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Reduced Thalamic NAA in Idiopathic Normal Pressure Hydrocephalus

A Controlled ^1H -MRS Study of Frontal Deep White Matter
and the Thalamus Using Absolute Quantification

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ABSTRACT

Introduction

Patients with idiopathic normal pressure hydrocephalus (INPH) frequently have a reduction of cerebral blood flow in the subcortical frontal lobe/basal ganglia/thalamic areas. With magnetic resonance spectroscopy the metabolism in the brain can be examined.

The aim of this study was to investigate if there was a compromised metabolism in the thalamus and in the subcortical frontal areas in INPH patients. This was done by measuring total creatine (tCr), myo-inositol (mIns), total choline (tCho), N-acetylaspartate (NAA), total N-acetylaspartate (tNA), glutamate (Glu), and lactate (Lac) levels. A comparison was made with healthy individuals (HI).

Subjects and Methods

Sixteen patients (9 M, 7 F, mean age 74 years, range 49-83) diagnosed as INPH and 15 HI (9 M, 6 F, mean age 74 years, range 62-89) were examined. ¹H-MRS (1.5 T, PRESS, TE/TR 30/3000 ms, VOI 2.5-3 ml) was performed in frontal deep white matter and in the thalamus. Absolute quantification with internal water as a reference was used.

Results

INPH patients had lower NAA (p=0.02) and lower tNA (p=0.05) concentrations in the thalamus compared to HI. NAA and tNA in the frontal deep white matter (FDWM) did not differ between patients and HI. The absolute metabolic concentrations of tCr, mIns tCho, tNA, Lac and Cr ratios in FDWM and in the thalamus were similar in INPH patients and HI.

Conclusion

Reduced thalamic NAA and tNA in INPH patients suggests a compromised metabolic neuronal function in these regions. Thus, the thalamus might have an important role in the pathogenesis of INPH.

INTRODUCTION

Normal Pressure Hydrocephalus (NPH) is a condition in which disturbed cerebrospinal fluid dynamics cause the typical symptoms of gait disturbance, cognitive impairment and urinary incontinence first described by Hakim & Adams and Adams *et al.* in 1965.(1,2) NPH is classified as idiopathic NPH (INPH), where no cause can be identified, and secondary NPH when it is preceded by a known disease.(3)

The symptoms and signs in INPH are generally recognized as being of subcortical origin.(4) Damage to myelin and axons, and signs of arteriosclerosis in these areas have been described.(5, 6) Furthermore, the incidence of cardiovascular risk factors is high and these are thought to be of importance for disease development.(7)

MR Spectroscopy (MRS) is a well-established method for studying metabolites in the brain non-invasively. The concentrations of a number of metabolites can be analyzed, including total N-acetyl compounds (tNA) consisting of N-acetylaspartate (NAA), and N-acetylaspartateglutamate (NAAG) which is highly concentrated in the mitochondria, and both of these are regarded as neuronal density markers.(8,9,10) NAA also occurs in white matter in an approximately equal amount.(8) Low NAA may be an indication of neuronal loss but may also indicate a reversible state.(8,11) Choline (Cho) is found in cell membranes and is believed to be a marker of membrane turnover and myelination.(8) Myo-inositol (mIns) is a constituent of membrane lipids and is also regarded as a marker of glia cells.(8) tCr is divided into creatine and phosphocreatine, the latter being three times higher in astrocytes.(12) Generally tCr is however considered as a marker of energy deposits. (8) Glutamate (Glu) is an amino acid that is highly concentrated in excitatory neurons.(8) Lactate accumulates when energy metabolism is subjected to anaerobic stress.(8)

In MRS-NPH studies, different techniques and placements of the voxels have been used, which unfortunately makes a direct comparison very difficult. Data on absolute metabolite concentrations have not yet appeared in NPH patients. Based on the hypothesis that INPH patients suffer from disturbed basal ganglia–thalamic–subcortical frontal circuits, our aim was to demonstrate that there was a reduced metabolism in the thalamus and the subcortical frontal areas in INPH patients in comparison with HI.

SUBJECTS AND METHODS

Patients

Twenty patients, 12 M and 8 F, mean age 74 (49-83) years, consecutively recruited from our outpatient clinic and diagnosed with INPH were included in the study. All fulfilled the following clinical and radiological inclusion criteria, modified from INPH guidelines.(13)

Clinical inclusion criteria were: Gait disturbance of both legs, unexplained by other conditions, disturbance of tandem walking, multistep turning, small steps and wide based gait, Mini Mental State Examination (MMSE) (14) score between 21-30, no evident aphasia, apraxia or agnosia, and bladder instability might be present.

Radiological inclusion criteria were: Symmetrical communicating quadric-ventricular enlargement without cortical infarcts or other clinical relevant parenchymal lesions except lacunar infarcts (< 1cc), Evan's index ≥ 0.3 and temporal horns and third ventricle should be relatively enlarged, mild to moderate cortical atrophy and leuco-araiosis might be present.

Exclusion criteria were: Intracranial pressure (ICP) higher than 18 mmHg, cerebrospinal fluid (CSF) changes not compatible with INPH, infarctions in the thalamus and in the frontal lobes, patients not able to cooperate, and finally, short expected survival time.

Medication was not a reason for exclusion.

Healthy Individuals

Sixteen healthy elderly people, 7 M, 9 F, mean age 73 (62-89) years were recruited consecutively as HI. They considered themselves healthy and no serious disease could be found by examination.

Reasons for exclusion were clinical obvious gait disturbance, MMSE <21 and infarction in the Volume of Interest (VOI). Periventricular hyperintensities and cortical atrophy were allowed.

Medication and previous diseases not impairing gait and cognition were not reasons for exclusion in HI.

All participants gave written consent the study was performed according to the Declaration of Helsinki.

Clinical assessment

The patients underwent a neurological examination by a neurologist (FL) and later on an MRI was performed. The motor functions were examined by a physiotherapist. Time for 10 metre walk in

seconds (w10mt) and number of steps (w10ms) at a self-selected speed with their usual walking aid were registered.(15) Timed up and go test in seconds and steps (TUGt,TUGs), which is a timed test for standing up from a chair, walking 3 metres, turning and walking back to the chair and sitting down.(16) Gait scale: 1, normal; 2, insecure; 3, insecure with cane; 4, bi-manualsupport; 5, aided; 6, wheelchair.(17) The balance test used was Romberg's, slightly modified from Blomsterwall et al.(15), and it was performed standing, with the feet together, eyes closed and hands on the chest. Seconds to correction up to 60 seconds were registered. An occupational therapist performed the MMSE test. Lumbar CSF pressure was measured either manually in the recumbent position or automatically if a computerized lumbar infusion study was performed. Cells, proteins and antibodies against *Borrelia burgdorferi* were analyzed.

The HI were examined by a neurologist (FL), including the test for 10 metre walking (measured in seconds), number of steps, and Romberg's test. MMSE was performed by an occupational therapist or a neurologist.

MR Acquisitions

All MR measurements were performed using an Achieva 1.5 T MR scanner (Philips, Best, The Netherlands), and an eight-channel SENSE head coil.

MRS data were acquired using Point Resolved Spectroscopy (PRESS), with an echo time of 30 ms and a repetition time of 3 s, 128 water-suppressed spectra were averaged, two MRS volumes of interest (VOI), one in the left thalamus and, one in the left FDWM were placed in each subject, each voxel with a volume of ca. 2.50 mL. In addition, spectra without water suppression were also obtained. In a later phase of the study, two additional MRS VOI were acquired, one in the right thalamus and one in the right FDWM (this was done for the ten final INPH patients and the six final HI).

Schematic placements of a single VOI in the FDWM and the thalamus of an INPH patient in the cardinal directions of the localizer images are shown in Fig. 1 and Fig 2 respectively. The MRS VOIs were placed in normal appearing matter avoiding hyperintensities. For quantification of R_2 ($1/T_2$), an axial multi-echo GRASE (ME-GRASE) pulse sequence was used to acquire data on the tissue water signal (= ME-GRASE-Volume). The parameters used for ME-GRASE were: matrix $2 \times 2 \times 4 \text{ mm}^3$; TE was 20, 40, 60, 80, 100, 120, 140, and 160 ms; TR 5.28 s).

To avoid errors due to patient movement the coronal localizer images were re-sampled prior to and after each MRS acquisition. If pre/post-MRS movement was detected and considered to be significant ($> 3 \text{ mm}$) the MRS data were discarded, and the data acquisition was repeated.

Quantification of the NMR data

1. MRS post processing: The MRS signals were analyzed using LCModel ver. 6.1-4G.(18) The unsuppressed water signals were used as an internal reference, and quantified using the water scaling function in LCModel, with the parameter attenuation of the NMR-visible water (ATTH20) = 1.00 (from 0.7). Thus the resulting concentrations were aqueous concentrations compensated for by differences in coil load and temperature, and differences in RF-amplification, etc in different subjects. The concentration values determined in this manner depended then only on differences in the relaxation, and different compartmentalization, between the metabolites and the internal water reference signal.

2. Estimation of quantitative R2 values: The R2-values were obtained from the ME-GRASE experiments by fitting an exponential function to the data. The amplitude M0 is a T1 and B1-weighted estimate of the proton density and R2 is the transversal relaxation rate.

3. Correction for CSF content with the MRS VOI: The fractions of CSF (P_{CSF}) in the MRS VOIs were determined using the lower tail of the distribution of the R2 values in the VOIs, together with an upper R2-limit of 2.5 s^{-1} . The concentrations were then scaled with $1/(1-P_{\text{CSF}})$.(19)

4. Scaling to absolute concentrations: In order to convert the LCModel estimates to absolute concentrations, we used a modified version of the method described in (20). The major difference was that the water scaling function in the LCModel was here used to scale the differences between the in-vitro measurements of the basis set, and the in-vivo measurement on the MR scanner used in this investigation.(21)

5. Exclusion: The spectra were inspected for residuals and large artefacts in the spectral region of interest. In order to avoid bias toward high concentrations, no measurements were excluded from the subsequent statistical analysis based only on the SD% values in LCModel.

Statistical Analysis

The statistical analysis used JMP 8.0 (SAS Institute Inc, USA). A full mixed linear model (MLM) was created with the three fixed factors “Group”, “Tissue”, “Lateral”, and the corresponding crossing effects. A random factor “Patient” nested with the “Group” factor was also included. The Restricted Maximum Likelihood (REML) was used to analyze the mixed model. The model gave no significant effects for the “Lateral” effect or any of its crossing effects. Therefore, the model was subsequently reduced to contain only the “Group”, “Tissue”, their crossing effect, and the “Patient” effect.

Group analysis

From the MLM mean values of the crossing Effect, Group and Tissue were calculated (HI-FDWM, HI-thalamus, INPH-FDWM, and INPH-thalamus). Differences in mean values were tested using a Tukey test, and p-values < 5% was considered significant.

Confounding factors

To avoid any eventual differences in metabolite concentrations or R² could be due the higher occurrence of vascular risk factors in the INPH than in the HI, six extended MLMs were created by adding fixed factors for Stroke/TIA, Hypertension, Heart disease, Diabetes, Smoking, Risk one by one and all their crossing effects (Risk=1 if any of the risk factors were 1). Factors were considered significant to the model at $p < 0.05$.

Regression analysis

Linear regression models were created using the motor variables: w10mt, w10ms, TUGt and TUGs as explanation variables for the metabolite concentrations NAA, tNA, Glu, tCr, tCho, Lac, mIns and the mean R² as response variables. For patients for whom two MRS measurements were made from the same tissue, the mean values were used as response variables. All models were created using the standard least square method in JMP. Variables were considered significantly correlated for $p < 0.05$.

RESULTS

Subjects

Four out of twenty patients were excluded from the study (due to neuroborreliosis, severe heart failure, unwillingness to participate and MMSE < 21). Sixteen patients (9 males and 7 females) were finally evaluated in the study. Age was similar between patients and HI. Hypertension was more common in the patients. The patients performed worse in motor functions, while MMSE was similar (Table I). One of the 16 HI was excluded as the MRS examination was lost due to data error, thus fifteen remained in the study. Twenty-six voxels were each placed in the FDWM and in the thalamus of the 16 patients; two voxels were excluded in the FDWM due to poor spectral quality. Twenty-one voxels were placed each in the FDWM and in the thalamus of the 15 HI. In the FDWM and in the thalamus one voxel each was excluded due to poor spectral quality. Because of lacunar infarctions in the thalamus in one HI the voxels in the thalamus were excluded. One HI was reinvestigated due to motion artefacts.

Cardiovascular risk factors did not influence the metabolic concentrations.

qMRI results

No significant differences between R2 in different tissues and subjects were observed. Estimated mean and standard error R2 for; HI FDWM 10.92 s^{-1} , (SE = 0.19), thalamus 10.93 , (SE = 0.19), for; INPH FDWM = 10.78 s^{-1} , SE = 0.17, thalamus = 10.66 s^{-1} , SE = 0.17. This suggests that there was no significant difference in the intracellular aqueous state between the groups and different tissues.

MRS results

The mean thalamic concentration of NAA was 11% lower ($p = 0.02$) and mean tNA were 6% lower ($p = 0.05$) in the patients compared to the HI (Table II, III). No significant differences between the groups were observed for the FDWM. Metabolite ratios to tCr were not significant different in either the FDWM or the thalamus compared to HI. An increased tCr in FDWM correlated to a reduced motor function. At a trend level there was a negative correlation, *i.e.* reduced NAA correlated to a reduced motor function, between NAA and the motor variables in the thalamus (Table IV). Elevated lactate could not be detected either in the thalamus or in the FDWM.

DISCUSSION

The main findings of this study are significant lower concentrations of NAA and tNA in the thalamus in INPH patients compared to HI. Data were obtained from absolute concentrations and indicate a neuronal dysfunction in the thalamus in INPH patients (Table III). Interestingly, no metabolic abnormalities were observed in the FDWM (Table II).

We believe that our patients are representative of the typical form of INPH patients since they showed classical motor symptoms, but cognitively they were relatively well preserved. The patients had approximately half the gait velocity of the HI. The cognitive performance measured with MMSE was similar to that of the HI. The reason for excluding patients with a profound cognitive deficit was to create a homogeneous group of patients representing INPH in a rather pure form and avoid other co-existing pathologies with impaired cognitive function related to FDWM, which would have influenced the results.(22,23)

MRS studies in NPH patients have presented conflicting results. In a heterogeneous hydrocephalic population ranging from neonates to the elderly, Braun *et al.* could not find any metabolic abnormalities in the periventricular white matter nor in the CSF compared to HI.(24) In a study by Lenfeldt *et al.* regarding INPH patients it was shown that patients had a significant lower NAA/Cr in FDWM compared to a control group and they also showed that patients with a positive Elongated Lumbar Drainage (ELD) had a higher NAA/Cr than those with a negative ELD. Their conclusion was that having enough functional neurons was a prerequisite for the CSF drainage to have an effect.(25) Based on the absolute concentration measurements of NAA and tCr in FDWM (Table

II), the hypothesis that the decreased NAA/Cr ratio was due to a lowered concentration of NAA can be rejected. This is particularly interesting as one then can conclude that the observed decreased NAA/Cr ratio can be fully explained by the increase in absolute tCr concentration. A non-controlled study by Del Mar Matarin *et al.* in INPH focusing on postoperative changes found an increase of NAA/Cr after a shunt operation in the frontal lobe and the postoperative value of NAA/Cr was correlated with the MMSE score.(26) They concluded that NAA/Cr may be reversible and that cognitive level is associated with the NAA/Cr levels in the subcortical frontal lobe.

The size of the voxels in FDWM used in our investigations were smaller compared to those used by Lenfeldt *et al.*(25) The size of the VOI was chosen to include as much of the thalamic area as possible, with a minimal inclusion of other tissues thus avoiding partial volume effects. A larger voxel would result in a better signal to noise ratio (SNR), but we found it impossible to combine larger spectral voxels with a sufficiently accurate tissue selection. Another difference was the placements of the voxels, which in our study were closer to the periventricular zone.

In contrast to all previous NPH-MRS studies we have used absolute quantification of the metabolite concentrations. Such an approach involves a slightly more complex procedure; however, it will provide much more informative results than procedures that are based on creatine ratios. Regional and individual variability of tCr as well as influences from systemic diseases may influence the tCr (27, 28), thereby affecting the concentration ratios. In addition, Li *et al.* found that the coefficient of variation (CV) in a large series of examined voxels was higher for ratios than for absolute quantification.(29) Schirmer *et al.* also found that a significantly lower CV, both intra- and inter-individually, favoured absolute quantification.(30)

No previous MRS study of NPH patients has involved examination of the thalamus. We detected a significant reduction of NAA and tNA in the thalamus compared to HI. This result of reduced NAA concentrations in the thalamus is very interesting since thalamus is of great importance in movement disorders. Previously the basal ganglia were thought to relay motor circuits to the motor cortex via thalamic slave neurons. We now know that in idiopathic Parkinson there is a significant neuronal loss in the caudal interlamina nuclei while the limbic parts are spared.(31) Thalamus is today regarded as a major regulator of basal ganglia function.(32) The motor as well as the cognitive symptoms, may from a strict neuro-anatomical point of view, originate from the thalamus. In schizophrenia as well as in Huntington's disease thalamus has been examined with MRS and NAA has found to be decreased.(33,34)

Our results may indicate a primary pathology or secondary to dysfunction elsewhere, e.g. the basal ganglia.

Though we expected to find changes also in the FDWM as Lenfeldt *et al.* did, this was not observed either using absolute quantification or tCr ratios. The most consistent finding of CBF studies is a decreased CBF in the frontal lobe.(35) However, Owler *et al.* did not find any changes in the frontal area using positron emission tomography.(36) Even if this is not directly coupled to our findings it is interesting that Owler and colleagues observed a normal CBF in the frontal lobe but a decreased CBF in the thalamus, and this is in line with our results. An alternative explanation for the apparently reduced NAA levels (as deduced from the tCr-ratios) in the frontal lobe compared to Lenfeldt *et al.* could be that they included patients with a lower cognitive function, who may have had a co-existing pathology, since it has been shown that cognitive function is correlated to NAA in the frontal lobe.(23)

As motor symptoms are of major interest in these patients, we separately performed a regression analysis between motor variables and the absolute concentrations of the metabolites, in addition to tCr ratios. A significant positive correlation of tCr concentrations in FDWM with motor function, *i.e.* higher tCr concentration was correlated with worse gait function, and no correlation were observed between NAA concentrations in FDWM and gait function. We interpret this result as being due to glial cell increase in the tissue without any effective density loss of neurons, similar to what has been reported by Vrenken.(37) However, more studies will be needed to confirm this important finding, preferably at higher field strengths. In addition, we found a significant negative correlation of NAA/tCr with motor variables in the FDWM. This we concluded was due to the positive correlation of tCr – and not to any change in NAA. This result clearly highlights the importance of using absolute concentrations instead of tCr ratios for investigations of tissue metabolism.

The trends of negative correlation between NAA concentration and motor variables in combinations with the clinical picture of the patients, and also an absolute reduction of NAA concentration in thalamus suggests that the thalamus is linked to the pathogenesis of INPH.

CONCLUSION

We demonstrated significantly lower NAA and tNA concentrations in the thalamus of INPH patients compared to HI, which indicates a neuronal dysfunction in the thalamus. In contrast, we were not able to find any evidence of neuronal dysfunction in the FDWM.

In order to further explore our hypothesis about a dysfunction in basal ganglia–thalamic–sub-cortical frontal circuits in INPH patients, future studies must also involve the basal ganglia. In addition, it would be valuable to investigate if there is reversibility of the decreased NAA and tNA in the thalamus following surgery.

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Competing interests: None

Ethics approval: The study was approved by the Regional Ethical Review Board in Linköping, Sweden

Patient consent: Obtained

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Table I. Clinical results for HI and INPH-patients in the study.

	HI	INPH
N	15	16
Male/female	6/9	9/7
Age, years mean (range)	74 (62-89)	74 (49-83)

Disease duration (mean months, range)	-	35 (12-96)
Vascular risk factors		
Stroke/ TIA	1	2
Hypertension	3	7
Heart disease	2	4
Diabetes mellitus	1	3
Smoking	1	1
ICP (Intracranial Pressure), baseline mmHg (mean, range)	-	10 (4-17)
Evan's index (mean, range)	0.28 (0.23–0.34)	0.38 (0.30-0.49)
Gait		
10 m (mean seconds, range)	7 (5-8)	16 (8-29)
10 m (mean steps, range)	10 (9-13)	26 (16-42)
TUGt (mean seconds, range)	-	21(8-36)
TUGs (mean steps, range)	-	28(12-43)
Gait scale 1-6	1	14 (1-6)
Balance		
Romberg 0-60 (mean seconds, range)	60 (60)	22 (0-60)
Urgency	-	11/16
Cognition		
MMSE 0-30	29 (27-30)	27 (24-30: n=15)

Table II. Mean and standard errors (SE) of metabolite concentrations in the FDWM in patients and HI. Concentration values are presented in units of mM aq. and as concentration ratios. LCM CR = LC Models Cramer Rao bounds

FDWM	HI (N=15)			INPH (N=16)		
	Mean	(SE)	LCM CR	Mean	(SE)	LCM CR
NAA	8.95	0.28	0.71	8.91	0.25	0.80
tNA	9.82	0.23	0.65	9.91	0.20	0.71
Glu	8.76	0.46	1.72	8.73	0.41	1.95
tCr	6.86	0.19	0.51	7.28	0.17	0.56

tCho	2.70	0.11	0.20	2.53	0.10	0.21
Lac	0.40	0.15	0.49	0.71	0.13	0.60
mIns	7.51	0.36	0.75	7.67	0.34	0.83
NAA/tCr	1.32	0.05		1.23	0.04	
tNA/tCr	1.45	0.04		1.37	0.03	
tCho/tCr	0.40	0.02		0.35	0.01	
mIns/tCr	1.10	0.05		1.06	0.05	

Table III: Mean and standard error of metabolite concentrations in the thalamus in HI and patients. Concentration values are presented in units of mM aq. and as concentration ratios. * = significant difference from HI at $p < 0.05$, ** = significant difference from HI at $p < 0.025$. LCM CR= LC Models Cramer Rao bounds

TH	HI (N=15)			INPH (N=16)		
	Mean	(SE)	LCM CR	Mean	(SE)	LCM CR
NAA	10.77	0.29	0.96	9.61**	0.24	0.99
tNA	12.71	0.24	0.77	11.88*	0.20	0.83
Glu	13.02	0.48	2.00	12.32	0.39	2.12
tCr	8.08	0.20	0.61	7.87	0.16	0.63
tCho	2.53	0.11	0.22	2.38	0.10	0.23
Lac	0.36	0.16	0.63	0.41	0.13	0.51
mIns	6.47	0.38	0.76	7.13	0.33	0.86
NAA/tCr	1.34	0.04		1.23	0.04	
tNA/tCr	1.58	0.04		1.52	0.03	
tCho/tCr	0.31	0.02		0.30	0.01	
mIns/tCr	0.79	0.06		0.91	0.05	

Table IV: Correlation coefficient r for linear regression models for gait parameters; w10mt, w10ms, TUGt, TUGs against tCr NAA and NAA/tCr

		tCr		NAA		NAA/tCr	
	Tissue	r	p	r	p	r	p
w10m t	FDWM	0.73	0.00	0.18	0.51	-0.51	0.04

w10m s	FDWM	0.54	0.03	0.03	0.92	-0.47	0.07
TUG t	FDWM	0.56	0.02	0.04	0.88	-0.50	0.05
TUG s	FDWM	0.53	0.04	0.04	0.88	-0.47	0.08
w10m t	TH	0.20	0.46	-0.36	0.17	-0.48	0.06
w10m s	TH	0.12	0.66	-0.46	0.07	-0.47	0.07
TUG t	TH	0.11	0.69	-0.41	0.11	-0.45	0.08
TUG s	TH	-0.10	0.73	-0.61	0.02	-0.36	0.18

w10mt=walk 10m in seconds, w10mt=walk 10m steps, TUGt=Timed Up and Go in seconds, TUGs=Timed Up and Go steps

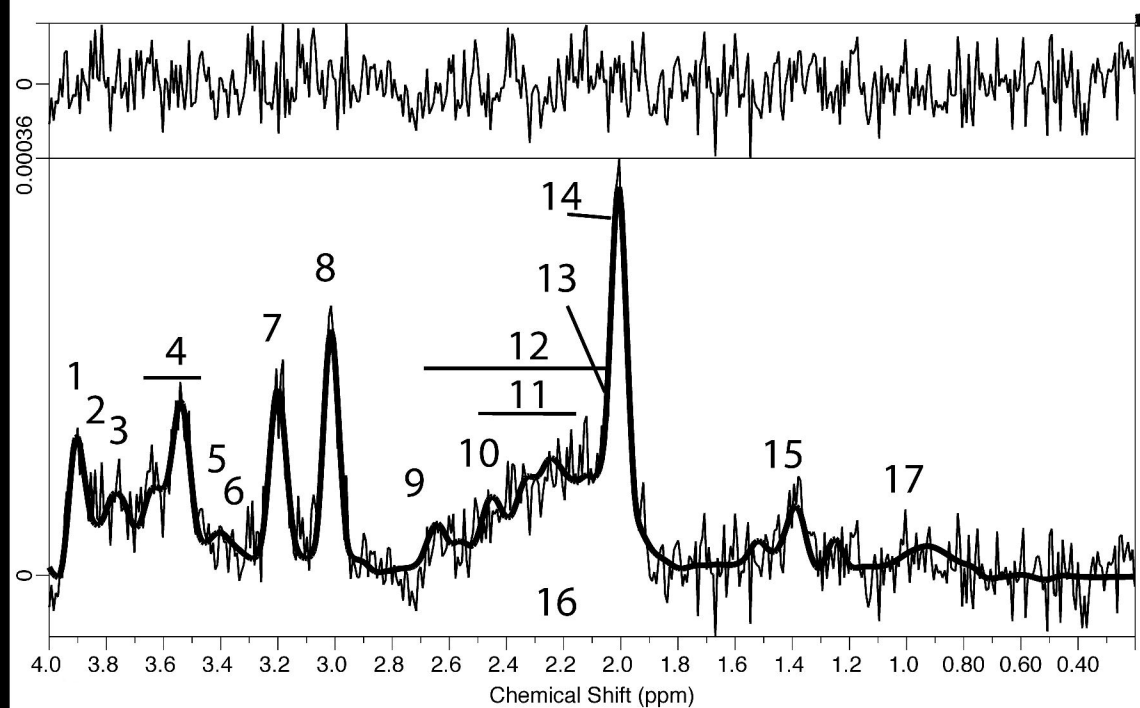
Figure legends

Figure 1: Schematic placement of a single VOI in the FDWM of an INPH patient in the cardinal directions of the localizer images. Assignments: 1. Cr-CH₂; 2. Glu a/Gln a; 3. Gln (multiplet from C2-C6); 4. myo-Ins; 5. Gln (multiplet from C2-C6); 6. scyllo-Ins; 7. Cho (CH₃)₃; 8. Cr (CH₃); 9. NAA; 10. NAA; 11. Glu g/b; 12. Gln g/b; 13. NAAG-methyl (singlet); 14. NAA-methyl (singlet); 15. Lac CH₃; 16. protein background ('baseline'); 17. unassigned.

Figure 2: Schematic placement of a single VOI in the thalamus of an INPH patient in the cardinal directions of the localizer images. For assignments, see Fig. 1.

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