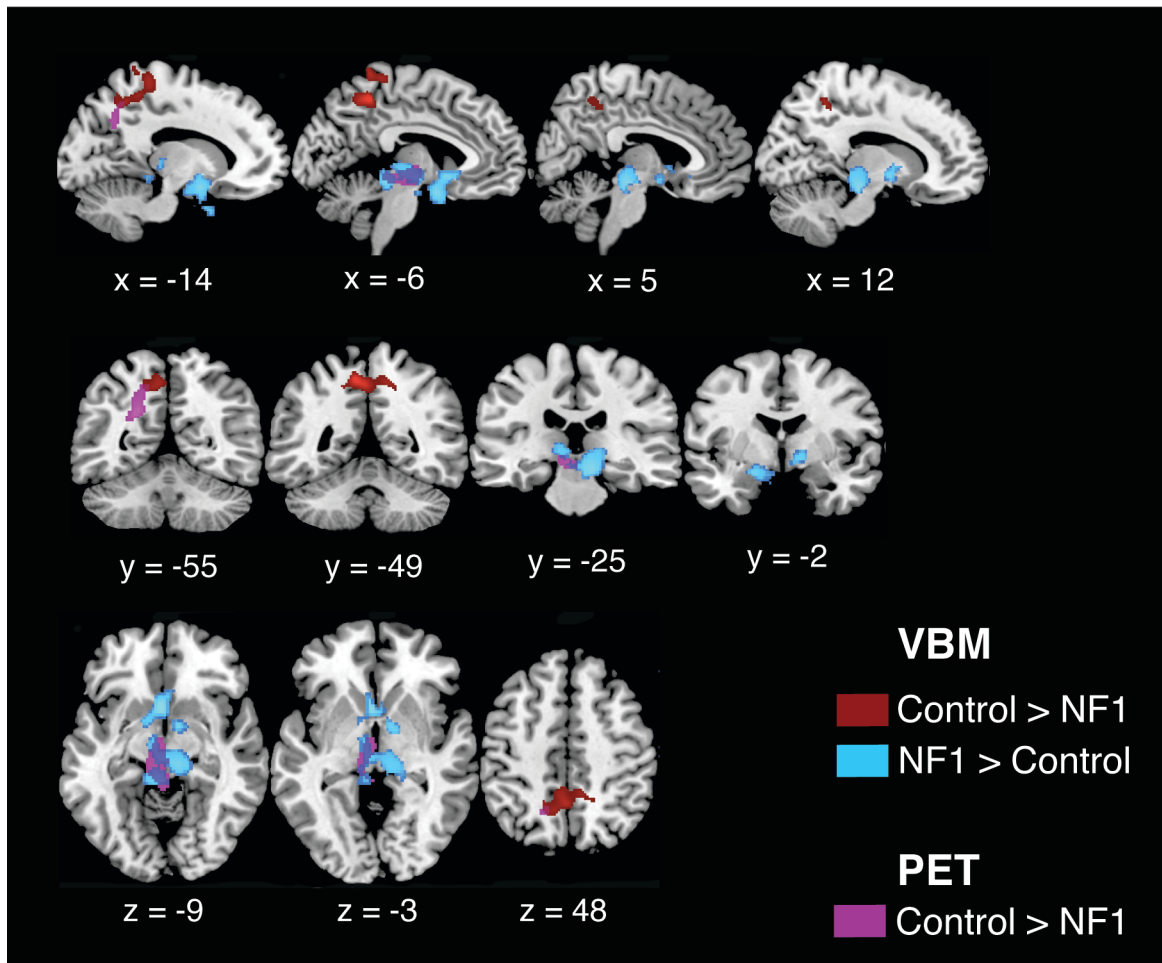


Figure e-2. Voxel-based morphometry (VBM) analysis between patients with NF1 and controls.

Areas of decreased grey matter volume in patients are displayed in red (Control > NF1) and areas of increased grey matter volume in patients are displayed in blue (NF1 > Control) at a level of $P < 0.05$ corrected (peak height threshold of uncorrected $P < 0.005$ with cluster size greater than 1352 contiguous voxels as estimated by 10,000 Monte Carlo simulations). Regions of decreased grey matter volume in patients (red) do not co-localize with regions of decreased [^{11}C]-Flumazenil PET binding (pink).

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4. la Fougere C, Grant S, Kostikov A, Schirmacher R, Gravel P, Schipper HM *et al.* Where in-vivo imaging meets cytoarchitectonics: the relationship between cortical thickness and neuronal density measured with high-resolution [18F]flumazenil-PET. *Neuroimage* 2011; **56**(3): 951-960.