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Title: Magnetic resonance spectroscopy investigations of functionally defined language areas in schizophrenia patients with and without auditory hallucinations

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Abstract: Background: Cerebral dysfunction occurring in mental disorders can show metabolic disturbances which are limited to circumscribed brain areas. Auditory hallucinations have been shown to be related to defined cortical areas linked to specific language functions. Here, we investigated if the study of metabolic changes in auditory hallucinations requires a functional rather than an anatomical definition of their location and size to allow a reliable investigation by magnetic resonance spectroscopy (MRS).
Methods: Schizophrenia patients with (AH; n=12) and without hallucinations (NH; n=8) and healthy controls (HC; n=11) underwent a verbal fluency task in functional MRI (fMRI) to functionally define Broca's and Wernicke's areas. Left and right Heschl's gyrus were defined anatomically.
Results: The mean distances in native space between the fMRI-defined regions and a corresponding anatomically defined area were 12.4±6.1 mm (range: 2.7-36.1 mm) for Broca's area and 16.8±6.2 mm (range:4.5-26.4 mm) for Wernicke's area, respectively. Hence, the spatial variance was of similar extent as the size of the investigated regions. Splitting the investigations into a single voxel examination in the frontal brain and a spectroscopic imaging part for the more homogeneous field areas led to good spectral quality for almost all spectra. In Broca's area, there was a significant group effect ($p = 0.03$) with lower levels of N-acetyl-aspartate (NAA) in NH compared to HC ($p = 0.02$). There were positive associations of NAA levels in the left Heschl's gyrus with total ($p = 0.03$) and negative ($p = 0.006$) PANSS scores. In Broca's area, there was a negative association of myo-inositol levels with total PANSS scores ($p = 0.008$).
Conclusion: This study supports the neurodegenerative hypothesis of schizophrenia only in a frontal region whereas the results obtained from temporal regions are in contrast to the majority of previous studies. Future research should test the hypothesis raised by this study that a functional definition of language regions is needed if neurochemical imbalances are expected to be restricted to functional foci.

Highlights

- Auditory hallucinations associated with circumscribed cortical language areas
- Magnetic resonance spectroscopy potentially requires functionally defined regions of interest
- Results support the neurodegenerative hypothesis of schizophrenia for Broca’s area

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Magnetic resonance spectroscopy investigations of functionally defined language areas in schizophrenia patients with and without auditory hallucinations

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Abstract

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Results: The mean distances in native space between the fMRI-defined regions and a corresponding anatomically defined area were 12.4±6.1 mm (range: 2.7–36.1 mm) for Broca's area and 16.8±6.2 mm (range:4.5–26.4 mm) for Wernicke's area, respectively. Hence, the spatial variance was of similar extent as the size of the investigated regions. Splitting the investigations into a single voxel examination in the frontal brain and a spectroscopic imaging part for the more homogeneous field areas led to good spectral quality for almost all spectra. In Broca's area, there was a significant group effect ($p = 0.03$) with lower levels of N-acetyl-aspartate (NAA) in NH compared to HC ($p = 0.02$). There were positive associations of NAA levels in the left Heschl's gyrus with total ($p = 0.03$) and negative ($p = 0.006$) PANSS scores. In Broca's area, there was a negative association of myo-inositol levels with total PANSS scores ($p = 0.008$).

Conclusion: This study supports the neurodegenerative hypothesis of schizophrenia only in a frontal region whereas the results obtained from temporal regions are in contrast to the majority of previous studies. Future research should test the hypothesis raised by this study that a functional definition of language regions is needed if neurochemical imbalances are expected to be restricted to functional foci.

Keywords: magnetic resonance spectroscopy; N-acetyl-aspartate; myo-inositol; schizophrenia; auditory hallucinations; language

Introduction

^{1}H Magnetic Resonance Spectroscopy (MRS) has been used in clinical and research investigations of human brain physiology and pathology for over two decades [1]. Since it is currently not possible to acquire whole brain MRS measurements with high spatial resolution in a timeframe that is endurable for (living) subjects, regions of interest (ROIs) are commonly defined. Most often, the region to be examined is chosen based on pathological structural abnormalities or in extended brain areas presumed to be involved in the targeted patho-physiologic processes. In both cases, the region of interest (ROI) is chosen using anatomic landmarks. However, from functional brain studies it is well known that besides substantial individuality of cortical morphology [2,3], there is considerable interindividual variability in terms of the exact location of cortical activation for specific brain functions [4,5,6,7]. Hence, if metabolic disturbances are hypothesized to be restricted to or most expressed in limited areas representing specific brain function, the ROIs for MRS investigations have to be defined functionally in each subject. Furthermore, because most MRS studies use either single voxel (SV) localization techniques or spectroscopic imaging (SI) of single slices where the ROIs must be known and defined at the time of data acquisition, the functional definition of the ROI must usually precede MRS. Alternatively, spectroscopic whole brain analyses by multislice or 3D techniques [8], where ROIs can be chosen during data analysis, could be performed without knowledge of the exact region to be targeted, but these techniques are usually not considered to be as reliable and not as time-efficient for small focused ROIs, in particular when using short echo times. Since previous studies have shown dysfunctions in circumscribed cerebral areas related to the language system we investigated auditory verbal hallucinations (AVH) as a potential case of local limited metabolic imbalance.

It is well known that AVH comprise a critical domain in schizophrenia, including a low quality of life since about 30% of patients do not sufficiently respond to medication [9,10]. Notably, 25% of patients with AVH had made serious suicide attempts driven by their voices [11]. Thus, a better understanding of the neural underpinnings of AVH might contribute to a better treatment regime [12,13]. However, an

obstacle of a more sophisticated understanding of AVH lies in the complexity of the process possibly including anatomical, functional and neurochemical aspects and the corresponding complexity of investigation approaches. Several imaging studies found an association of speech- and language related cerebral regions and AVH. In an early functional magnetic resonance imaging (fMRI) study we showed that the cerebral activity in the primary auditory cortex during the experience of internal voices is comparable to the activation caused by external auditory stimuli [14]. We propose that this dysfunctional activity may account for the belief of externality of the voices the patients hear, which may be further supported by the measurement of auditory evoked potentials in EEG, where we found higher EEG activity in left temporal regions and lower AEP amplitudes during AVH compared to phases without AVH [15]. Thus, we suggested that pre-activation of the primary auditory cortex competes for processing resources with the external stimuli [15] and that these alterations might be the microstructural basis responsible for the dysfunctional primary auditory cortex activity. A processing failure in Wernicke's area may promote a disturbed feedback loop reflected in altered connectivity of Broca's and Wernicke's area [16] and a pathological activation of Heschl's gyrus.

However, EEG and MRI cannot assess the molecular basis of schizophrenia and psychotic symptoms, and our understanding of the molecular changes in schizophrenia is essentially based on the observed effects of psychoactive drugs. MRS offers a tool to measure neurochemical alterations, here with respect to AVH. With MRS, the chemical composition of cerebral tissue is determined non-invasively by measuring the magnetic resonance signal of hydrogen in specific metabolites [1,17]. The metabolite N-acetyl-aspartate (NAA) is considered a marker of neuronal viability and/or integrity. NAA concentrations are reduced in affected areas in neurodegenerative diseases [18] and correlate with cognitive performance [19]. With respect to schizophrenia, MRS may allow to disentangle the meaning of white matter and gray matter volume reductions which may stand for neuronal loss or dysfunction [20]. Although there are inconsistencies in the literature [21], most previous research found that in schizophrenia NAA is decreased in frontal and temporal regions and in the thalamus [22]. NAA concentrations in the thalamus and the

duration of positive symptoms were negatively correlated in schizophrenia patients [23]. Martinez-Grandoz et al. found a decreased ratio of NAA to choline ratio in the thalamus of schizophrenia patients compared to healthy controls and in the right thalamus of hallucinating patients compared to not hallucinating patients and controls [24]. In addition, hippocampal NAA levels have been reported to be decreased in hallucinating patients [25].

The metabolite myo-inositol (mI) has been shown to be present in glial but not neuronal cells in vitro [26] and since gliosis may lead to increased mI concentrations, mI has been found to be increased in neurodegenerative diseases such as Alzheimer's disease [27]. With respect to schizophrenia, an increase in mI concentrations compared to healthy controls would be compatible with the neurodegenerative hypothesis but has not been found as of yet [28]. Instead, most studies have shown that mI levels in schizophrenia are largely unaltered (as reviewed in [29]). In addition, there do not seem to be any published investigations into potential relations of mI content and presence of AVH, so investigations of this metabolite in AVH can be done only in an exploratory fashion.

In the current study, we used a verbal fluency (VF) task in fMRI to functionally define speech related ROIs (Broca's and Wernicke's area) in each subject for a subsequent neurochemical analysis with MRS in two patients groups (AH: schizophrenia patients with AVH; NH: schizophrenia patients without AVH for the last 12 months) and a healthy control group (HC). In addition, left and right Heschl's gyrus (HG) were defined anatomically. Thus, the four target ROIs for MRS were Broca's and Wernicke's area (both on the left side) and left and right HG. Since Broca's area lies in the frontal part of the brain, where optimizing the magnetic field homogeneity is challenging, but the other 3 areas are in relative proximity with less stringent difficulties for field homogenization, the MRS signal acquisition was split into two parts. First, Broca's area was investigated by single voxel MRS, then the other 3 ROIs were examined by SI of a single slice, the position and orientation of which was defined by these 3 ROIs.

In accordance with previous findings of NAA being decreased in schizophrenia in frontal and temporal regions and in the thalamus [22,24], we hypothesized to find a relative reduction of NAA in the

functionally defined Wernicke's area in hallucinating schizophrenia patients compared to patients without hallucinations and controls. In an additional exploratory analysis, we tested for metabolic changes in further metabolites, like mI, choline (Cho) and glutamate (Glu) or glutamate plus glutamine (Glx).

Methods and materials

Participants

The investigation was conducted in accordance with the Declaration of Helsinki and approved by the local ethics committee (Kantonale Ethikkommission Bern). To ensure informed consent, the investigator explained the aims and procedure of the study to the potential subject verbally, providing all important information. Then the potential subject was given written instructions as well as time to ask any questions. Only then the decision whether or not to participate in the study was made, and subjects provided written consent to participate in the study.

Three groups were investigated in the current study: AH (n=12), NH (n=8), and HC (n=11). General inclusion criteria for patients were: aged between 25 and 50 years, right handedness, ICD-10 diagnostic criteria for schizophrenia or schizo-affective disorder, a history for schizophrenia of more than 5 years, no substance use other than nicotine, the ability to give written informed consent. AH could but did not have to perceive AVH at the time of the measurement. According to their anamnesis (patients' files and clinical interview) these patients perceived AVH in most of their acute exacerbations (90 %). NH did not experience AVH at the time of investigation nor in the previous 12 months and did not perceive AVH in most of their exacerbations (less than 10 %). Individual psychopathology was assessed in a clinical interview involving the assessment of the Positive and Negative Syndrome Scale (PANSS) [31]. All 3 groups were age- and gender-matched. Inclusion criteria for HC were: no current or previous neurological or psychiatric disorder, no severe somatic-medical disorder, no substance use other than nicotine, the ability to give written informed consent, and – valid for all subjects - no contraindications for MRI.

Statistical analysis of behavioral data

PANSS scores were compared between AH and NH using two-sample t-tests. In addition, positive symptoms within the PANSS scores were compared using two-sample t-tests. The statistical threshold for these contrasts was set at $p < 0.05$, two-tailed. SAS 9.2 (SAS Institute Inc., Cary, NC, USA) was used for all analyses. The means of the data are reported with their standard deviations.

MRI examinations

All examinations were performed at 3 T on a Siemens Trio scanner using a 12-channel receive head coil. MRI included multiple quick localizer images to check for gross patient motion, and the manufacturer's auto-align procedure (referencing the individual brain to a prescription in an atlas brain) was used to ensure consistency in scan prescription (used primarily for slice orientations in MRI). For blood oxygen level dependent (BOLD)-sensitive functional MRI (fMRI), thirty-five axial slices (slice thickness = 3 mm, interslice distance = 0.75 mm), parallel to the plane crossing the anterior and posterior commissure (AC/PC) and covering the whole supratentorial brain, were acquired with a 2D echo planar imaging sequence (repetition time/echo time [TR/TE] = 2.66 s/30 ms, flip angle [FA] = 90°, field of view [FOV] = 192 × 192 mm^2, matrix = 64 × 64, voxel size = 3 × 3 × 3 mm^3, with 0.75 mm gap between slices). 80 scans were measured. For morphologic evaluation and brain segmentation, a 3-D gradient echo sequence (mdeft [31], TR/TE/TI 7.92 ms/2.48ms/910ms, FA 16°) was measured initially providing 176 sagittal slices with a nominal 1 mm^3 voxel size, 256 mm × 224 mm field of view (FoV) and a matrix size of 256 × 192 pixel resolution.

fMRI task and analysis

Prior to MRS, but in the same session all subjects performed a VF language task that has been shown to reliably activate Wernicke's and Broca's area (Figure 1). During the scan, subjects were exposed to visual stimuli on an LCD-Monitor eyeglass (Resonance Technology, Inc. Northridge, USA). Conditions alternated between a rest (R) and a VF condition, each lasting 30 s. The task started with the R-condition

(gray screen with a fixation cross). In the VF condition, a letter was displayed and subjects were instructed to silently produce as many verbs starting with the shown letter.

At the scanner console, a one-sample t-test was computed after motion correction to reveal brain regions that were significantly activated during the VF-blocks compared to the R-blocks. The resulting statistical parametric maps were used to identify Broca's area (left side) and Wernicke's areas (left side) for the MRS scan in each subject individually. Left and right HG were anatomically identified in each subject. We used the stringent criterion of $p < 0.001$ (uncorrected) for the threshold value. However, we decreased the threshold to $p < 0.01$ in subjects where not enough activity was present near the expected ROI.

Offline MRI and fMRI data analysis was realized using Matlab (Matlab version 7, release 2010b; The MathWorks, Inc., Natick, MA, USA), SPM8 (Wellcome Trust Centre for Imaging, London, England; www.fil.ion.ucl.ac.uk/spm8), and the toolbox aslm [32].

Post-hoc preprocessing of the images included slice-scan time correction, removing low-frequency drifts, 3-D motion detection, and spatial smoothing with a Gaussian Kernel (8-mm full width at half maximum, FWHM). Co-registration of the 2-D functional to the 3-D structural images was performed. The anatomical and functional data sets were then transformed into normalized MNI space. Each subject's individual stimulation condition was modeled by a boxcar function that was convolved with a double gamma hemodynamic response function to create the design matrix for the general linear model (GLM). The design matrix included motion parameters as covariates. A contrast was computed by calculating a one-sample t-test on the difference between VF-predictors and R-predictors. The statistical threshold for these analyses was set at a cluster-level threshold of $p < 0.05$, whole-brain corrected for familywise error.

ROI placement: interindividual differences in location of activation

To document interindividual variation of the activation areas and to test whether a functional definition of Broca's and Wernicke's area had indeed been necessary, activation foci were determined in each subject in postprocessing and compared to the coordinates of a likely anatomically prescribed ROI (pars

opercularis and pars triangularis of the left inferior frontal gyrus for Broca'a area and the posterior section of the left superior temporal gyrus for Wernicke's area) in native space. However from these areas no MRS data were acquired except if these overlapped with the functional definition.

MRS data acquisition

SV MRS using an optimized implementation of short TE Point-RESolved Spectroscopy (PRESS) [33] was performed in the frontal lobe centered in the functionally defined Broca's area (TE 22 ms, TR 3 s, 16 step phase rotation, single slice outer volume suppression, 128 scans, 14x14x14 mm^3, 2000 Hz bandwidth, 2048 time points, water presaturation; a minimum TE of 22 ms realized by using a shorter excitation pulse and shorter but stronger gradient pulses compared with the product PRESS sequence that allowed a minimum TE of 30 ms). If the functionally defined ROI happened to lie very close to the skull, the ROI for SV MRS was rotated and moved slightly away from the skull such as to best avoid subcutaneous lipids, but to still include the fMRI-proven active area. In addition, to optimize spectral quality for this ROI close to the skull, it was important to set the transmit frequency at the center of the spectrum (2.5 ppm, preventing unnecessarily large relative chemical shift induced shifts of the excited ROI), to use a B1 map in order to use proper flip angles with best ROI profiles and to verify that the gradient polarity of the slice-selective pulses was such that the effective ROI for lipid resonances was shifted away from the skull.

2D short TE PRESS SI (TE 30 ms, TR 1.7 s, 12 outer volume suppression slices, 4 averages, weighted acquisition, matrix 20x20 with circular k-space sampling, FOV 160 mm, thickness 15 mm, individually adjusted volume, 1200 Hz bandwidth, 1024 time points, water presaturation) was used to obtain spectra from Wernicke's area, and the left and right HG. The plane prescription parameters were calculated from the coordinates of these three ROIs obtained from the anatomic and functional maps. Fast spin echo MRI's were obtained coplanar and orthogonal to the SI slice to individually define the size of the

localization area, including the ROI's with extra space to prevent edge effects in excitation, and to place four outer volume saturation slices to prevent fold in of lipid signals from subcutaneous fat.

MRS data processing

SV data was processed using jMRUI [34] in version 5.0. Individually stored acquisitions were averaged and the residual water signal was removed by Henkel Lankos Singular Value Decomposition (HLSVD) before modeling with QUEST [35]. The following metabolites were included as basis spectra obtained from spectral simulation also using jMRUI (NMRscopeA, pulse sequence with ideal RF pulses assumed): NAA, Ch (as 1 : 2 mixture of phosphocholine : glycerophosphocholine), Cr (as 1 : 1 mixture of creatine and phosphocreatine), mI, Glu, glutamine, aspartate, glutathione, and taurine. In addition, an experimental macromolecule baseline, obtained with metabolite nulling in GM, was included to model the baseline. Details on the acquisition parameters for the macromolecular baseline using metabolite nulling can be found in [36]. (HLSVD was used to eliminate the residual water from the metabolite nulled spectrum and HLSVD was also used to parameterize the baseline to be used as noiseless basis spectrum, which in addition had been narrowed by the expected Lorentzian linewidth in order to be compatible with the QUEST algorithm that enforeced identical linewidths for all base spectra, including the macromolecular baseline). The QUEST algorithm with inbase background handling and 6 truncated points was used to model further baseline features. Area estimates with fitting errors above 50 % were excluded from further analysis.

SI measurements yielded multiple spectra within the PRESS localization volume and processing included spatial apodization and spatial zerofilling to 32x32. The spectra were assigned to brain structures using co-registered MRIs for anatomical guidance.

In order to relate metabolite values to WM, GM and CSF voxel contributions, tissue segmentation was used. GM, WM, and CSF composition of all examined ROIs was approximated from the segmented mdeft

images neglecting the exact ROI shape (given that the exact cuboid profile and SI ROI size is unknown, it was approximated by a sphere.)

Spectra from a single SI voxel of the SI dataset were selected at the activated Wernicke's area, and the left and right HG. For Wernicke's area, the voxel closest to the center of activation was selected. Only in very few cases, when the center of activation was located at the border between ROIs, a half-voxel shift was performed. The spectra were processed separately, also using jMRUI. The data were analyzed using AQSES as quantitation method [37]. The same metabolites as for the SV spectra were included as basis spectra (NAA, Ch, Cr, mI, Glu, glutamine, aspartate, glutathione, and taurine) and also the macromolecule baseline was included. In contrast to the SV spectra analysis, the residual water was not removed, but the fit restricted to a frequency region upfield from water. While SV spectra were fitted assuming common damping for all metabolites (i.e. same line broadening), line widths of resonances were fitted individually for SI spectra.

Results for both, SV and SI spectra were expressed as ratios with regard to creatine without any relaxation corrections. Linewidth and SNR values were obtained to control for potential systematic differences between subject groups.

Statistical analysis of MRS data

MRS data of each metabolite of interest was pooled to comprise data for each region of interest (ROI) measured with SI. Wernicke's area, left and right HG were defined as ROI, as well as Broca's area, which, however, had been separately assessed in SV MRS. Metabolites of prime interest were NAA and mI. To account for the repeated measurements in the same subjects, full factorial linear mixed models with restricted maximum likelihood estimations were used to examine the effects of various outcome measures. Schwarz's Bayesian criteria were used to determine the best fitting covariance structure for each set of measures because the typical compound symmetry approach used by ANOVA did not provide the appropriate structure for the data. The effects of group (3 levels; AH, NH, HC), region (3 levels;

Wernicke's area, left and right HG) and their interaction on the NAA levels and mI levels were assessed with linear mixed models with an autoregressive covariance structure. The effect of group on Broca's area was assessed in a separate model because of the different method of measurement. Additional computations included the effect of psychopathology (PANSS total, positive, negative scores) for each ROI to assess the association of NAA and mI levels and psychopathology. All models included the percentage of GM as covariate of no interest. Subject numbers were included as additional effects to account for the repeated measurements in the SI models. Post hoc t-tests involved a Tukey-correction for multiple comparisons. The significance thresholds for these contrasts were set at alpha = 0.05, two-tailed. SAS 9.2 (SAS Institute Inc., Cary, NC, USA) was used for all analyses. The means of the data are reported with their associated standard deviations.

Results

Behavioral data

Sample characteristics with mean values and standard deviations are detailed in Table 1. The mean levels of psychopathology as measured with the PANSS total score in AH and NH were 67.4 ± 19.0 and 53.4 ± 8.5, respectively ($t [1, 18] = 1.95$, $p = 0.07$). The mean levels of positive symptoms within the PANSS in AH and NH were 22.3 ± 6.0 and 13.5 ± 3.2, respectively ($t [1, 18] = 3.83$, $p = 0.001$). There was no significant difference in medication levels in terms of chlorpromazine equivalents (CPZE) between AH and NH. AH were medicated with 638 ± 541 mg CPZE, NH with 2389 ± 4111 mg CPZE ($p = 0.16$).

fMRI data: group analysis and ROI placement

Typical activation maps from 2 AH subjects are presented in Figure 1 illustrating the interindividual spatial variance in activated Broca's area in native space.

Group analysis

In 3 subjects (2 AH, 1 NH), the ROIs had to be defined anatomically because of a lack of activation near the expected areas of Broca's or Wernicke's area. In the remaining 28 subjects, the mean distances in native space between the individually defined ROIs using fMRI and a corresponding anatomical definition were 12.4 ± 6.1 mm (range: 2.7 – 36.1 mm) for Broca'a area and 16.8 ± 6.2 mm (range: 4.5 – 26.4 mm) for Wernicke's area, respectively.

MRS data: general results

Data quality was acceptable in almost all subjects (one SV spectrum from Broca was excluded because of strong motion effects along with 6 SI spectra (4 spectra from Wernicke's area (2 from AH and 2 from NH) and 2 spectra from right HG of AH). Typical SI spectra for the HG and Wernicke's ROIs are reproduced in Figure 2, while group averaged spectra are given for Broca's area in Figure 3. Mean line widths and SNR values did not differ significantly between groups in any of the ROIs. Overall, mean (± 1 SD) line widths for Broca, Wernicke's, HG left and HG right were 6.9±2.2 Hz, 9.6±1.9Hz, 9.1±1.8Hz, 9.3±1.8Hz, respectively. Individual linewidths were different for NAA in HG left and in HG right between HC and NH (HG left: 8.2±2.1Hz vs. 10.2±1.4Hz, p = 0.03; HG right: 8.8±2.2Hz vs. 10.8±1.6Hz, p = 0.03, both without corrections for multiple comparisons), and for Cr in HG right between AH and NH (10.3±1.7Hz vs. 11.9±1.2Hz, p = 0.03). None of these differences were significant after correcting for multiple comparisons.

ROI composition in terms of WM and GM percentage was not different between groups for any ROI. Mean GM percentages as determined by segmentation ranged between 54±9% in HG and 61±9% in Wernicke's area.

MRS data: NAA/Cr

The mean NAA ratios with standard errors for each region and each group are shown in Figure 4a. In Broca's area, there was a significant group effect ($F [2, 26] = 3.94$, $p = 0.03$) that was attributable to lower

levels in NH compared to HC (t [1, 26] = 2.8, p = 0.02). For the NAA levels measured with SI, there was an effect of region at trend level (F [2, 49] = 2.83, p = 0.07). Post-hoc tests revealed that NAA/Cr was higher at a trend level in left HG compared to Wernicke's area (t [1, 49] = 2.27, p = 0.07). In the SI data, there was no significant group-effect (F [2, 49] = 1.21, p = 0.31), nor a significant group-by-region interaction (F [4, 49] = 1.81, p = 0.14).

Correlation analysis showed that there was a positive association of the NAA levels in the left HG with the total PANSS scores (F [1, 15] = 5.92, p = 0.03; Figure 4C) and the negative PANSS scores (F [1, 15] = 10.36, p = 0.006; Figure 4C), but no significant relation with the positive PANSS scores.

MRS data: mI/Cr

The mean mI levels with standard errors for each region and group are shown in Figure 4b. mI levels measured with SI did not show a significant effect of region (F [2, 49] = 0.39, p = 0.68), group (F [2, 49] = 1.2, p = 0.31) nor group-by-region interaction (F [4, 49] = 0.74, p = 0.57).

In Broca's area, there was no group-effect (F [2, 26] = 0.03, p = 0.97) either, but a negative association of the mI levels with the total PANSS scores (F [1, 15] = 9.35, p = 0.008; Figure 4D) and a trend towards significance in a negative association of mI levels and the negative PANSS scores (F [1, 15] = 3.54, p = 0.08; Figure 4D).

MRS data: further metabolites

In an exploratory fashion, metabolites beyond those involved in our original hypotheses (NAA and mI) were evaluated. The analysis was restricted to those metabolites that had Cramer Rao uncertainties in individual spectra from Broca's below 50 % for the large majority of subjects. These included total choline (all ROIs), glutamate (SV), glutamate plus glutamine (Glx, SI), and taurine (SV). None of these metabolites (as ratios vs. Cr) showed any significant group effects.

Discussion

This study assessed possible neurochemical and functional differences between patients with AVH, patients that had not had AVH for the last 12 months, and healthy control subjects. Four regions within the cerebral language system were chosen for a neurochemical analysis. However, it is well established that there are crucial neuroanatomical variations across subjects in terms of location and function [2,3] that persist to a substantial degree after normalization into a common anatomical space [33,38,39,40] and that are particularly relevant with respect to the functional specification of cerebral language regions [41]. A possible reason for inconsistencies between functional imaging studies of the language system is the application of group analyses in most of the investigations, which might have resulted in an underestimation of individual differences [41]. In addition, it has been shown that only sensory processes of a rather low-level can be linked reliably to macroanatomy, and functional variability increases systematically if cognitive processes of higher order are involved [42]. Thus, the current study used fMRI to functionally define two ROIs of the cerebral language system for subsequent MRS analysis. This approach allowed taking interindividual variability of functional areas within the language system into account. It is well known that two of the assessed ROIs, Broca's area and Wernicke's area, show a considerable amount of variation among healthy subjects. We therefore compared the functional loci with the most likely anatomically defined loci in each subject and found that the mean distance of the functional and the corresponding anatomical loci for Broca'a area (12 mm) and Wernicke's area (17 mm) was in the same range as in previous studies and larger or of the same size as the individual activation areas. If these ROIs had been prescribed anatomically for the MRS investigation the investigated volume (14×14×14 mm) would only rarely have covered most of the functional activation area. Inversely, to guarantee the functional activity focus to be within the MRS ROI, one would have to increase the voxel size considerably, which would come with an enormous dilution effect with areas of different function and also with more white matter. However, the current study could not statistically test the difference between neurochemical results in functionally defined language regions (Broca's area and Wernicke's area) versus

those that could have been obtained in the corresponding regions defined by anatomical landmarks since this would have substantially prolonged the study procedure in a population of particularly vulnerable patients with limited resilience and compliance. The spectral quality was quite good for all spectra and ROIs, even though one ROI (Broca's) was in a frontal location that is known to show significant B_0 field inhomogeneities. Hence, the strategy to split the investigations into a single voxel examination in the 'difficult' frontal brain and a spectroscopic imaging examination for those brain areas with a more homogeneous field distribution paid out leading to a very limited number of rejected spectra. In addition to reduced field homogeneity and broader lines, a multi-slice or volumetric SI investigation would have been longer and probably subject to more motion-related artifacts.

In spite of the functional definition of two of the MRS ROI's, no neurochemical imbalances were found to support the hypotheses of a relative reduction of NAA in Wernicke's area in hallucinating schizophrenia patients compared to patients without hallucinations and controls. Instead, NAA ratios as measured with SV in Broca's area revealed a group-difference between NH and HC that was attributable to lower NAA ratios in NH. In addition, a regional difference was found for NAA ratios as measured by SI, with higher levels in left HG compared to Wernicke's area across the whole sample, but no group-differences. In terms of correlations between neurochemical levels and patients' symptoms scores, there were positive associations between the total as well as the negative PANSS scores and the NAA levels in left HG for all patients (AH, NH). Both patient groups also showed negative associations of psychopathological symptoms as measured with the total and the negative PANSS score and mI levels in Broca's area.

The current study used a combined approach to measure functional and neurochemical parameters in the same subjects. A verbal fluency task was used to functionally define ROIs in each subject individually that were then assessed with MRS. This approach tries to overcome some of the inherent limitations that have been discussed with respect to an anatomical definition of ROIs. In addition, the combination allows testing conflicting hypotheses in the same subjects [43]. However, our neurochemical results are largely conflicting with what we had hypothesized a priori. The assessment of neurochemistry within functionally

and anatomically defined ROIs revealed a group-difference in NAA concentrations in Broca's area only, where NH showed lower concentrations compared to HC. This finding is in contrast to our a-priori hypothesis that there would be a reduction in NAA concentrations in Wernicke's area of AH compared to NH and HC. Overall, we found decreased NAA only in Broca's area in our patient population, which is also in contrast to most of the current literature where generally it is found (though more at long echo time, see below) that NAA in schizophrenia is decreased in frontal and temporal regions and in the thalamus [22,24,25], although there are a number of conflicting results as summed up in a meta-analysis by Steen et al. [21]. Specifically, there is some evidence that the effects on NAA levels in schizophrenia patients are dependent on antipsychotic medications in that typical antipsychotic medication is associated with lower NAA levels and atypical neuroleptics are possibly associated with less of a reduction [44,45,46] (reviewed in [21]) or even elevated NAA concentrations [47]. Furthermore, Bracken et al. recently concluded that the apparent NAA deficit in schizophrenia might mainly be caused by changes in the transverse relaxation time of NAA, rather than the NAA content, which would explain why NAA deficits are primarily reported for studies using long echo times [48]. In addition, the detected positive correlation between NAA in left HG and psychopathology in all patients is also in contrast with the hypothesis that schizophrenia might be a progressive neurodegenerative disease involving left temporal regions. However, we have recently suggested that a gray matter deficit found in language and primary auditory areas in AH compared to NH may reflect a loss of neuropil (compatible with experience-dependent modeling of the cortical column architecture) rather than neuronal cell degeneration [49]. In addition, all of the above interpretations of NAA levels solely base on the putative role of NAA as a marker for the density of healthy neurons, but NAA levels may also reflect mitochondrial activity, osmolytic balance and has other putative roles, which led K.K. Bhakoo recently to conclude in his review on NAA metabolism that "… its function/s remains an enigma" [50].

With respect to mI, the values in the collapsed patients group showed a negative association with the psychopathology in Broca's area. This result is incompatible with a neurodegenerative hypothesis of

schizophrenia [28] but may indicate either decreased glial content or more dysfunctional glia in more severe disease [30].

Several limitations should be noted. Our sample size was small, particularly with respect to the NH group. Remarkably, it has been noted that this confound of a small sample size is particularly evident in previous studies using the categorical distinction between AH and NH [43] (though we did not even find a trend to support the original hypothesis of a relative reduction of NAA in Wernicke's area in hallucinating schizophrenia patients). To evaluate the designed and actual power of the study, we calculated the minimal detectable difference for the sample sizes of AH and NH in the NAA levels of Wernicke's area for two cases; first for the ideal case with the variability as found in the healthy control group and full group size, and second for the actual situation as found post hoc with the larger variability found in the patient groups. At 80 percent probability and two-sided 0.05 significance level the study would have detected a difference in NAA levels in Wernicke's area between AH and NH of 0.23 units (i.e., about 21% of the normal level) for the ideal case and 0.48 units (about 44% of the normal level) for the actual situation. However, the actually measured difference in the mean NAA levels in AH and NH was 0.09 units only. In addition, we studied patients on medication, which means that medication confounds of antipsychotics have to be taken into account. However, we tried to take this possible variable into account by matching as well as possible AH and NH by their CPZE, so there was no significant difference in CPZE in AH and NH. Furthermore, the VF-task during fMRI did not include any behavioral measure, so we do not have a control for the subjects' compliance. We also had to weaken our initial threshold defining a NH patient as someone who had not had experienced hallucinations in the past 5 years to a more lenient criterion of 12 months without AVH. In addition, we did not assess the psychopathology (i.e. AVH) during the MRI scan which means that patients may have experienced AVH during the MRI procedure. Instead, AVH and other psychopathological symptoms were assessed before the MRI procedure. In terms of methodology, it should be noted that creatine levels were taken for concentration reference, as used by a large fraction of the published studies on schizophrenia. The use of CSF-corrected

water signal would be a better suited reference, but is not available for this and most SI studies in general because of time reasons. However, given the information from both large meta-analyses [21,22], there is only very limited reason to suspect relevant alterations of creatine content in schizophrenia patients, though there is some speculation of increased metabolism with associated increased creatine levels in temporal areas [22].

In conclusion, the present study supports the neurodegenerative hypothesis of schizophrenia only in a frontal region whereas the results obtained from temporal regions do not support this hypothesis and are in contrast to the majority of previous studies that have found NAA reductions in temporal and frontal regions. In addition, the difference we documented between a functional and an anatomical definition of regions to be studied by MRS suggests that future research should test the hypothesis that a functional definition of language regions is needed if neurochemical imbalances are expected to be restricted to functional foci.

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Table and Figure legends

Table 1

Subject characteristics of schizophrenia patients with persistent auditory hallucinations (AH; n = 12), schizophrenia patients without persistent auditory hallucinations (NH; n = 8), and healthy controls (HC; n = 11). Mean values and standard deviations are reported. Abbreviations: f = female, m = male; PANSS = Positive and Negative Syndrome Scale; y = years; mo = months; CPZE = Chlorpromazine equivalent.

Figure 1

Functional activity from a verbal fluency task overlaid on axial and coronal views of structural images as determined in 2 schizophrenia patients with AVH. The spatial variability of the activation in the left inferior frontal region demonstrates the individual location of Broca's area. The images are plotted in native space.

Figure 2

Sample spectra from SI shown as obtained from a AH patient to demonstrate the setup for data acquisition and to show data quality in a single subject. The anatomical MR image shows and was prescribed to contain the targeted ROIs, Heschl's gyri (HG left, HG right) and Wernicke's area (W). The image also shows the position and size of the localized SI PRESS-box (in blue) and the field of view of the SI data set. MR signal visible in the anatomic images outside the head originates from the goggles used to display the functional task.

Figure 3

Group-averaged single voxel spectra from Broca's area, which had been defined by a preceding fMRI scan. To ease visual comparison, the spectra have been matched in linewidth and in intensity to reach equal width and peak intensity for the creatine methyl peak.

Figure 4

A. The mean NAA levels with standard errors for each region and group (AH, NH, HC). **B.** The mean mI levels with standard errors for each region and group. **C.** A positive association of the NAA ratio in the left Heschl and the total PANSS score ($r = 0.59$, $p = 0.007$) and the negative PANSS score ($r = 0.6$, $p = 0.006$). **D.** A negative association of the mI ratios in Broca's area and the total PANSS score ($r = -0.52$, $p = 0.02$) and a trend towards significance in the negative association of mI levels and the negative PANSS score ($r = -0.44$, $p = 0.06$).

Table 1

	AH (n=12)	**NH (n=8)**	**HC (n=11)**	**p-Value**
Gender, m/f	7/5	7/1	6/5	0.35
Age, y	42.3 (9.7)	41.2 (14.7)	43.1 (10.4)	0.94
Age of onset, y	25.9 (7.8)	26.2 (7.0)	n.a.	0.92
Duration of illness, mo	161.0 (80.3)	157.5 (127.2)	n.a.	0.94
PANSS total	67.4 (19.0)	53.4 (8.5)	n.a.	0.07
PANSS positive	22.3 (6.0)	13.5 (3.2)	n.a.	**0.001**
PANSS negative	16.2 (8.0)	16.7 (4.5)	n.a.	0.87
CPZE, mg	638 (541)	2389 (4111)	n.a.	0.16

Figure 1

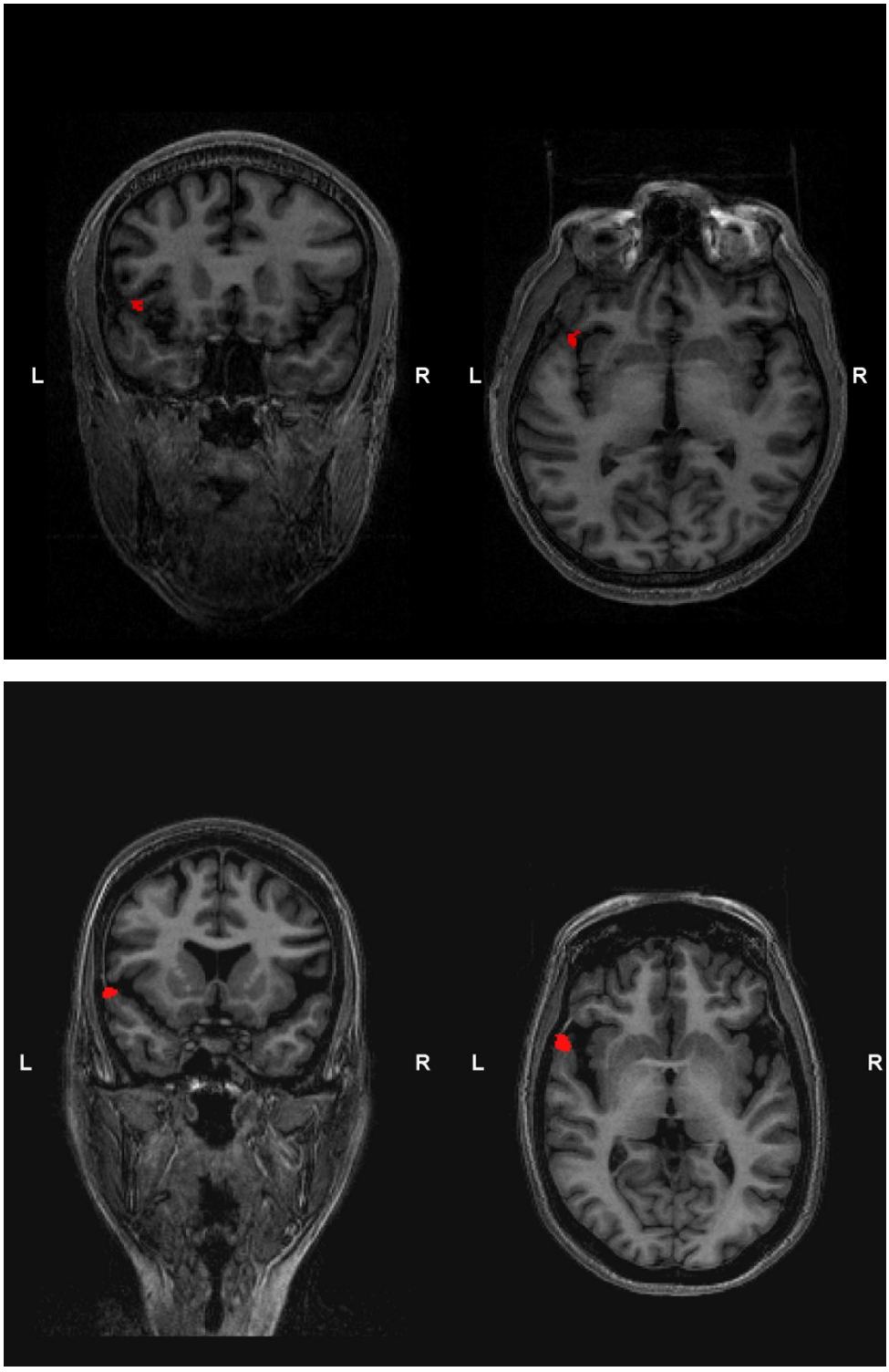

Figure 2

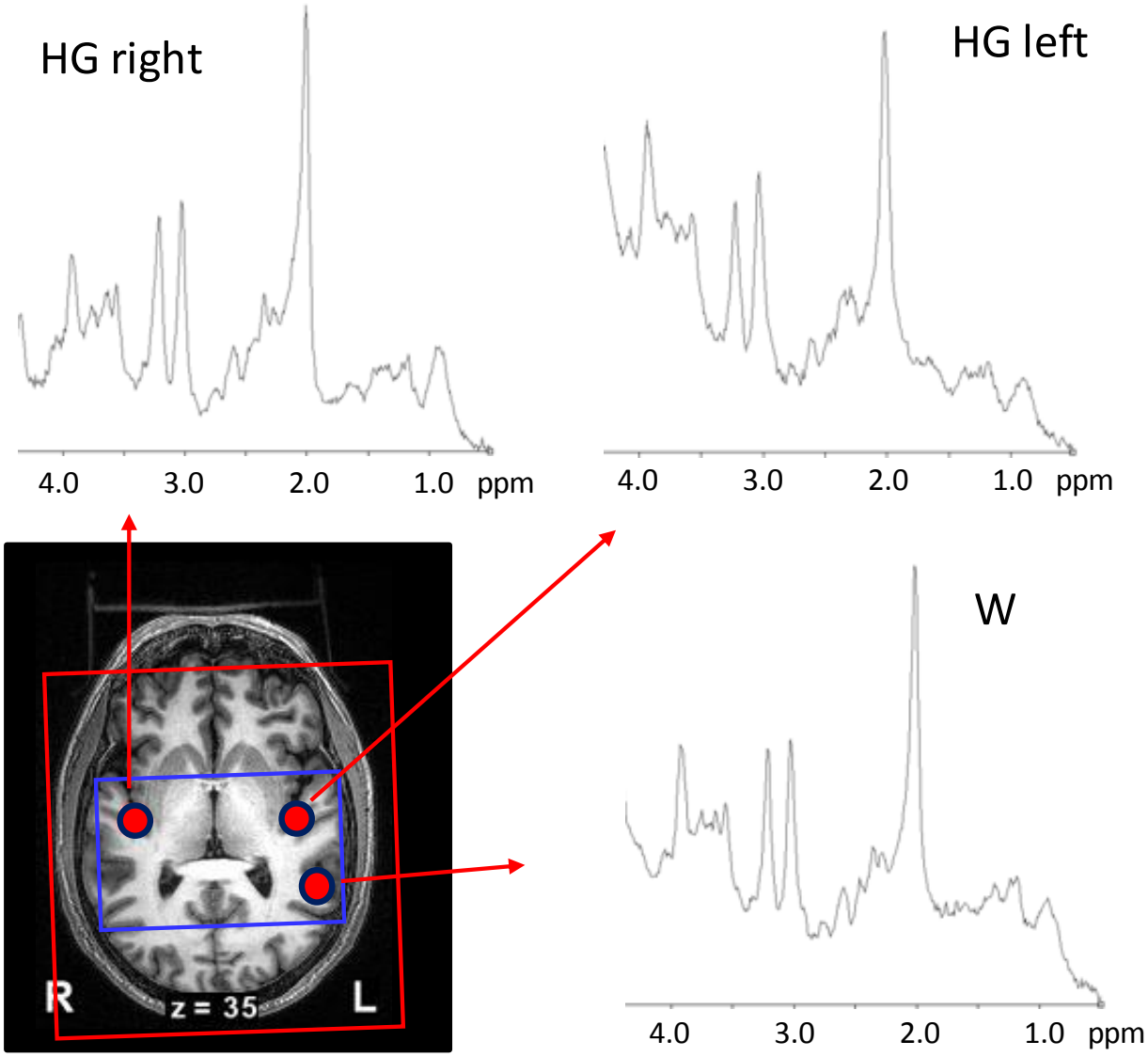
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Figure 3

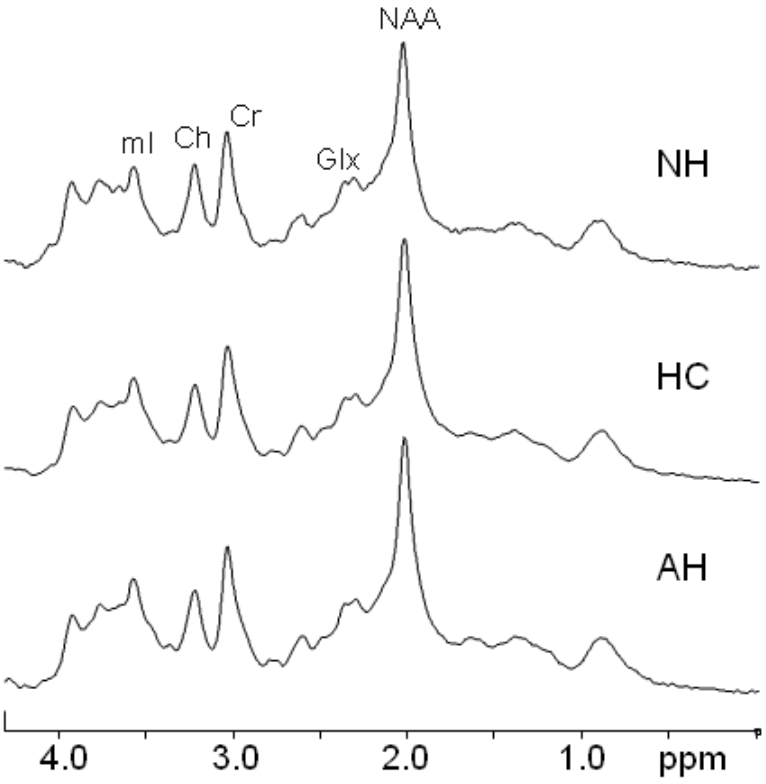
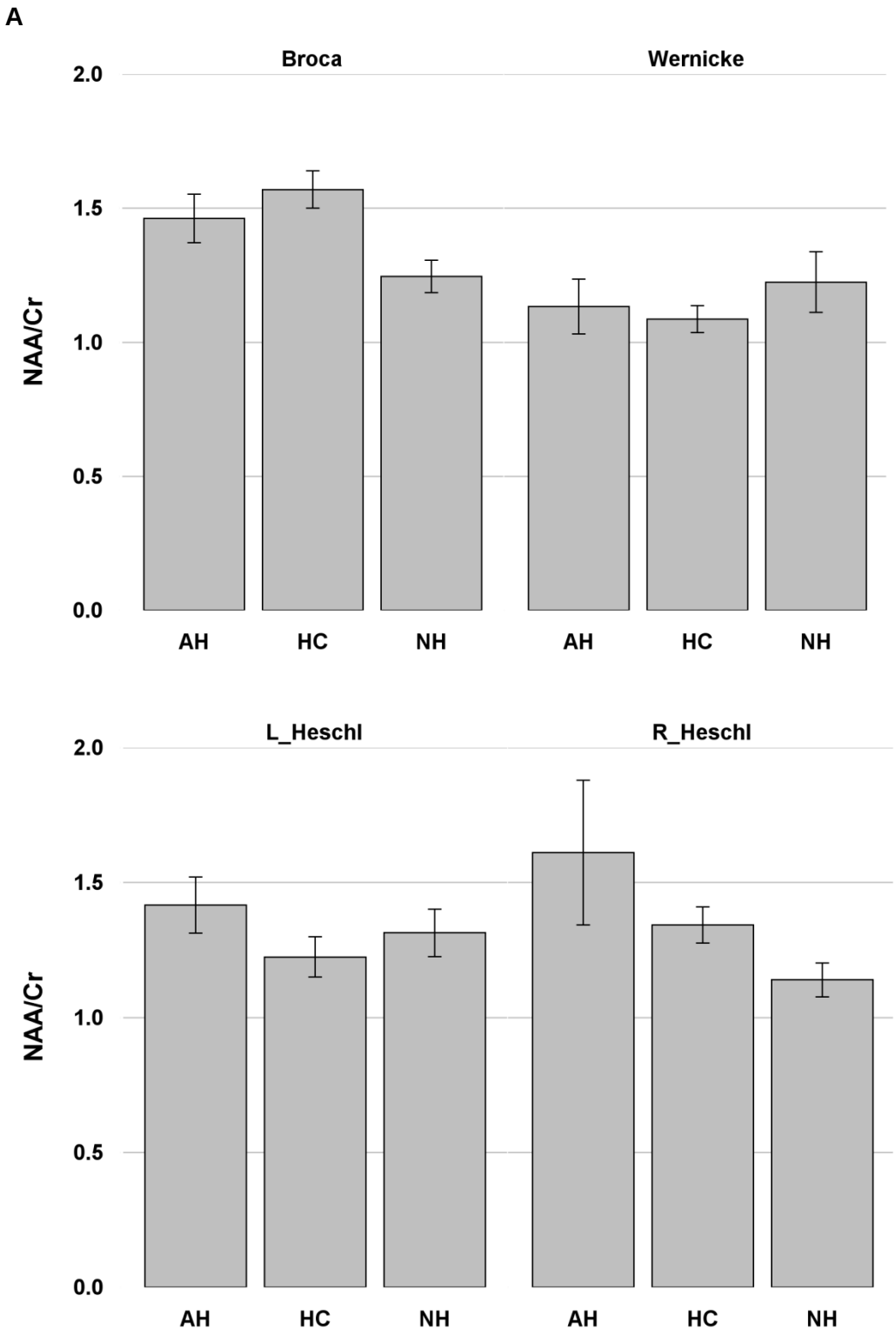


Figure 4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Broca
Wernicke
NAA/Cr
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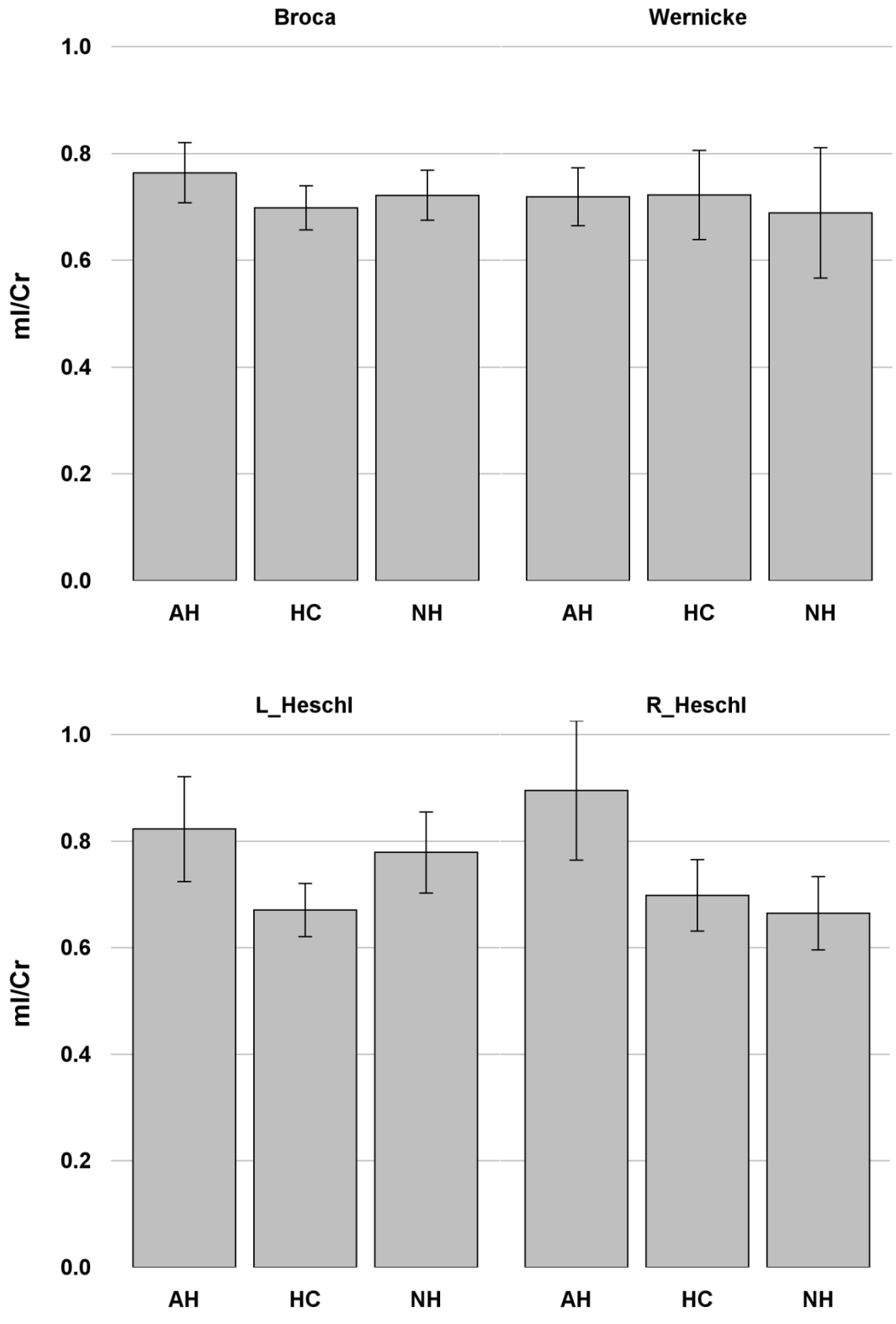
1.5
1.0
0.5
0.0
AH
HC
NH
AH
HC
NH
L_Heschl
R_Heschl
NAA/Cr
2.0
1.5
1.0
0.5
0.0
AH
HC
NH
AH
HC
NH

B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0.4
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ml/Cr
AH
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NH
AH
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NH
L_Heschl
R_Heschl
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ml/Cr
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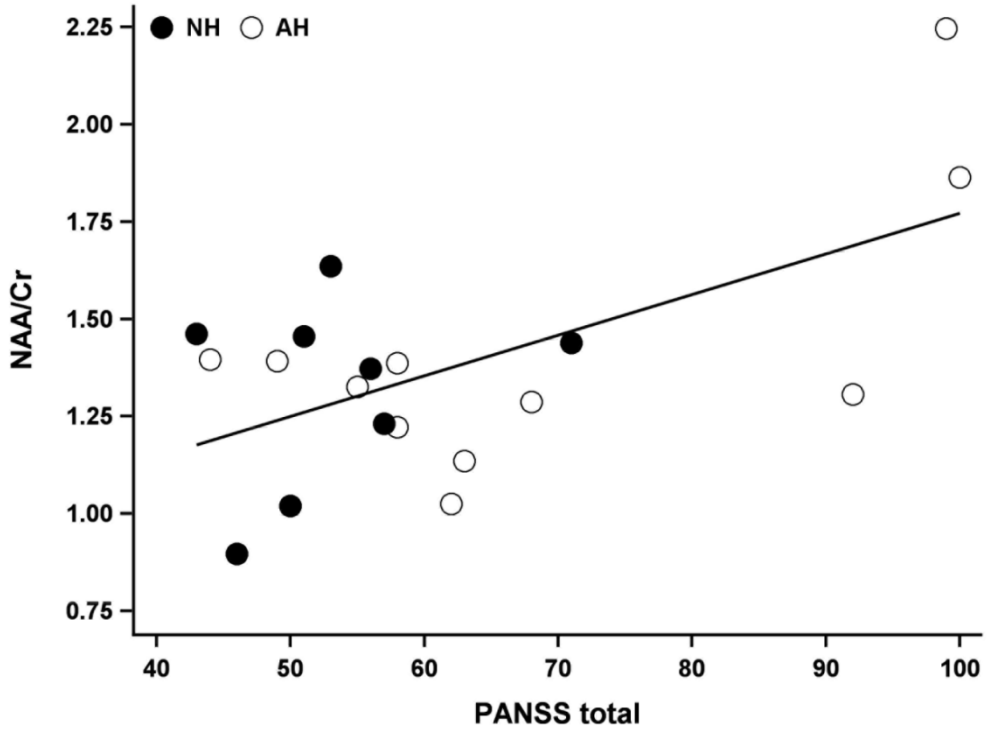
HC
NH
AH
HC
NH

C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AH
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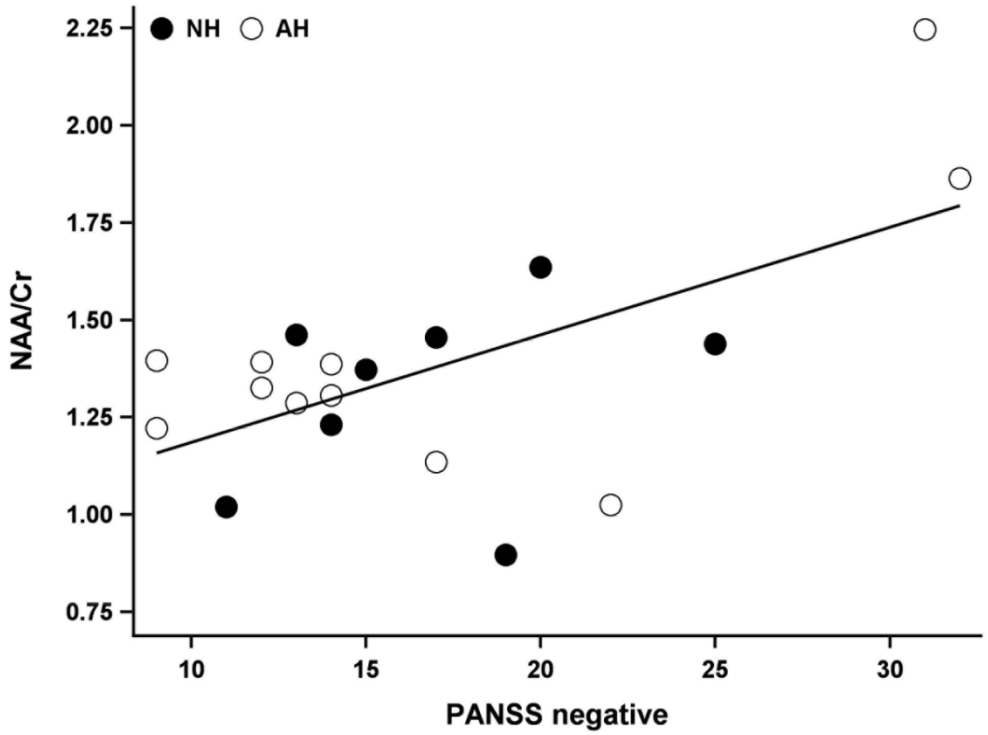
1.00
0.75
NAA/Cr
40
50
60
70
80
90
100
PANSS total


NH
AH
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1.00
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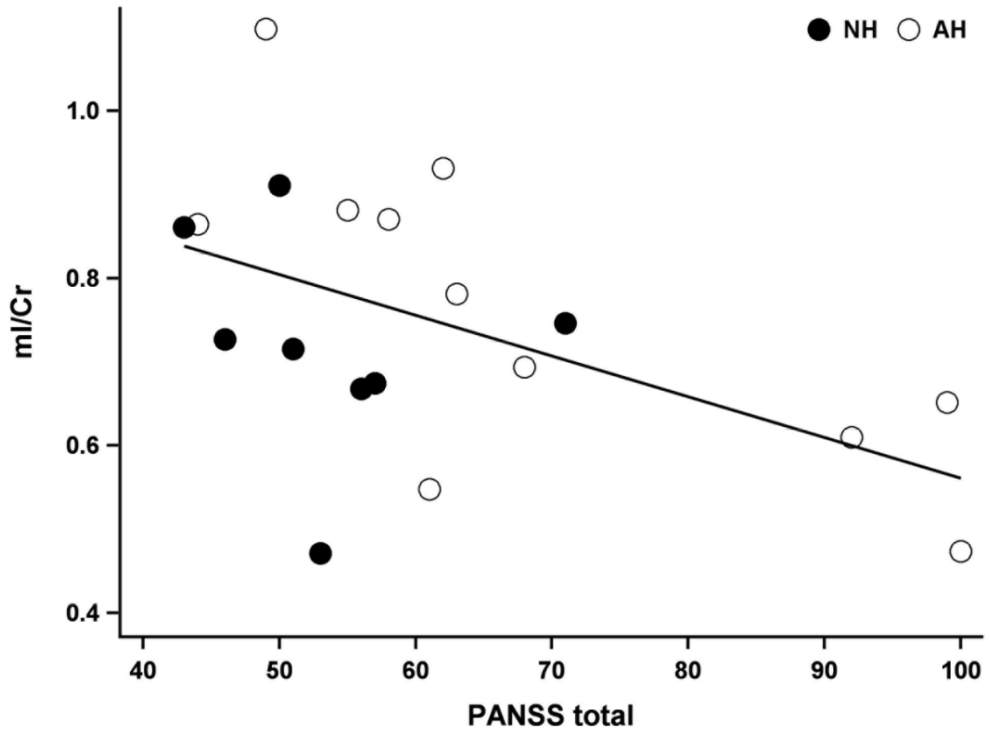
NAA/Cr
10
15
20
25
30
PANSS negative

D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ml/Cr
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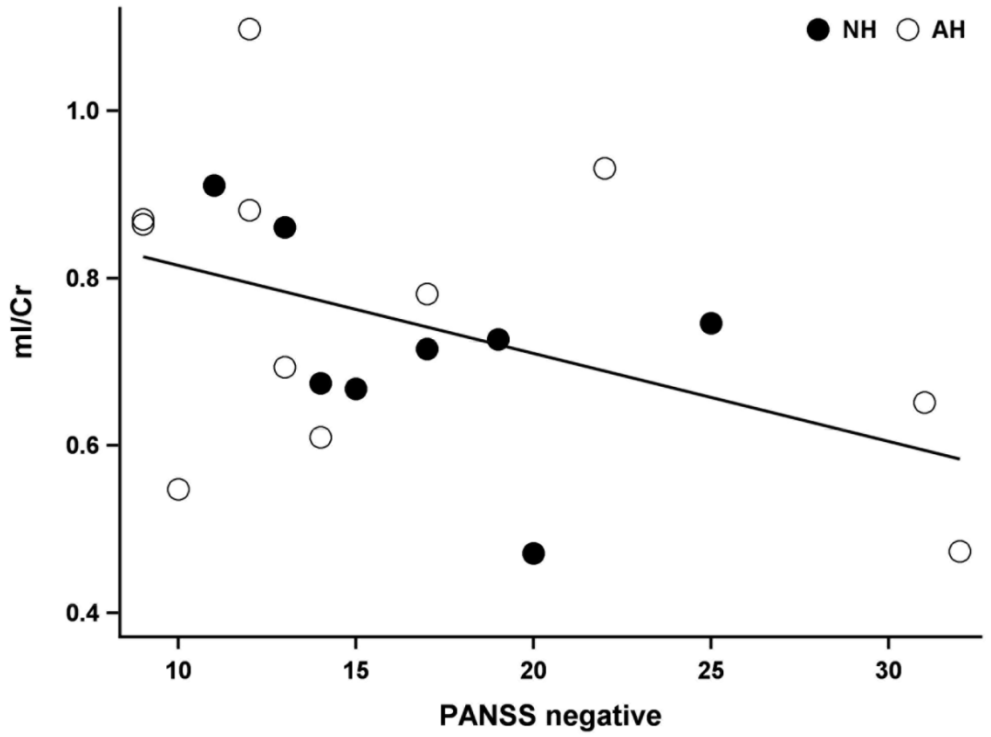
50
60
70
80
90
100
PANSS total


NH
AH
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0.8
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ml/Cr
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PANSS negative