



## Reduced hippocampal N-acetyl-aspartate (NAA) as a biomarker for overweight<sup>☆</sup>



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### ABSTRACT

**Objective:** We previously demonstrated an inverse relationship between both dentate gyrus neurogenesis – a form of neuroplasticity – and expression of the antiapoptotic gene marker, BCL-2 and adult macaque body weight. We therefore explored whether a similar inverse correlation existed in humans between body mass index (BMI) and hippocampal N-acetyl-aspartate (NAA), a marker of neuronal integrity and putatively, neuroplasticity. We also studied the relationship of a potentially neurotoxic process, worry, to hippocampal NAA in patients with generalized anxiety disorder (GAD) and control subjects (CS).

**Methods:** We combined two previously studied cohorts of GAD and control subjects. Using proton magnetic resonance spectroscopy imaging (<sup>1</sup>H MRSI) in medication-free patients with GAD (n = 29) and a matched healthy control group (n = 22), we determined hippocampal concentrations of (1) NAA (2) choline containing compounds (CHO), and (3) Creatine + phosphocreatine (CR). Data were combined from 1.5 T and 3 T scans by converting values from each cohort to z-scores. Overweight and GAD diagnosis were used as categorical variables while the Penn State Worry Questionnaire (PSWQ) and Anxiety Sensitivity Index (ASI) were used as dependent variables.

**Results:** Overweight subjects (BMI ≥ 25) exhibited lower NAA levels in the hippocampus than normal-weight subjects (BMI < 25) (partial Eta-squared = 0.14) controlling for age, sex and psychiatric diagnosis, and the effect was significant for the right hippocampus in both GAD patients and control subjects. An inverse linear correlation was noted in all subjects between right hippocampal NAA and BMI. High scores on the PSWQ predicted low hippocampal NAA and CR. Both BMI and worry were independent inverse predictors of hippocampal NAA.

**Conclusion:** Overweight was associated with reduced NAA concentrations in the hippocampus with a strong effect size. Future mechanistic studies are warranted.

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### 1. Introduction

Whereas the hippocampus is commonly associated with memory and learning, a critical role in emotional control and mood and anxiety disorders has also been noted (Apfel et al., 2011). An important but less recognized role for the hippocampus is in the control of food intake and energy balance. For instance, amnesic humans with brain

damage that includes the hippocampus have been reported to exhibit insensitivity to signals of hunger and satiety (Hebben et al., 1985; Rozin, 1998), an effect that has also been observed in rats with highly selective lesions that are confined to the hippocampus (Davidson and Jarrard, 1993). Also, obese and post-obese patients tasting a liquid meal showed a decreased activity on positron emission tomography in the posterior hippocampus compared to lean control subjects (DelParigi et al., 2004). Collectively, these results suggest that hippocampal damage might interfere with appetite.

The hippocampus may also play a critical role in the brain's ability to regulate body weight through learning processes (Benoit et al., 2010). Investigators (Davidson et al., 2007) hypothesize that “hippocampal-dependent learning and memory mechanisms translate neurohormonal

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signals of energy balance into adaptive behavioral outcomes involved with the inhibition of food intake". Conversely, the same group proposes the hypothesis "that excessive caloric intake and obesity may be produced by dietary and other factors that are known to alter hippocampal functioning." Reduced hippocampal function and plasticity is observed in rats maintained on diets high in fat and sugar (Kanoski et al., 2007; Liu et al., 2004; Molteni et al., 2002; Monteggia et al., 2004; Wu et al., 2003; Yamada and Nabeshima, 2003). Impaired hippocampal neurogenesis occurs in male rats fed on high-fat diet for 4 weeks (Lindqvist et al., 2006). By contrast, Walker et al. demonstrated that both neonatal leptin treatment and exposure to high-fat diet during the perinatal period increase neurogenesis and neuronal survival in the hippocampal dentate gyrus of young animals, an effect attributed to a reduction of apoptotic processes (Walker et al., 2008).

More recently our own studies found an inverse relationship between body mass in non-obese male nonhuman primates and dentate gyrus doublecortin (reflective of immature neurons) and dentate gyrus expression of BCL-2, an anti-apoptotic gene product (Perera et al., 2011). Ki-67, a marker for precursor proliferative cells, did not correlate with body mass, suggesting that correlations with body mass were maturational rather than proliferation related. Our non-diabetic animals were fed standard monkey chow with occasional fruit treats excluding the potential confound of a high lipid diet causing hippocampal dysfunction. We found markedly high correlations between fasting blood sugar and dentate gyrus BCL-2 ( $r = 0.99$ ) and doublecortin ( $r = 0.99$ ), supporting the premise that one form of dentate gyrus neuroplasticity was involved in metabolic control (Perera et al., 2011).

In light of the overweight/obesity epidemic in the United States (King, 2013; Mitchell et al., 2011), we wished to extend our preclinical studies into the clinical realm, using a non-invasive neuroimaging modality. Proton magnetic resonance spectroscopic imaging ( $^1\text{H}$  MRSI) is well-suited to examine regional alterations in tissue concentrations of neurochemicals that are indicative of brain metabolism (Coplan et al., 2006; Lyoo and Renshaw, 2002). We recently proposed that N-acetylaspartate (NAA), an accepted marker of neuronal integrity, serves as a putative marker of neuroplasticity in GAD (Abdallah et al., 2013), positively tracking hippocampal volume alterations in response to the antiepileptic agent, riluzole, in the treatment of GAD.

Since we had observed that neurogenesis rates vary inversely with body mass in nonhuman primates, and our recent studies suggest that NAA may track neurotrophic processes in the hippocampus, we hypothesized that, to the extent neurogenesis is representative of neurotrophic processes in the hippocampus in general, relative elevations in BMI would predict relative reductions in hippocampal NAA levels. However, other possibilities besides the neurogenesis changes restricted solely to the dentate gyrus may be relevant in determining hippocampal NAA, such as synaptic changes, dendritic remodeling or glial cell changes. Although prefrontal cortical volume had previously been associated with reduction in glial cell number (Rajkowska, 2000), recent work by the same group (Cobb et al., 2013) did not demonstrate reductions in glial cell number associated with reduced hippocampal volume in major depressive disorder. Thus the neuropathology associated with lower NAA may have causes other than reduced neurogenesis, which remain to be determined.

We also focus on choline containing compounds (CHO), in part reflective of membrane turnover, which we have shown to be reduced in the centrum semiovale (CSO) of patients with GAD versus healthy volunteers (HV) (Coplan et al., 2006). We examine concentrations of the metabolites of Creatine + phosphocreatine (CR), a potential index of brain metabolism, which we have shown also to be reduced in the CSO of patients with GAD versus HV (Coplan et al., 2006). One study by Massana et al. reported reduced CR in the right medial temporal lobe in patients with panic disorder (Massana et al., 2002). In this study, we wished to examine in a substantial number of subjects combining two cohorts, whether the findings of the Massana et al. study were specific to panic disorder or would be evident in other anxiety

disorders. We also examined if parametric effects were evident between CR and measures of worry as measured by the PSWQ.

Thus, the primary aim of our study was to test the hypothesis in humans that an inverse relationship existed between BMI and a marker of hippocampal neuronal integrity, reflected by NAA, on proton magnetic resonance spectroscopy. We might then detect a central biomarker of overweight, facilitating understanding and treatment. Secondly, we sought to examine the influence, if any, of the diagnosis of GAD, or if not, of worry itself, on the hypothesized relationship between BMI and NAA. Should a relationship between NAA and BMI be observed, it may pave the way for improving our understanding of the hippocampal contribution to the pathophysiology of overweight and the metabolic syndrome.

## 2. Methods

### 2.1. Subjects

$^1\text{H}$  MRSI data were obtained from two previous studies (Mathew et al., 2008, 2009) for a total of 51 subjects—32 women and 19 men, 19 in the former study and 32 in the latter study. 22 control subjects (eight men, fourteen women; mean age  $\pm$  SD, 33.7 years  $\pm$  10.4) and 29 medication-free GAD patients (eleven men, eighteen women; mean age  $\pm$  SD, 35.1 years  $\pm$  11.9) were recruited by advertising or clinician referral.

All patients met the DSM-IV-TR Criteria for GAD as established by the Structured Clinical Interview for DSM-IV (SCID) (First et al., 1995). None of the GAD patients had been taking any psychotropic medication for at least 2 weeks before the MRSI scan. GAD patients had at least moderate worry severity (mean baseline Penn State Worry Questionnaire [PSWQ] (Meyer et al., 1990) score: 64.9  $\pm$  8.1) and severe anxiety sensitivity (Keller, 2002) (mean baseline Anxiety Sensitivity Index [ASI] score: 32.2  $\pm$  11.5). Comorbid diagnoses, determined by SCID, included panic disorder ( $n = 7$ ), social anxiety disorder ( $n = 7$ ), dysthymia ( $n = 6$ ), specific phobia ( $n = 4$ ), depressive disorder not otherwise specified ( $n = 2$ ), adjustment disorder with mixed depressed and anxious mood ( $n = 1$ ), and Bipolar II disorder; most recent depressed in partial remission ( $n = 1$ ).

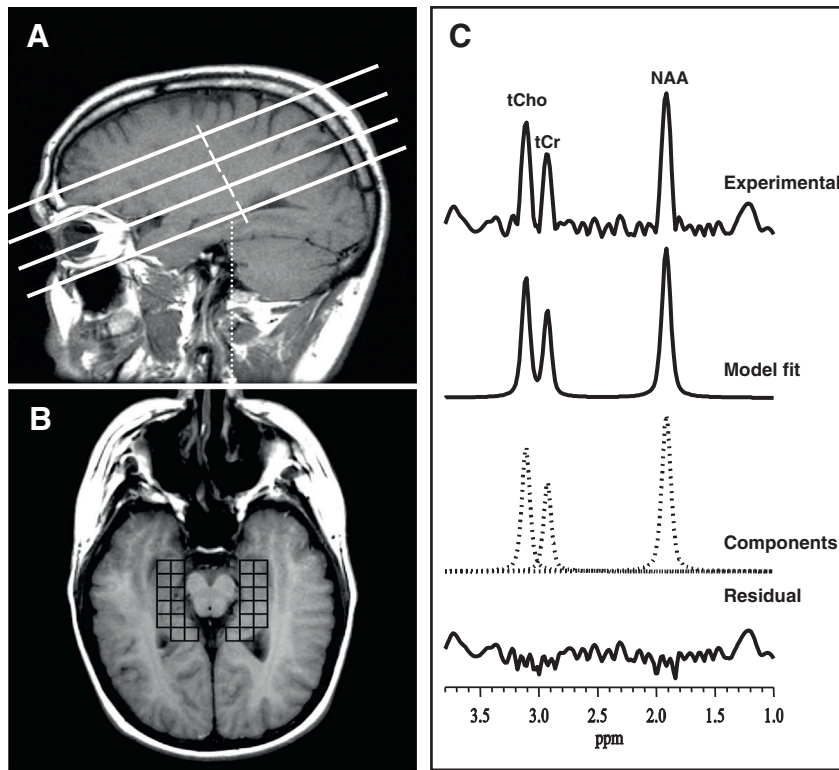
Exclusion Criteria for GAD patients included the following: major depressive episode or substance abuse/dependence within 6 months of study entry; lifetime histories of psychosis, bipolar disorder, obsessive-compulsive disorder (OCD), eating disorder, or posttraumatic stress disorder (PTSD); or significant medical or neurologic conditions requiring daily medication treatment. In addition, subjects who were pregnant or who had any condition precluding clinical magnetic resonance examination (e.g. pacemaker, metallic prosthesis) were excluded.

Control subjects did not have any current medical conditions or any lifetime history of Axis I psychiatric disorders, according to the SCID-NP interview (Spitzer, 1996). All participants had unremarkable screening laboratory evaluations, including urine toxicology. Written informed consent was obtained and all study procedures were approved by the Institutional Review Board.

### 2.2. $^1\text{H}$ MRSI data acquisition protocol

Neuroimaging studies were conducted on a 1.5-T GE Horizon 5.x Signa MR system in one study (Mathew et al., 2008), and on a 3.0 T GE MRI system using a standard quadrature head coil, in the other study (Mathew et al., 2009). Voxels that best covered the primary regions of interest "ROIs" (right and left hippocampi) in each subject were selected on the basis of their location on the matching high-resolution MR localizer images.

Following sagittal scout images, a four-section T1-weighted axial/oblique localizer imaging series, angulated parallel to the Sylvian fissure (Fig. 1A), was acquired, with a slice thickness of 15 mm and an interslice



**Fig. 1.** Voxel placement for hippocampus. (A) MRI depicting the location of the four oblique brain sections investigated; (B) coronal view from left and right grids of hippocampal voxels of interest. (C) Example of an MR spectrum from a hippocampal voxel in the grid shown in (B). NAA, N-acetyl-aspartate; tCho, total choline containing compounds; tCr, total Creatine + phosphocreatine.

gap of 3.5 mm, matching the subsequent multislice  $^1\text{H}$  MRSI scan. Next, the  $^1\text{H}$  MRSI scan was performed using the method of Duyn et al. (1993), with TE/TR of 280/2300 ms, field of view of 240 mm,  $32 \times 32$  circularly sampled k-space phase-encoding steps with one excitation per phase-encoding step, and 256 time-domain points. The strong pericranial lipid resonances from the skull, scalp, and calvarial marrow were suppressed using octagonally tailored outer-volume suppression pulses, and water was suppressed with a single chemical shift-selective pulse followed by spoiler gradients. The entire neuroimaging protocol required approximately 60 min to complete. The raw data were separated into individual slices and then processed by the standard fast Fourier transform algorithm, as previously described (Mathew et al., 2004). The actual MRSI voxel, estimated from the integral of the point-spread function (PSF) following spatial filtering with a Hamming window and Fermi window and then Fourier transformation, was  $1.13 \text{ cm}^3$  or approximately 40% larger than the nominal voxel size that would be derived from the acquisition parameters (Mathew et al., 2008).

### 2.3. $^1\text{H}$ MRSI data analysis and quantification

The raw MRSI data were processed and analyzed voxel by voxel offline on a Sun Microsystems (Mountain View, CA) work station, using the Interactive Data Language (IDL, ITT Visual Information Solutions, Boulder, CO) software package developed in-house by two of the investigators (XM, DCS). Voxels that best covered the primary ROIs (right and left hippocampi) in each subject were selected on the basis of their location on the matching high-resolution MR localizer images (Fig. 1B). Fig. 1C shows a representative spectrum and sample spectral fit for a hippocampal MRSI voxel. Data analysis was performed by a trained investigator blinded to diagnosis and scan number. The mean of the peak areas for each metabolite within the ROIs was computed from fitted spectral data. The a priori measure of

interest was the concentration of NAA. Concentrations of Creatine + phosphocreatine (CR) and choline-containing compounds (CHO, an index of myelin turnover) were also obtained. Peak areas derived from spectral fitting were converted to “absolute” (i.e., molar) metabolite concentrations using the phantom replacement methodology (Mathew et al., 2008; Soher et al., 1996).

### 2.4. Hippocampal volume determination

Data were only available on 17 subjects from the 1.5 T MRI study (Mathew et al., 2008). MRI images were collected using a Sagittal T1-gradient echo volumetric acquisition protocol (TE/TR = 2/9 ms, voxel size =  $0.9 \times 0.9 \times 1.5 \text{ mm}$ , flip angle =  $7^\circ$ , FOV = 240 mm, 1.5-mm thickness with no gaps, totalizing 256 slices per slab, matrix size  $256 \times 256$ , NEX = 1). Images were converted to ANALYZE format using MRICro. Hippocampal segmentation was performed with the fully automated Freesurfer image analysis package (<http://surfer.nmr.mgh.harvard.edu/>). This processing includes removal of non-brain tissue using a hybrid watershed/surface deformation procedure, automated Talairach transformation, segmentation of the subcortical white matter and deep gray matter volumetric structures (including the hippocampus), intensity normalization, tessellation of the gray matter–white matter boundary, automated topology correction, and surface deformation following intensity gradients to optimally place the gray/white and gray/cerebrospinal fluid borders at the location where the greatest shift in intensity defines the transition to the other tissue class (for additional details, please see Fischl and Dale, 2000).

### 2.5. Statistical analysis

Statistical analyses were conducted using Statistica Version 10. NAA, CR, and CHO regional concentrations in the hippocampi (from both

studies) were separately z-scored for standardization. Independent t-tests as well as chi-square tests were applied to compare age, body mass index, sex distribution, PSWQ and ASI among the diagnostic groups (GAD versus control subjects).

### 2.5.1. Categorical analyses

We implemented a general linear model (GLM) with hippocampal metabolite concentrations (NAA, CR, and CHO) as dependent measures. Hippocampal metabolite concentrations (left and right) were used as a repeated measure. Independent variables included the categorical variables of diagnostic group (GAD or Control Subjects), BMI status [normal weight (BMI < 25) or overweight (BMI ≥ 25)], the interaction term of diagnostic group \* BMI status and sex. Age was included as a covariate. “Study Number” was not included as a formal covariate because neurometabolites had been standardized across studies. Nevertheless, all categorical analyses were run with “Study Number” as a covariate after the formal analysis. Significant results were unchanged and “Study Number” was not a significant covariate in any of the analyses. All three multivariate analyses (NAA, CR, and CHO) were followed by univariate analyses focusing on each side of the hippocampus. Since nine of the 16 overweight (BMI ≥ 25) subjects were in fact obese (BMI ≥ 30), a three group comparison (normal weight, overweight and obese) was performed for NAA and tested with post-hoc Newman–Keuls testing. In order to confirm the NAA findings, we computed means from the raw data by combining the 1.5 T and 3.0 T MRSI studies and using BMI status, study, diagnosis, the BMI status \* diagnosis interaction and sex as factors in the analysis and age and sex as a continuous variable.

### 2.5.2. Correlational analyses

Normality of distribution was validated using the Kolmogorov–Smirnov test and the Lilliefors probability for non-standardized variables. Pearson’s Product-Moment correlations were performed relating BMI to each metabolite concentration for each side (N = 51). Hippocampal volume (HV) data from the Mathew et al. study (Mathew et al., 2008) were correlated with NAA, CHO and CR and BMI (N = 17) for GAD and CS was combined. The HV correlational analyses then examined GAD subjects alone and CS alone. Then PSWQ scores were correlated to each metabolite concentration for each side and BMI (N = 46 as 5 control subjects’ values were missing) and finally the same analysis was performed for the ASI. Scatterplots were examined for outliers.

Additional exploratory analyses were permitted to model respective contributions of variance of specific variables and the computation of variance of overall models. Correction for multiple testing was not performed as the primary hypotheses examined the relationship between BMI and NAA, where NAA served as a marker of neuronal integrity

and putative neuroplasticity. Other findings were considered to be of a secondary nature. All tests were two-tailed, with significance level set at  $p \leq 0.05$ .

## 3. Results

### 3.1. Diagnostic groups

There was no age difference between the GAD and CS groups and the ratio of women to men did not differ between the diagnostic groups (Table 1). BMI, height and body mass did not differ between GAD and control subjects (Table 1). GAD subjects exhibited markedly greater scores on the Penn State Worry Questionnaire (PSWQ) and the Anxiety Sensitivity Index (ASI) in comparison to CS, confirming the former’s symptomatic status.

### 3.2. N-Acetylaspartate (NAA)

Employing a general linear model (GLM) using z-scores of absolute NAA of bilateral hippocampus, subjects with a BMI ≥ 25 exhibited reduced NAA in the hippocampus in comparison to subjects with a BMI < 25 [Overweight NAA z score = −0.61 (95% CI: −1.03 to −0.19) (n = 16) versus normal weight NAA z score = 0.25 (95% CI: −0.02 to 0.53) (N = 35):  $F_{(1,45)} = 7.92$ ;  $p = 0.007$  (partial Eta-squared = 0.14)]. Effects were not attributable to age, sex or diagnosis nor was there an interactive between BMI status and diagnostic grouping. Women overall exhibited relative elevations of hippocampal NAA concentration, compared to males [ $F_{(1,45)} = 5.15$ ;  $p = 0.028$ ]. However we controlled for gender by including sex as a covariate in the GLM. Univariate analyses indicated that the BMI effect was confined to the right hippocampus [ $F_{(1,45)} = 12.04$ ;  $p = 0.001$ ; partial Eta-squared = 2.0] whereas the sex effect was confined to the left hippocampus [ $F_{(1,45)} = 6.00$ ;  $p = 0.018$ ]. On the right side, z-score transformed hippocampal NAA concentrations were significantly lower (indicated by \*) in overweight subjects in both the GAD group ( $p = 0.036$ ) and control subjects ( $p = 0.0032$ ) using Newman–Keuls post-hoc testing (Fig. 2). In contrast, there were no significant BMI differences for diagnostic grouping on the left hand side.

When including an obese group (N = 9), overweight group (N = 7) and normal weight group (N = 35) in the identical model, an overall group effect for NAA is noted [ $F_{(2,43)} = 3.99$ ;  $p = 0.03$ ]. Effects were again confined to right hippocampus [ $F_{(2,43)} = 6.23$ ;  $p = 0.004$ ]. However, Newman–Keuls post-hoc testing indicated that although the normal weight group exhibited significantly higher hippocampal NAA concentrations than the overweight group ( $p = 0.02$ ) and obese group ( $p = 0.038$ ), there was no difference between the overweight and obese groups for NAA ( $p = 0.88$ ).

**Table 1**

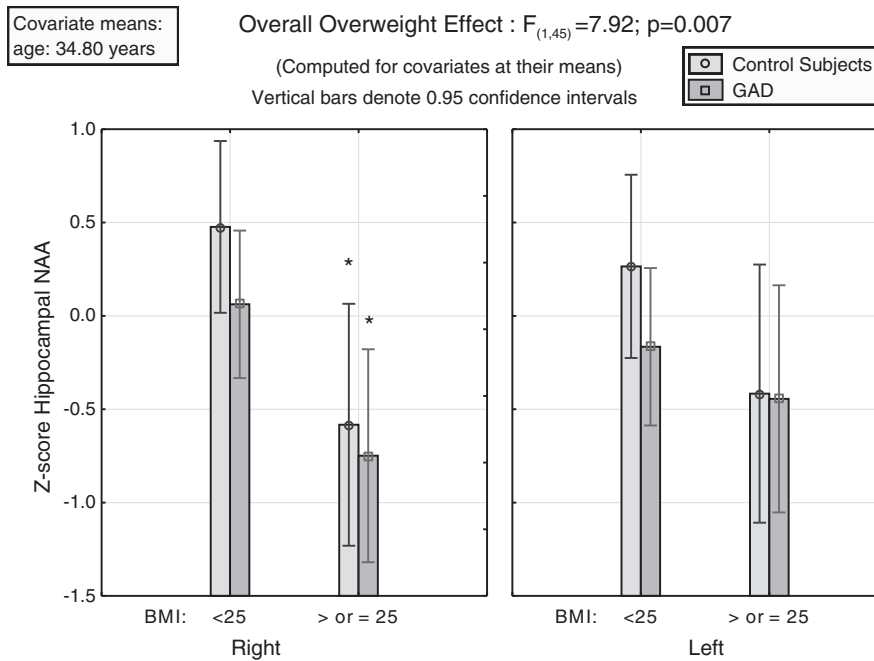
Baseline characteristics of GAD patients and control subjects.

	GAD patients N = 29	Control subjects N = 22	t-Value	p-Value
Age (years)	36 (Wu et al., 2003)	34 (Monteggia et al., 2004)	−0.51	0.61
Female	18*	14*	*Chi-squared (df = 1) = .01; $p = 0.90$	
Body Mass Index	24.64 (6.04)	25.14 (4.40)	0.32	0.74
Height (cm)	170.31(9.05)	168.53(10.07)	−0.66	0.51
Weight (kg)	71.68(18.99)	71.87(16.60)	0.03	0.97
PSWQ	64.41 (7.86)	30.11 (7.86) <sup>a</sup>	−14.77	$p \leq 0.0001$
ASI	32.89 (11.03)	9.21 (7.49) <sup>a</sup>	−8.19	$p \leq 0.0001$

There was no age differences between the GAD and CS groups and the ratio of females to males did not differ between the groups. BMI, height or body mass did not differ between GAD and healthy controls. GAD subjects exhibited markedly greater scores on the Penn State Worry Questionnaire (PSWQ) and the Anxiety Sensitivity Index (ASI) in comparison to CS, confirming their symptomatic status.

Standard deviations are in parentheses. GAD = Generalized Anxiety Disorder; PSWQ = Penn State Worry Questionnaire; ASI = Anxiety Sensitivity Index.

<sup>a</sup> Scores on five control subjects for the PSWQ and ASI were missing.



**Fig. 2.** Comparison of BMI groups: Normal weight  $<25$  versus Overweight  $\geq 25$  for left and right hippocampal NAA Z-scores in patients with GAD and control subjects. Using a general linear model (GLM) using standardized z-scores of absolute NAA of bilateral hippocampus, subjects with a BMI  $\geq 25$  exhibited reduced NAA in hippocampus in comparison to subjects with BMI  $< 25$  [ $F_{(1,46)} = 7.92$ ;  $p = 0.0071$ ]. Effects were not attributable to age, sex or diagnosis nor was there an interactive effect between BMI grouping and diagnostic grouping. On the right side, z-score transformed hippocampal NAA concentrations were significantly lower (indicated by \*) in overweight subjects in both the GAD group ( $p = 0.036$ ) and control subjects ( $p = 0.0032$ ) using Newman–Keuls post-hoc testing. In contrast, there were no significant BMI differences for diagnostic grouping on the left hand side. BMI = body mass index. NAA = N-acetylaspartate.

Using raw NAA concentration scores, overweight subjects exhibited a mean NAA concentration of 20.16 [95% CI: 17.93 to 22.39;  $N = 16$ ] versus normal weight subjects mean NAA concentration = 24.21 [95% CI: 22.72 to 25.71;  $N = 35$ ;  $F_{(1,44)} = 9.14$ ;  $p = 0.004$ ]. According to the overall model, right hippocampal NAA concentrations accounted inversely for 50% of the BMI group variance [ $F_{(6,44)} = 9.25$ ;  $p = 0.000001$ ] whereas left hippocampal NAA concentrations inversely accounted for 33% of the BMI group variance [ $F_{(6,44)} = 5.19$ ;  $p = 0.0004$ ]. Hippocampal NAA concentrations were significantly greater in the 1.5 Tesla study in comparison to the 3.0 Tesla study with a marked study effect [ $F_{(1,44)} = 28.91$ ;  $p = 0.000003$ ] but this difference was controlled for in the analysis.

### 3.3. Choline-containing compounds (CHO)

For hippocampal CHO concentrations (Fig. 3), there was a repeated measure (left and right) by diagnostic status (GAD versus control subjects) effect in the hippocampus [ $F_{(1,45)} = 4.69$ ;  $p = 0.036$ ]. In the left hippocampus, GAD subjects – in comparison to controls – exhibited lower CHO whereas in the right hippocampus, GAD subjects showed higher CHO. Univariate analyses were not significant.

### 3.4. Creatine (CR)

In the general linear model, there was a sex effect [ $F_{(1,45)} = 11.72$ ;  $p = 0.001$ ; males  $<$  females] but no BMI group, diagnostic group or interactive effects. On univariate analysis, there was a reduction in CR concentration in GAD subjects versus controls in the left hippocampus [GAD mean z score CR =  $-0.32$  (95% CI:  $-0.60$  to  $-0.04$ ) ( $N = 29$ ) versus CS mean z score CR =  $0.33$  (95% CI:  $-0.14$  to  $0.80$ ) ( $N = 22$ );  $F_{(1,45)} = 4.65$ ;  $p = 0.036$ ] (Fig. 4) as well as an age effect (older subjects had lower CR) and a sex effect (males with lower CR). On the other hand, subjects with BMI  $\geq 25$  exhibited lower CR than

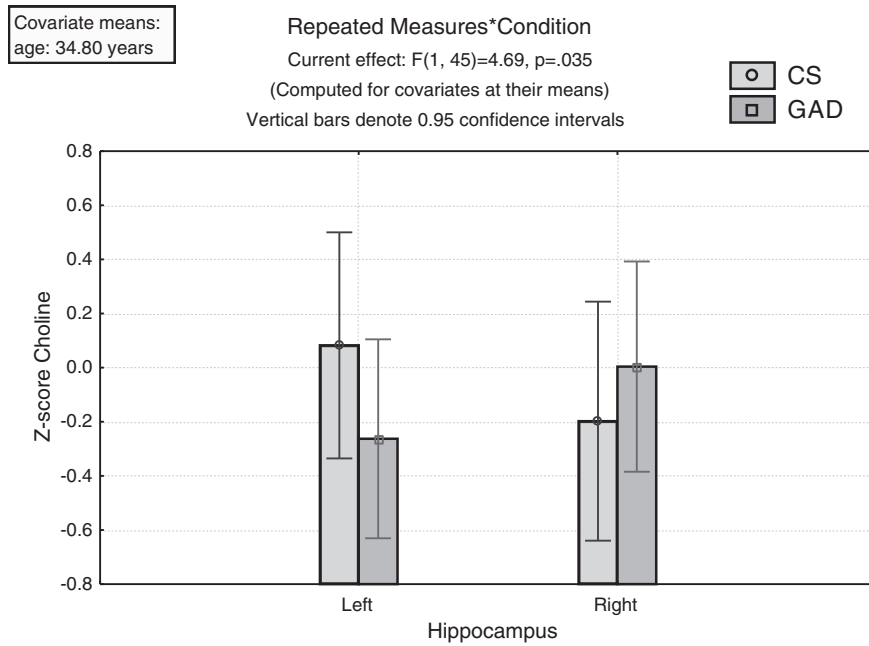
subjects with BMI  $< 25$  in the right hippocampus [Overweight mean z score CR =  $-0.48$  (95% CI:  $-0.95$  to  $-0.01$ ) ( $N = 16$ ) versus normal weight mean z score CR =  $0.16$  (95% CI:  $-0.17$  to  $0.50$ ) ( $N = 35$ );  $F_{(1,45)} = 4.90$ ;  $p = 0.031$ ].

### 3.5. Correlational analyses—BMI data

An inverse correlation was noted in all subjects between the right hippocampal NAA and BMI ( $r = -0.41$ ;  $N = 51$ ;  $p = 0.0026$ ) (Fig. 5) although this effect was not significant on the left ( $r = -0.26$ ;  $N = 51$ ;  $p = 0.066$ ). Of note, BMI scores were normally distributed (Kolmogorov–Smirnov  $d = 0.19$ , Lilliefors  $p \leq 0.01$ ). An additional analysis was performed to evaluate the impact of the right hippocampal NAA on BMI. Right hippocampal NAA accounted (adjusted multiple  $R^2$ ) for 17.6% [ $F_{(3,47)} = 3.36$ ,  $p = 0.026$ ] of the variance of BMI when controlling for age and sex.

### 3.6. Hippocampal volume (HV) analyses

There were no significant correlations between right or left HV and ipsilateral NAA, CHO or CR or between BMI and left or right HV when combining GAD patients and CS [ $n = 17$ ; Appendix Table 1]. Similar negative results were encountered in patients with GAD ( $N = 11$ ) but a significant positive correlation was noted between right HV and right CHO in CS ( $r = .86$ ,  $n = 6$ ,  $p = 0.02$ ). We then used the data generated from the 17 available subjects to determine the N required to detect a two-tailed significant effect of  $p \leq 0.05$  with a power of 0.8 for a right hippocampal volume/BMI relationship or a right hippocampal volume/right hippocampal NAA relationship (see Appendix B). The power analysis did not support a type II error for the absence of either correlations, indicating that an excess of 175 subjects would be required to detect the significant effects for both relationships, far in excess of the



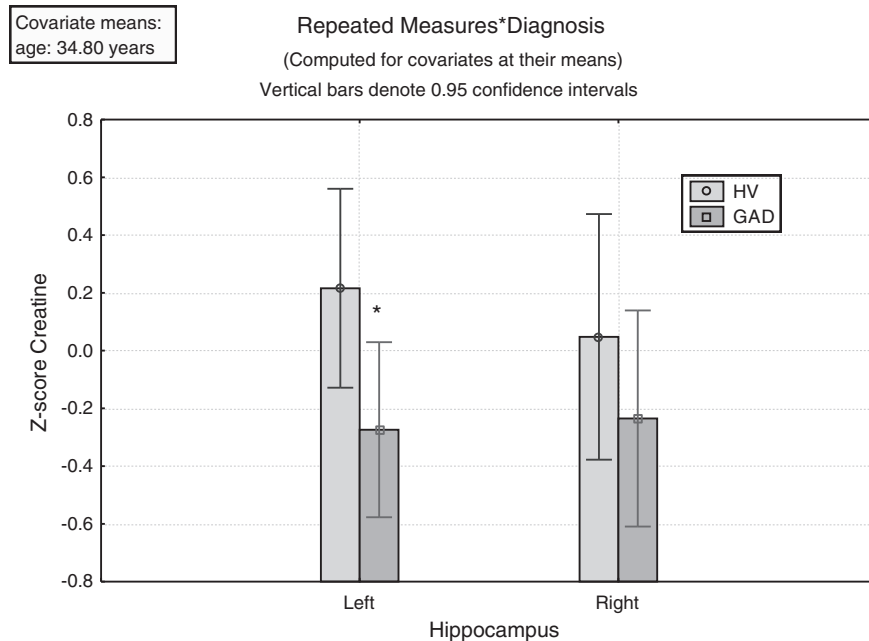
**Fig. 3.** Left versus right hippocampal choline z-scores in patients with GAD in comparison to control subjects. For CHO concentrations, there was a repeated measure (left and right) by diagnostic status (GAD versus controls) effect in hippocampus [ $F_{(1,45)} = 4.69$ ;  $p = 0.036$ ]. In left hippocampus, GAD subjects – in comparison to controls – showed lower CHO whereas in right hippocampus GAD subjects showed higher CHO. Univariate analyses were not significant. CHO = total choline compounds, GAD = generalized anxiety disorder, CS = control subjects, R1 = repeated measure, “status” = diagnostic group.

N of the subjects of the current study, assuming hippocampal volumetrics were available on all subjects.

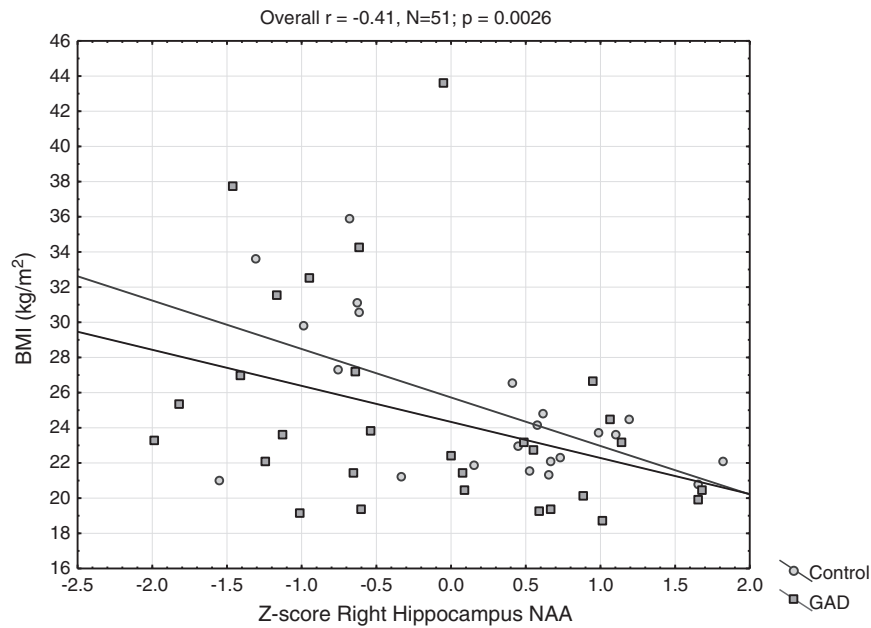
3.7. Correlational analyses—*anxiety rating scales*

For the Penn State Worry Questionnaire (PSWQ) scores ( $N = 46$ ), distribution of scores were normal (Kolmogorov–Smirnov  $d = 0.15$ , Lilliefors  $p \leq 0.01$ ) despite being derived from two diagnostic groups

(patients with GAD and CS). PSWQ scores were inversely correlated with left ( $r = -0.31$ ;  $p = 0.038$ ) and right ( $r = -0.31$ ;  $p = 0.037$ ) hippocampal NAA concentrations. A multiple regression analysis was performed to examine whether the variance contributed to right hippocampal NAA by BMI vis-à-vis PSWQ was an independent source of variance. A significant Beta was noted for BMI [ $\beta = -0.38$ ;  $F_{(1,43)} = 8.26$ ;  $p = 0.006$ ] as was the case for PSWQ scores [ $\beta = -0.32$ ;  $F_{(1,43)} = 5.63$ ;  $p = 0.022$ ]. The combination of Worry and BMI



**Fig. 4.** Left versus right hippocampal creatine and phosphocreatine z-scores in patients with GAD in comparison to control subjects. On univariate analysis, there was a reduction in CR concentration in GAD subjects versus controls in left hippocampus [ $F_{(1,45)} = 4.65$ ;  $p = 0.036$ ]. \*Asterisk indicates significant effect. CR = Creatine and phosphocreatine, GAD = generalized anxiety disorder, CS = control subjects, R1 = repeated measure, “status” = diagnostic group.



**Fig. 5.** Relationship between BMI and right hippocampal NAA z-score. An inverse correlation was noted in all subjects between right hippocampal NAA and BMI ( $r = -0.41$ ;  $n = 51$ ;  $p = 0.0026$ ) although this effect was not significant on the left ( $r = -0.26$ ;  $n = 51$ ;  $p = 0.066$ ), BMI = body mass index. NAA = N-acetylaspartate.

accounted for 21% of the adjusted variance of the overall model [ $F_{(1,43)} = 6.83$ ;  $p = 0.0027$ ], PSWQ scores were also inversely correlated with left ( $r = -0.43$ ;  $p = 0.003$ ) and right ( $r = -0.33$ ;  $p = 0.024$ ) hippocampal CR. These effects were not changed by controlling for study. The only within-group effect that was noted was for right hippocampal CR in GAD subjects ( $r = -0.41$ ;  $N = 29$ ;  $p = 0.028$ ). PSWQ was not correlated to BMI. No correlations were noted between PSWQ scores and CHO.

Anxiety sensitivity index scores were not normally distributed. Therefore they were log transformed (Log ASI, Kolmogorov–Smirnov  $d = 0.24$ , Lilliefors  $p \leq 0.01$ ). The normalized ASI scores correlated inversely with left hippocampal CR ( $r = -0.36$ ;  $p = 0.015$ ). No study effects were noted and normalized ASI scores did not correlate with BMI.

### 3.8. Neurometabolite correlations

The overall data suggest that high BMI was associated with low NAA or reduced hippocampal neuronal viability which is associated with altered hippocampal metabolism, as reflected by relatively low levels of the metabolite CR. Thus, combining all subjects ( $N = 51$ ) right hippocampal NAA correlates positively with right hippocampal CR ( $r = 0.57$ ;  $p < 0.001$ ) and left hippocampal NAA correlates positively with left hippocampal CR ( $r = 0.63$ ;  $p < 0.001$ ). Thus, low NAA or decreased neuronal viability is associated with low CR or altered hippocampal metabolism.

## 4. Discussion

### 4.1. BMI findings

Our data are the first, to our knowledge, reporting that relatively high BMI is associated with relatively low concentrations of a marker of neuronal viability, NAA, in the human hippocampus, with right hippocampal NAA accounting for 17.6% of the variance of BMI. We moreover demonstrate a central correlate of overweight (BMI  $\geq 25$ ) as reflected by low hippocampal NAA with a strong effect size. The effect was independent of age, sex and psychiatric diagnosis. These findings

corroborate our group's earlier findings in non-obese adult bonnet macaques demonstrating an inverse relationship between dentate gyrus neurogenesis rates (reflected by the immature neuronal marker, doublecortin) and expression of the anti-apoptotic gene factor, B-cell lymphoma 2 "BCL-2" and metabolic parameters (body weight) (Perera et al., 2011). Given a role for the hippocampus in appetite (Davidson et al., 2007), the mechanism for the relationship between low NAA and relatively high BMI remains to be determined. Interestingly, obese subjects did not exhibit reduced NAA in comparison to overweight subjects. It is of note that an inverse relationship between hippocampal neuronal integrity and body mass index exists in a non-lesioned, medically stable population. This view is supported by animal studies demonstrating that a high lipid diet may impair hippocampal neurogenesis (Lindqvist et al., 2006). Moreover, our recent data (Abdallah et al., 2013) would suggest that not only does NAA reflect neuronal integrity but also appears to positively mirror alterations that occur in hippocampal volume during riluzole treatment of GAD and thus may also serve, at least in part, as a putative marker of neuroplasticity. These data taken together, both preclinical and clinical, would suggest a significant role of hippocampal neuronal viability in metabolic regulation and weight control.

Although studies have examined the relationship between BMI and NAA, they have not focused on the hippocampus (Gazdzinski et al., 2010a). A cogent issue – the specificity of the BMI findings for the hippocampus – is considered. To analyze the relationship of BMI to NAA in all available regions of interest (ROIs) would be problematic for a number of reasons. First: we have an a priori hypothesis for the involvement of the hippocampus—we have only been able to locate one other study describing an inverse relationship between BMI and NAA in the anterior but not the posterior cingulate cortex in cognitively normal elderly (Gazdzinski et al., 2010a). Another study relates BMI inversely to frontal lobe NAA in alcohol-abusing subjects (Gazdzinski et al., 2010b) although alcohol itself may confound determination of NAA and alcohol independently contributes to obesity. Second: the large number of ROIs when using the Duyn et al. method (Duyn et al., 1993), increases the risk of a type I error, i.e. finding a spurious effect. Third: correcting for multiple testing might abrogate our current primary finding, i.e. a type II error. Nevertheless, in the

absence of testing the relationship of NAA and BMI in other ROIs, the specificity of the effect for hippocampus has to be viewed with caution.

A question arises as to whether the NAA findings in the current study are dependent on hippocampal volume reductions. An inverse relationship has previously been established between BMI and hippocampal volume (Ursache et al., 2012) specifically in obese subjects (Raji et al., 2010), although another large MRI volumetric study failed to find a relationship between hippocampal volume and BMI (Orsi et al., 2011). Hippocampal volume data on a subset of subjects who participated in the overall study were available. Although the N of subjects was substantially less than the overall study, there was no indication of any significant relationships between hippocampal volume and ipsilateral NAA for either right or left hippocampus for patients with GAD and control subjects combined, for patients with GAD or control subjects observed alone. Moreover, we were not able to replicate the inverse BMI-hippocampal volume in the current study. Possible reasons are that our study represented a relatively representative portion of the population whereas the cited studies specifically examined obese subjects. Another reason for the failure to replicate other studies was that the small N available may have reduced the power to detect BMI/right hippocampal volume effects and right hippocampal NAA/right hippocampal volume effects. However, the possibility of a type II error for these effects was not supported by a power analysis on the 17 available subjects (see Appendix B).

Regarding laterality effects, findings were generally significant for the right hippocampus and only marginally less prominent on the left. We controlled for potential hemispheric effects in the repeated measures (with side as the repeated measure) component of the general linear model analyses. We did not detect a single instance of a side by independent variable interaction for NAA. Previously we demonstrated hippocampal asymmetry of neurometabolites in relation to body mass and metabolic syndrome markers in nonhuman primates (Coplan et al., 2011) but were unable to replicate this finding in humans. Moreover, cross-species comparison between our human and nonhuman primate MRSI data is complicated by the use of “ratios” in the latter whereas our human study utilized absolute quantification.

#### 4.2. GAD findings

Hippocampal NAA demonstrated little value as a biomarker of the GAD diagnosis per se, which is consistent with other negative studies (Hetteema et al., 2012; Mathew et al., 2008). However, of note, we were able to demonstrate a significant inverse relationship between PSWQ scores and NAA in both hippocampi. This finding was not specific to either group but became evident when patients with GAD and CS were combined. Interestingly, the distribution of PSWQ scores is normal suggesting a continuum of worry despite strong group differences for the measure. These data are consistent with one previous study in healthy volunteers demonstrating an inverse relationship between NAA and trait anxiety scores (Gallinat et al., 2005). We were able to demonstrate that worry and BMI contribute inverse yet independent sources of variance to right hippocampal NAA and the overall model accounts for 21% of the NAA variance.

Our finding of reduced CR concentration in the left hippocampus of GAD subjects might indicate an altered metabolic state as previously reported in the CSO of patients with GAD (Coplan et al., 2006). The finding is in accordance with an earlier report of significantly lower CR concentration in the right medial temporal lobe region of panic disorder patients compared to healthy subjects (Massana et al., 2002). Of note, Hetteema et al. recently conducted a twin imaging study and demonstrated that lifetime GAD was associated with increased creatine levels in the amygdalae and was associated with smaller left hippocampal volume (Hetteema et al., 2012). PSWQ scores also inversely predicted CR bilaterally and ASI inversely predicted left

hippocampal CR. Of note, the inverse relationship between worry and CR was evident in patients with GAD in the right hippocampus. Thus, worry appears to trigger altered metabolism of the hippocampi and a proportionate depletion of CR. The finding that our overweight subjects exhibit reduced right hippocampal concentrations of both NAA and CR and, increased BMI appears to be associated with a reduction in both hippocampal neuronal integrity and altered hippocampal metabolism. This view is supported by the highly positive correlations between NAA and CR in both hippocampi.

Regarding justification of the anxiety ratings scales used: in generalized anxiety disorder worry is the cardinal symptom and is effectively scored using the Penn State Worry Questionnaire (PSWQ) (Keller, 2002). Patients with generalized anxiety disorder in comparison to high worry controls are more prone to “more negative beliefs about worry, a greater range of worry topics, and more frequent and severe negative thought intrusions” (Hirsch et al., 2013). The anxiety sensitivity index was devised to assess the fear of physical symptoms, fear of publicly observable anxious symptoms, and fear of cognitive “dyscontrol” (Reiss et al., 1986). The ASI-fear of cognitive dyscontrol dimension has been shown to be strongly associated with GAD (Rector et al., 2007). We chose not to use the Hamilton Anxiety Rating Scale (Hamilton, 1967) because the scale was primarily developed for depression.

#### 4.3. Study limitations

Limitations of the study include the combination of two studies of GAD and CS. One study was performed on a 1.5 T and the second, as our capabilities and technology improved, on a 3 T machine. To counter this limitation all neurometabolites' absolute values of each study were converted to z-scores and then combined. Other factors that potentially could differ between studies such as BMI were used as raw values without covariation, as BMI is a consistent measure from study to study. On the other hand, the combining of the two studies rendered one of the largest <sup>1</sup>H MRSI studies that we are aware of in GAD. Although the field strength differed for the two studies, the in-house analytic software used was identical across scanners, as was the data analyst, and all neuroimaging procedures. The greater signal to noise ratio of the 3 T MR compared to 1.5 T does not negate the use of z-score analysis. Moreover, we are able to obtain similarly significant results when using raw NAA concentration values while controlling for study as a factor.

Interpretation of NAA as a marker of neuronal integrity is complicated by the view that it also reflects neuronal metabolism. <sup>13</sup>C MRS studies have shown a strong correlation between NAA levels and neuronal glucose oxidative metabolism (Boumezbeur et al., 2010; Lin et al., 2003). These findings are further supported by in-vitro studies showing an association between NAA synthesis, energy production, and oxygen consumption (Bates et al., 1996); as well as by animal and human studies associating NAA with neuronal activity and metabolism (Benarroch, 2008; De Stefano et al., 1999; Moffett et al., 2007). Thus, the complexity of NAA as a marker of neuronal viability should be acknowledged.

Extensive debate exists on what the best measure of overweight is, and what indicators are most sensitive to society's looming metabolic syndrome. Thus, our study could have been strengthened by measures such as abdominal circumference and sagittal abdominal diameter, and measures of insulin sensitivity and blood lipids as we have used in our nonhuman primate metabolic studies, a goal for future studies.

Thus, one hypothesis is that frank ablation of the hippocampus is not required for alterations in satiety signaling and weight control. Further studies may shed light on the directionality of the relationship between BMI and hippocampal NAA. Our nonhuman primate data suggest that dentate gyrus neurogenesis itself is associated with metabolic control.



## Appendix A

**Appendix Table 1**

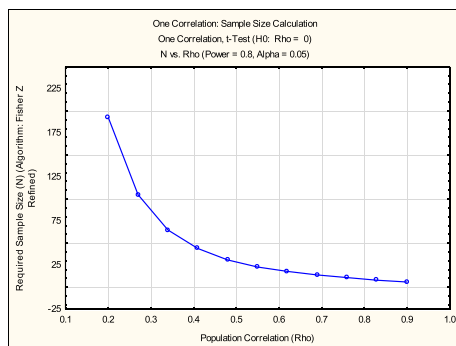
Tables of relationship between hippocampal volume to neurometabolites and BMI in patients with GAD and control subjects (Analyses bolded are  $p \leq 0.05$ ).

Variable	Left NAA	Left CR	Left CHO	Right NAA	Right CR	Right CHO	BMI
<i>All subjects combined (N = 17):</i>							
Left hippocampus	-.20 p = .43	-.26 p = .32	.32 p = .19				-.06 p = .81
Right hippocampus				-.07 p = .77	.15 p = .55	-.16 p = .53	-.08 p = .73
<i>In patients with GAD only (N = 11)</i>							
Left hippocampus	-.12 p = .71	-.05 p = .86	.28 p = .39				.05 p = .86
Right Hippocampus				-.09 p = .77	.00 p = .98	-.20 p = .55	.01 p = .96
<i>In control subjects only (N = 6)</i>							
Left hippocampus	-.61 p = .19	-.62 p = .18	.63 p = .17				-.38 p = .44
Right hippocampus				-.02 p = .96	.69 p = .12	<b>.87</b> <b>p = .02</b>	-.31 p = .53

## Appendix B. Power analysis of the relationship between right hippocampal volume and BMI and right hippocampal volume and right hippocampal NAA

We sought to clarify further the speculation that no relationship between right hippocampal volume and BMI and right hippocampal volume and right hippocampal NAA was evident because of the low number of subjects with volumetric data. We conducted power analyses on the data generated from the 17 available subjects to determine the N required to detect a two-tailed significant effect of  $p \leq 0.05$  with a power of 0.8 for the aforementioned relationships.

Using the data for the relationship between BMI and right hippocampal volume (the side on which significant BMI/NAA correlations had been observed) for all subjects [ $r = -.08$ ] it is evident in the graph below that for a rho less than 0.2, an N of over 175 subjects would be required to achieve statistical significance, far in excess of the N of the subjects of the current study, assuming hippocampal volumetrics were available on all subjects.



We then used data generated from the 17 available subjects to determine the N required to detect a two-tailed significant effect of  $p \leq 0.05$  with a power of 0.8 between right hippocampal volume and right hippocampal NAA [ $r = -.07$ ]. The graph is identical to that depicted above and again it is evident that for a rho less than 0.2, an N of over 175 subjects would be required to achieve statistical significance, far in excess of the N of subjects of the current study.

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