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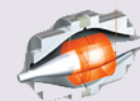
NAA and NAAG variation in neuronal activation during visual stimulation

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Abstract

N-acetyl-aspartyl-glutamate (NAAG) and its hydrolysis product N-acetyl-aspartate (NAA) are among the most important brain metabolites. NAA is a marker of neuron integrity and viability, while NAAG modulates glutamate release and may have a role in neuroprotection and synaptic plasticity. Investigating on a quantitative basis the role of these metabolites in brain metabolism *in vivo* by magnetic resonance spectroscopy (MRS) is a major challenge since the main signals of NAA and NAAG largely overlap. This is a preliminary study in which we evaluated NAA and NAAG changes during a visual stimulation experiment using functional MRS. The paradigm used consisted of a rest period (5 min and 20 s), followed by a stimulation period (10 min and 40 s) and another rest period (10 min and 40 s). MRS from 17 healthy subjects were acquired at 3T with TR/TE = 2000/288 ms. Spectra were averaged over subjects and quantified with LCModel. The main outcomes were that NAA concentration decreased by about 20% with the stimulus, while the concentration of NAAG concomitantly increased by about 200%. Such variations fall into models for the energy metabolism underlying neuronal activation that point to NAAG as being responsible for the hyperemic vascular response that causes the BOLD signal. They also agree with the fact that NAAG and NAA are present in the brain at a ratio of about 1:10, and with the fact that the only known metabolic pathway for NAAG synthesis is from NAA and glutamate.

Key words: Proton magnetic resonance spectroscopy; Functional experiments; Brain activation; Brain energy metabolism; N-acetyl-aspartate; N-acetyl-aspartyl-glutamate

Introduction

N-acetyl-aspartate (NAA) is one of the molecules present at highest concentrations in the central nervous system (CNS) (1). Due to its prominent signal in magnetic resonance spectra (MRS), NAA has been used as a diagnostic marker for many neuropathologies, including Canavan's disease, ischemia and stroke, Alzheimer's disease, epilepsy, brain tumors, multiple sclerosis, traumatic brain injury, and schizophrenia. Most of these pathologies, with the exception of Canavan's disease, show a decrease of the NAA peak in brain MRS. At first, this decrease was thought to be an irreversible neuronal loss. However, at present there is evidence that the decrease in NAA concentration can also be associated with mitochondrial dysfunction, which in some cases could be reversible. In the specific case of Canavan's disease, which is a demyelinating disease (2), NAA levels in the CNS are increased due to absence of the aspartoacylase enzyme, responsible for the metabolization of this compound (1). This fact has suggested that high NAA levels in the CNS can also have harmful effects.

N-acetyl-aspartyl-glutamate (NAAG) is a neuron-specific dipeptide, present at the highest concentration in the brain (1). An important confounding factor in NAA-NAAG MRS analyses is that the NAAG peak appears as a shoulder of the NAA peak. Although NAA is responsible for the largest contribution to the MRS peak at 2 ppm, NAAG can contribute by 10 to 20% to this signal, but very frequently the entire peak is attributed only to NAA (3,4). This means that decreases in this peak, attributed to NAA, associated with all the aforementioned neuropathologies, either involve a joint decrease in NAA and NAAG levels, or underestimate the decrease in NAA levels in cases where NAAG levels increase or stay constant (1).

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Indeed, separate measurements of the NAA and NAAG contributions by the MRS technique are difficult due to the severe overlap of their respective spectra. In order to separate these contributions, post-processing methods such as LCModel (5), which adjust the measured spectrum using a linear combination of isolated metabolite spectra, have been used by some investigators (3,6,7). However, the efficacy of LCModel in separating these contributions has been contested (8). More recently, Edden et al. (9) used a MEGA-PRESS pulse sequence to separately measure the contribution of these metabolites in a "static" (conventional) MRS experiment. Nevertheless, up to now this has been the only study reporting this type of measurement.

The aim of the present study was to evaluate NAA and NAAG changes during a visual stimulation experiment using functional MRS (fMRS). There are only two other reports in the literature that have presented significant results regarding NAA variations during a brain activation task (10,11), and none that has presented results regarding NAAG variations. On the other hand, there are many reports of variations in other metabolites and constancy of the NAA peak in similar functional experiments (see Ref. 12). The discrepancy among these results has generated a still inconclusive discussion about possible NAA variations in experiments of this nature, showing the need for further investigation of this subject.

Material and Methods

All data were acquired with a 3.0T Philips Achieva scanner (Philips, The Netherlands) using an 8-channel SENSE head coil. The fMRS protocol used single-voxel localization with PRESS, TR/TE = 2000/288 ms, 2048 data points, spectral width of 2000 Hz, 8 spectral averages (16 s/spectrum), 8 phase cycles, and voxel size 2 x 2 x 2 cm^3. The visual stimulus consisted of a black-white radial checkerboard pattern covering the entire visual field and flickering at 8 Hz, which was programmed using the E-Prime software (Psychology Software Tools, USA, http://www.pstnet.com/), and was shown to the subjects on a monitor inside the MR scanner using the Eloquence system (*InVivo*, USA, http://www.invivocorp.com/fmri/eloquence.php). Before running the fMRS protocol, three anatomical T2-weighted images in the axial, coronal and sagittal views were acquired for voxel positioning. This was followed by a functional magnetic resonance imaging protocol, consisting of a block design (4 rest blocks and 3 stimulus blocks of 10 s each) with the same visual stimulus used for the fMRS experiment, to detect the activated area. The activation map was superposed on the T2 images, and the MRS voxel was positioned in the right hemisphere (in order to avoid contamination with cerebral-spinal fluid) on the occipital lobe, in such a way as to cover as much as possible the activated area. Next, fMRS acquisition was carried out. The fMRS paradigm consisted of one rest (off) block (duration of 5 min and 20

s, consisting of 20 spectra), followed by one stimulus (on) block (10 min and 40 s, 40 spectra) and another rest (off) block (10 min and 40 s, 40 spectra), for a total scan duration of 26 min 40 s (100 spectra).

Seventeen healthy subjects (mean age 28 ± 4 years, range 21-39 years, 7 women) participated in the study. The project was approved by the Comitê de Ética em Pesquisa of Universidade Estadual de Campinas (UNICAMP), and all tested subjects gave written informed consent.

In addition to the subjects' scans, six scans were also performed on a phantom that emulated main metabolite concentrations in the brain, in order to supervise the quality of our fMRS measurements (Table 1). These scans were performed with the same MR parameters and the same total scan duration, on the same days the subjects were scanned.

Data processing and analysis

First, the last two spectra of the first off block, and the first and last two spectra of the on and second off blocks were discarded due to a small asynchronicity between the stimulus generator and the scanner. The spectra were then phase corrected (frequency correction was not needed since the Achieva scanner parameter "frequency stabilization", which performs real-time frequency drift correction during the acquisition, was turned on). Next, 18-spectra-averages (4 min and 48 s) were obtained for every subject (and every phantom acquisition). The averaged spectra were quantified and the relative metabolite concentrations (with respect to the first off block) were averaged over subjects (or phantom acquisitions). We also tested averaging the 18-spectra-averages over subjects (or phantom acquisi-

Table 1. Metabolite concentrations used in the magnetic resonance spectroscopy phantom.

Metabolite	Concentration (mM)
NAA	10
Creatinine	8
Choline	3
Lactate	1
Myo-inositol	6
Glutamate	10
Glutamine	5
DSS	2
Na_2HPO_4	72
NaCOOH	200
NaH_2PO_4	28
Sodium azide	1.54
Total volume	0.3 L

NAA = N-acetyl-aspartate; DSS = dimethyl-sila-pentane-sulfonate; NaCOOH = sodium formate.

tions), and then quantifying the resulting all-subjects' (or all-phantoms') spectra.

All spectra were quantified with LCModel (5). The LCModel method uses a linear combination of model spectra (characteristic spectra of every metabolite of interest) to fit a given measured spectrum, instead of fitting individual resonance peaks from specific chemical groups. Since the spectra of most metabolites usually contain more than one peak, in theory, this method should be able to separate contributions of metabolites whose spectra severely overlap in some region(s), but do not overlap so much in other region(s). The method also has model terms to describe the baseline, which mainly results from lipid and macromolecule resonances. In the present study, a basis set containing 87 model spectra from metabolites, lipids and macromolecules was used. However, for long echo times (i.e., lasting more than 100 ms), as in the present case, LCModel only fits the spectral region between 1.0 and 4.0 ppm, excluding spectra that fall outside this region. Therefore, only 14 metabolite and 9 lipid/macromolecule spectra were used in our spectral fittings.

In all cases, only metabolites that were quantified by LCModel with Cramer-Rao bounds (13) smaller than 30%

were considered.

Figure 1 shows an example spectrum averaged over subjects. Figure 1a shows the measured spectrum (black line) and respective LCModel fit (red line), and Figure 1b shows the corresponding residue.

Results

As mentioned earlier, two types of analyses were performed with the present data: one consisting of separate quantification of each subject's spectra followed by averaging the quantification results over subjects (quantification prior to estimation of mean value), and the other consisting of averaging spectra over subjects followed by quantification of the mean spectrum (quantification after estimation of mean value). The quantification prior to estimation of mean value provided spectra with a very low signal-to-noise ratio, which resulted in changes that did not correlate with the paradigm and that were surpassed by error bars (data not shown).

With the quantification after estimation of mean values, NAAG was reliably quantified separately. Figure 1c and d show separate NAA and NAAG fits, respectively, for the
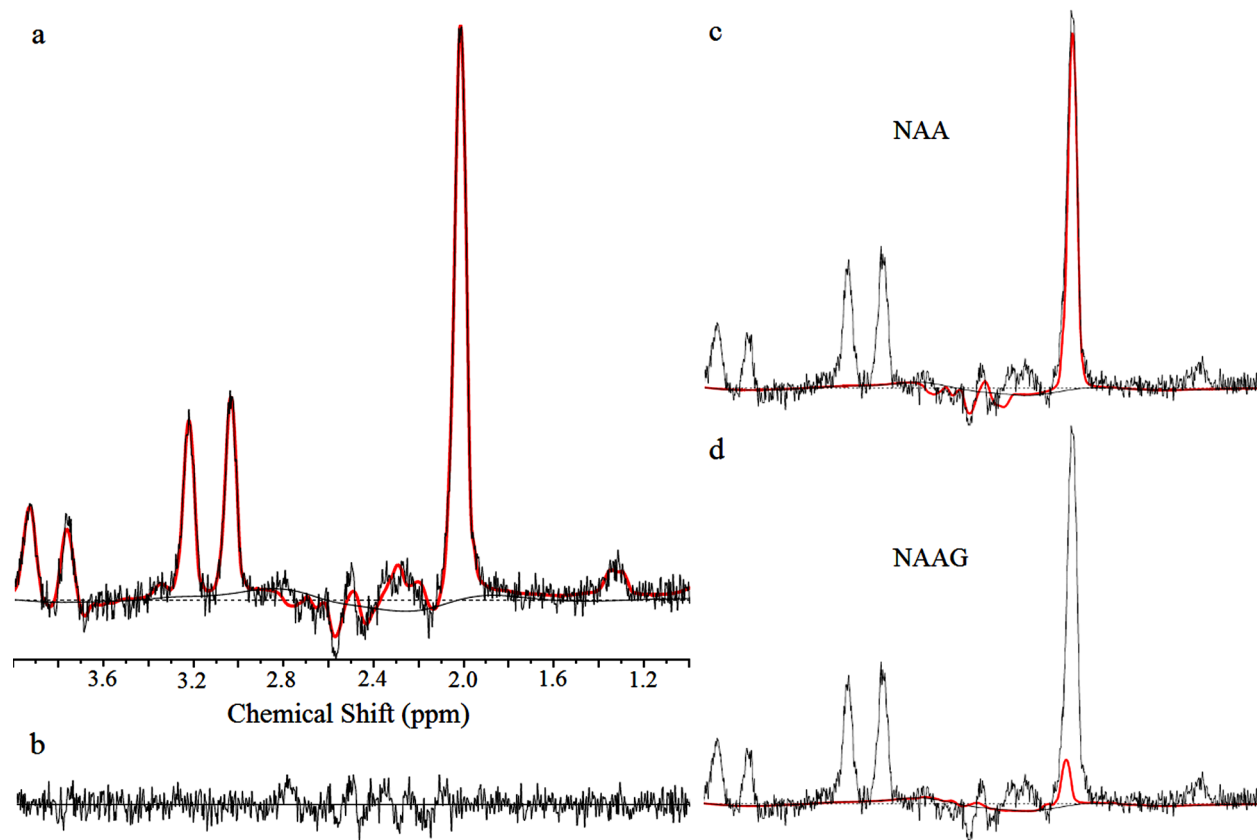

Figure 1. Example of averaged-over-subjects spectrum. *a*, Measured spectrum (black line) and LCModel fit (red line). *b*, Corresponding residue. *c*, Individual N-acetyl-aspartate (NAA) fit for the spectrum in *a*. *d*, Individual N-acetyl-aspartyl-glutamate (NAAG) fit for the spectrum in *a*.

averaged-over-subjects spectrum of Figure 1a. Figure 2 shows plots for the relative variations of NAA, NAAG, and NAA+NAAG concentrations along the paradigm. From these plots, we see that these metabolites follow a variation pattern
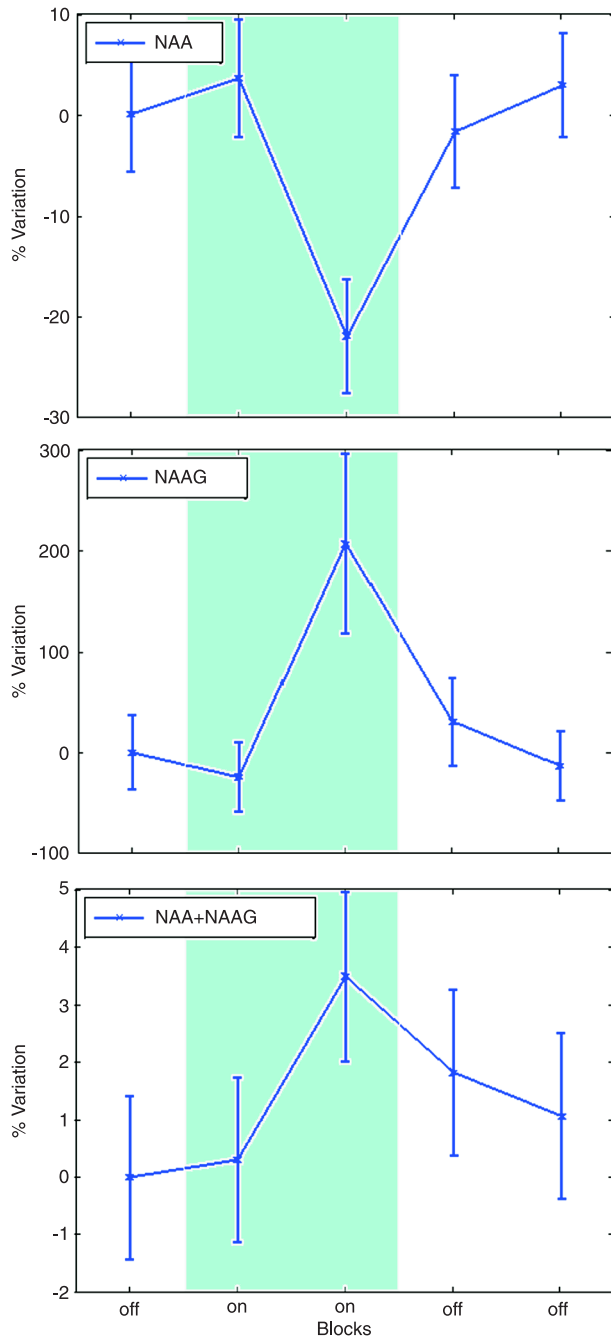
along the paradigm that is larger than the error bars. This was thus the only type of analysis that allowed assessing metabolic variations associated with the stimulus.

For all phantom acquisitions, none of the quantified metabolites varied by more than 2% along the acquisition, attesting the stability of the scan during the experiments.

Discussion

The results associated with NAA and NAAG are remarkable, given that for these metabolites the variations associated with the stimulus are much larger than the error bars. NAA and NAAG are usually quantified as a group due to the large overlap of their spectra. However, as mentioned, LCModel has been successfully used to separate the contributions of these metabolites in "static" MRS experiments (3,6,7). In the plots shown in Figure 2, we see that NAA decreased by about 20% with the stimulus, while NAAG concomitantly increased by about 200%. On the other hand, the NAA+NAAG plot shows that the sum of these metabolites changed by about 3%, which is around the percent variations found for phantom fluctuations, i.e., the sum of these metabolites virtually did not change. Such variations fall into models for the energy metabolism underlying neuronal activation, that point to NAAG as being responsible for the hyperemic vascular response that causes the BOLD signal (4,14). They also agree with the fact that NAAG and NAA are present in the brain at a ratio of about 1:10, and with the fact that the only known metabolic pathway for NAAG synthesis is from NAA and glutamate (4). These results are also in line with the fact that NAA has been shown to participate in the neuronal energy metabolism (1), and NAAG is known to mediate neurotransmissions (2).

To the best of our knowledge, there are only two other literature reports that point to a similar result. Both studies used visual stimuli and paradigms similar to ours. Sarchielli et al. (10) found a decrease in NAA levels during visual stimulation for three groups of subjects: 22 patients with migraine with aura, 22 patients with migraine without aura, and 10 healthy subjects. The decrease was more marked for the first group (14.6% for the patients with migraine with aura). Based on this, the authors suggested that there is a smaller efficiency of the mitochondrial functions for these patients, given that NAA is an indicator of the functional integrity of the neuronal mitochondrial metabolism (2).

Baslow et al. (11) found a similar decrease in NAA levels at the end of stimulation for healthy subjects (13.1%). They proposed that the NAA decrease was due to the fact that this metabolite works as a water pump, expelling into the extracellular fluid the water created in the neuron due to glucose metabolism. This glucose metabolism would be caused by neuronal activation resulting from visual stimulation. This explanation has been contested, given that up to now no protein has been found that would act to

Figure 2. Changes of N-acetyl-L-aspartate (NAA), N-acetyl-aspartyl-glutamate (NAAG) and NAA+NAAG in response to stimulations. Shaded regions correspond to stimulation periods. Error bars correspond to propagation of the LCModel quantification error.

transport NAA and water out of the neurons (1). In addition, at present there is little knowledge about the propagation means that specifically regulate NAA liberation from neurons (1). Therefore, considering the investigations carried out so far, NAA does not have the necessary features to fulfill the water pump function (1). On the other hand, the result obtained by Baslow et al. (11) was challenged by Mangia and Tkac (15), who claimed that in their research using a similar stimulus and paradigm, but performed with a 7 Tesla system (12), they were not able to detect any significant NAA variation. They only found significant changes of lactate, glutamate, glucose, and aspartate. In spite of the differences between the two studies (11,12), such as the intensity of the magnetic field, Mangia and Tkac (15) stated that this divergence between results should not exist. Baslow et al. (16) argued that the way Mangia et al. (12) computed their spectral averages would not allow detecting NAA variations larger than about 2%. In addition, according to the reply of Baslow et al. (16), Mangia et al. (12) took it for granted that the NAA signal should remain stable (rather than considering it a hypothesis to be verified) and presented combined data for two different (short and long) paradigms (12), while only the long paradigm is comparable to the results of Baslow et al. (11).

When we compare our results with those obtained by Sarchielli et al. (10), Baslow et al. (11) and Mangia et al. (12), we have two possible scenarios. On the one hand, our results seem to agree with those of Sarchielli et al. (10) and Baslow et al. (11), given that both reported decreases in the NAA peak associated with the stimulus, a fact that qualitatively corresponds to our result for this metabolite. It is important to remember, however, that both studies effectively measured the combined variation of NAA+NAAG, whereas our analysis allowed us to separate the contributions of these metabolites. On the other hand, if we assume that the results reported by Mangia et al. (12) also refer to the sum of NAA+NAAG (which is not explicit in their report), then our conjugated result for these metabolites is consistent with theirs.

In short, we presented here preliminary results of experiments performed using the innovative fMRS technique to study possible changes of NAA and NAAG under visual stimulation. We compared these results with findings of similar studies in the literature and highlighted controversial aspects associated with them. In the center of this controversy is the fact that while it is virtually the only method for measuring changes in NAA and NAAG in the human brain *in vivo*, the fMRS technique still presents considerable technical challenges, such as low sensitivity, which results in low spatial and temporal resolution. Thus, the effective fMRS signal ends up by being the result of averages performed on relatively large brain regions, compromising the reliability desirable to infer what is happening at the molecular level.

In addition, the differentiation process of the NAA and NAAG signals is particularly difficult. We know of only one study with positive results for the separation of these signals at a field strength of 3 Tesla (9). Given that such a procedure is fundamental for the analysis of NAA and NAAG changes, this will be the next step in our research using ^{1}H-fMRS in order to confirm (or refute) the results discussed in the present paper.

Acknowledgments

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