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NEUROCHEMICAL ALTERATIONS IN SPINOCEREBELLAR ATAXIA TYPE 1 AND THEIR CORRELATIONS WITH CLINICAL STATUS

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Abstract

Robust biomarkers of neurodegeneration are critical for testing of neuroprotective therapies. The clinical applicability of such biomarkers requires sufficient sensitivity to detect disease in individuals. Here we tested the sensitivity of high field (4 tesla) proton magnetic resonance spectroscopy (¹H MRS) to neurochemical alterations in the cerebellum and brainstem in spinocerebellar ataxia type 1 (SCA1). We measured neurochemical profiles that consisted of 10–15 metabolite concentrations in the vermis, cerebellar hemispheres and pons of patients with SCA1 (N=9) and healthy controls (N=15). Total NAA (N-acetylaspartate + N-acetylaspartylglutamate, tNAA) and glutamate were lower and glutamine, *myo*-inositol and total creatine (creatine + phosphocreatine, tCr) were higher in patients relative to controls, consistent with neuronal dysfunction/loss, gliotic activity and alterations in glutamate-glutamine cycling and energy metabolism. Changes in tNAA, tCr, *myo*-inositol and glutamate levels were discernible in individual spectra and the tNAA/*myo*-inositol ratio in the cerebellar hemispheres and pons differentiated the patients from controls with 100% specificity and sensitivity. In addition, tNAA, *myo*-inositol and glutamate levels in the cerebellar hemispheres and the tNAA and *myo*-inositol levels in the pons correlated with ataxia scores (Scale for the Assessment and Rating of Ataxia, SARA). Two other biomarkers measured in the cerebrospinal fluid (CSF) of a subset of the volunteers (F₂-isoprostanes as a marker of oxidative stress and glial fibrillary acidic protein (GFAP) as a marker of gliosis) were not different between patients and controls. These data demonstrate that ¹H MRS biomarkers can be utilized to non-invasively assess neuronal and glial status in individual ataxia patients.

Keywords

SCA1; MRS; ataxia; cerebellum; neurochemical profile

Spinocerebellar ataxias (SCAs) are a clinically and genetically heterogeneous group of movement disorders characterized by loss of cerebellar Purkinje cells in combination with neuronal loss in other regions, such as the dentate nucleus and pons.^{1, 2} Spinocerebellar ataxia type 1 (SCA1) was the first SCA for which the genetic defect was uncovered³ and is one of nine polyglutamine disorders identified so far.⁴ It is caused by the expansion of an unstable CAG repeat that results in a polyglutamine expansion in ataxin-1.²

Although there are currently no treatments for SCAs⁵, neurodegeneration is reversible in SCA1 mouse models by suppressing the expression of the mutant ataxin-1.^{6, 7} Successful implementation of such treatments will be facilitated by the establishment of objective, robust biomarkers. Biochemical changes associated with neuronal dysfunction/loss can be quantified by proton magnetic resonance spectroscopy (¹H MRS) and may serve as biomarkers that directly gauge neuronal and glial status. Prior studies implicated MRS as a powerful technique to detect cerebellar biochemical alterations in SCAs.^{8–10} However, many grouped several SCA subtypes together, resulting in a pathogenically heterogeneous sample. Following the establishment of genetic testing for SCAs, two MRS studies performed at 1.5 tesla (T) reported lower cerebellar N-acetylaspartate (NAA) and NAA/creatine in SCA1.^{9, 10} At higher fields, MRS enables quantification of 10–18 neurochemicals (“neurochemical profile”).^{11, 12} Furthermore, metabolites can be quantified with higher precision enabling detection of differences with smaller sample sizes. Therefore,

we hypothesized that multiple neurochemical abnormalities that reflect the clinical status would be detectable at 4T in the cerebellum and brainstem of patients with early-to-moderate stage SCA1. We evaluated the specificity and sensitivity of these MRS biomarkers to distinguish patients from controls. We further measured two other biomarkers in cerebrospinal fluid (CSF) of a subset of the volunteers to compare to the MRS findings. First, we quantified F₂-isoprostanes as markers of oxidative stress¹³ which has been suggested as a pathogenic factor in polyglutamine diseases¹⁴, and also specifically in SCA1.¹⁵ Second, we measured glial fibrillary acidic protein (GFAP), which is a gliosis marker.^{16, 17} Our goal was to compare F₂-isoprostane levels to the putative MRS oxidative stress markers glutathione and vitamin C and GFAP levels to the putative MRS gliosis markers *myo*-inositol and glutamine.

Methods

Patients, control participants and study design

Fifteen healthy volunteers and 11 patients with early-to-moderate stage SCA1 (Table 1), matched for age range, participated in the study after giving written informed consent using procedures approved by the Institutional Review Board: Human Subjects Committee of University of Minnesota. Smoking and diabetes were exclusion criteria as both may cause oxidative damage which could confound our findings. Volunteers were asked to discontinue antioxidant supplements for 3 weeks prior to the study since the most common supplemental antioxidants are eliminated over this time.^{18, 19}

All subjects underwent MR scanning; a subset of the volunteers also underwent lumbar punctures and was evaluated by a standardized ataxia rating scale (Scale for the Assessment and Rating of Ataxia, SARA)²⁰ within one day of MR scanning. MR spectra were acquired from 3 volumes-of-interest (VOI): vermis, cerebellar hemisphere and pons. Vermis and cerebellar hemisphere data were acquired from 9 patients and pons data from 8 patients. Useful MRS data could not be collected from two patients due to restless leg syndrome in one case and claustrophobia in the other. Vermis data were acquired from 13 controls, cerebellar hemisphere data from 11 and pons data from 10. All patients and 10 healthy controls were evaluated by the SARA to seek associations between the MRS markers and clinical status. CSF was obtained from 5 patients and controls by lumbar puncture via the L3-L4 intervertebral space under local anesthesia. Routine cell count, glucose, lactate (Table 1) and protein levels were normal in all CSF samples.

MR protocol

All studies were performed with a 4T magnet (Oxford Magnet Technology, Oxford, UK) with an INOVA console (Varian, Palo Alto, CA) as described before.²¹ Briefly, sagittal, coronal and transverse multi-slice images were obtained with a fast spin echo sequence (repetition time TR = 4.5 s, echo train length = 8, echo spacing = 15 ms, 9 slices, 2 averages). Proton spectra from the vermis ($1.0 \times 2.5 \times 2.5 \text{ cm}^3$), cerebellar hemisphere ($1.7 \times 1.7 \times 1.7 \text{ cm}^3$) and pons ($1.6 \times 1.6 \times 1.6 \text{ cm}^3$) were acquired with an ultra-short echo stimulated-echo acquisition mode (STEAM) sequence (echo time TE = 5 ms, TR = 4.5 s, 128 averages). Single shot spectra were averaged over 4–8 scans for frequency and phase correction and to exclude those that showed evidence for motion prior to summing. Unsuppressed water spectra were acquired at a series of TE values (TE = 5 – 5000 ms; TR = 15 s for full relaxation) to evaluate the CSF contribution to the VOI.²²

Metabolite quantification

Metabolites were quantified using an automated deconvolution program (LCModel)²³ as described previously.²¹ The model spectra for alanine (Ala), aspartate (Asp), ascorbate/

vitamin C (Asc), glycerophosphocholine (GPC), phosphocholine (PC), creatine (Cr), phosphocreatine (PCr), γ -aminobutyric acid (GABA), glucose (Glc), glutamine (Gln), glutamate (Glu), glutathione (GSH), *myo*-inositol (*myo*-Ins), lactate (Lac), N-acetylaspartate (NAA), N-acetylaspartylglutamate (NAAG), phosphoethanolamine (PE), *scyllo*-inositol (*scyllo*-Ins) and taurine (Tau) were generated based on previously reported chemical shifts and coupling constants.^{24, 25} Macromolecule spectra were acquired using an inversion recovery sequence. Metabolite concentrations were obtained relative to an unsuppressed water spectrum acquired from the same VOI assuming a water content of 72% for cerebellar hemispheres (primarily white matter) and 82% for vermis and pons.²⁶ The metabolite concentrations were corrected for the amount of CSF (obtained by fitting the integrals of the unsuppressed water spectra at different TE values with a biexponential decay²²) assuming zero concentration of metabolites in CSF²⁷, except for glucose and lactate. The tissue concentrations of glucose and lactate were obtained based on the % CSF contribution to the VOI and a CSF concentration of 3.2 mM for glucose and 1.8 mM for lactate (Table 1). Metabolites quantified with Cramér-Rao lower bounds (CRLB, estimated error of metabolite quantification) > 50% were classified as not detected. Only metabolites quantified with CRLB $\leq$ 50% in at least half of the spectra from a brain region were included in the final neurochemical profile. Ala and PE in the vermis, Ala, Asp, PE and *scyllo*-Ins in the cerebellar hemisphere and Ala, Asp, GABA, Gln and PE in the pons were excluded from final analysis based on these criteria. If the correlation between two metabolites was consistently high (correlation coefficient < -0.5) in a given region, their sum was reported.²⁸ Thus, total creatine (tCr, Cr+PCr) and total choline (tCho, GPC+PC) were reported in all regions and total NAA (tNAA, NAA+NAAG) and Glc + Tau were reported in the cerebellar hemispheres and pons. Therefore, 15 statistically independent concentrations were evaluated in the vermis (NAA, tCr, tCho, *myo*-Ins, Glu with average CRLB $\leq$ 7%, Gln, GSH, Tau with average CRLB < 20%, Asc, Asp, GABA, Glc, Lac, NAAG, *scyllo*-Ins with average CRLB $\leq$ 35%), 11 in the cerebellar hemispheres (tNAA, tCr, tCho, *myo*-Ins with average CRLB < 7%, Glu with average CRLB of 11%, Asc, GABA, Gln, GSH, Lac, Glc + Tau with average CRLB < 7 35%) and 10 in the pons (tNAA, tCr, tCho, *myo*-Ins with average CRLB < 7%, Glu with average CRLB of 12%, Asc, GSH, Lac, *scyllo*-Ins, Glc + Tau with average CRLB < 35%).

Ataxia rating scale

The SARA consists of 8 quantitative exam features for gait, stance, sitting, speech disturbance and limb kinetic functions²⁰ and yields a composite ataxia score in the range of 0 (no ataxia) – 40 (most severe ataxia).

Isoprostane and GFAP measurements in CSF

CSF was centrifuged to remove cells and the supernatant frozen in aliquots at -80°C. F₂-isoprostane levels were measured with a negative ion chemical ionization GC/MS based method as described previously.^{29, 30} The limit of detection was 10 pg/mL. GFAP levels were assayed by a commercially available ELISA kit which is a biotin labeled antibody based sandwich enzyme immunoassay (BioVendor, LLC, Candler, NC) specific for human GFAP with a limit of detection 0.1 ng/mL. Each sample was assayed in triplicate.

Statistical Analysis

MRS data from the two groups were compared using ANCOVA, adjusting for age and gender, for each neurochemical concentration in each region separately. Secondary analyses using a linear mixed model to account for the potential correlation across regions within person were not different from results shown here. Because of a few outliers and skewness for some measures, analyses were repeated using a Wilcoxon test; results were again not different from those shown here. Receiver operating characteristic (ROC) analyses were

used to compute sensitivity and specificity of each neurochemical in distinguishing patients from controls. Linear regression analyses were performed to evaluate the relationship between neurochemical concentrations and ataxia scores. P-values shown have not been adjusted for multiple testing. Measures of spectral quality (signal-to-noise ratio, SNR, and linewidth) and CSF data from the two groups were compared using the two-tailed, unpaired student's t-test.

Results

Spectral quality in patients and controls

Proton MR spectra with good SNR and spectral resolution were consistently obtained from both patients and controls (Fig. 1). The only difference in spectral quality was a slightly higher spectral linewidth in the cerebellar hemispheres of patients resulting in lower SNR (Table 1). Cerebellar atrophy was apparent by a higher CSF contribution to the vermis and cerebellar hemisphere VOI in patients.

The spectral patterns were characteristic of each of the three brain regions (Fig. 1, left) and the high spectral quality permitted the observation of neurochemical alterations in individual patients (Fig. 1). Specifically, decreased NAA and Glu and increased *myo*-Ins and tCr relative to controls were discernible in the patient spectra.

Neurochemical alterations in patients with SCA1

Multiple metabolites were significantly different in patients than controls (Fig. 2). In the vermis, NAA and Glu were lower and Gln higher in patients. The Glu/Gln ratio identified patients with 89% sensitivity and 100 % specificity (no false positives) based on ROC analysis. In addition, trends for lower Asp ($p = 0.08$) and higher *myo*-Ins ($p = 0.1$) were observed in this region and confirmed by quantifying spectra summed over all patients and controls (46% lower Asp, 16% higher *myo*-Ins in patients). The cerebellar hemispheres were more extensively affected than the vermis based on the significance levels of neurochemical alterations (Fig. 2). Thus, tNAA and Glu levels were lower and *myo*-Ins, tCr and Glc + Tau were higher here. Interestingly, GABA was not detectable (CRLB > 50%) in most patient spectra and Gln in most control spectra from the cerebellar hemispheres (Fig. 2), and analysis of summed spectra confirmed 32% lower GABA and 44% higher Gln in patients than controls. In the pons, tNAA and Glu were lower and *myo*-Ins higher in patients. Summed spectra further revealed lower Asp (55%) and higher Gln (125%) levels in the pons of patients than controls.

Consistent with unchanged levels of the antioxidants GSH and Asc, there was no difference in F₂-isoprostane levels in CSF (Table 1). CSF GFAP levels were also not different between patients and controls (Table 1) despite changes in the putative gliosis markers *myo*-Ins and Gln in the cerebellum and brainstem. Interestingly though, the patient with the highest GFAP level (2.1 ng/mL) was also the patient with the highest *myo*-Ins concentration in the vermis and one of the highest *myo*-Ins levels in the cerebellar hemispheres and pons. The average GFAP level of the remaining 4 patients was identical to that of the controls (0.9 ± 0.1 ng/mL).

The most robust biomarkers that separated the patients from controls were tNAA, *myo*-Ins, Glu and tCr in the cerebellar hemispheres and tNAA, *myo*-Ins and Glu in the pons. Plotting their levels against each other separated the groups with no or almost no overlap (Fig. 3). In fact, the tNAA/*myo*-Ins ratio in both regions classified the patients with 100% specificity and sensitivity.

Correlations of neurochemical levels with clinical status

The tNAA, Glu and *myo*-Ins levels in the cerebellar hemispheres and tNAA and *myo*-Ins in the pons significantly correlated with the ataxia scores (Fig. 4). These correlations were also apparent when only the patient data (without the controls) were plotted. tNAA displayed the most robust correlations with SARA. These correlations were detectable despite the limited range of disease stage among the patients studied (the highest SARA score was 14.5 of the maximum of 40).

Discussion

We report numerous neurochemical alterations in the cerebellum and brainstem of patients with SCA1 relative to healthy controls. By utilizing a 4T MR scanner we were able to detect highly statistically significant changes (Fig. 2), some of which were discernable in spectra from individuals (Fig. 1). tNAA, *myo*-Ins and Glu levels allowed complete separation of patients from controls (Fig. 3) and correlated with the SARA score indicating their applicability as robust biomarkers in SCAs.

We also investigated two CSF biomarkers because CSF measurements closely reflect biochemical and cellular processes active in the brain. We did not detect any indication for oxidative stress in SCA1 both by F₂-isoprostane levels in CSF and the antioxidant levels measured by MRS. On the other hand, gliosis was implied by MRS, but not GFAP levels, except in one patient. Therefore, gliotic activity may need to reach a certain threshold before GFAP levels increase significantly in CSF. Taken together, these data demonstrate the advantage of direct, non-invasive measurements in the brain and suggest that cellular alterations may be too localized to cause changes in CSF.

The MRS approach has the potential to monitor multiple aspects of pathology simultaneously. The most frequently studied MRS biomarker NAA is localized exclusively to neurons and is indicative of both neuronal cell number and viability.³¹ NAA was decreased in all regions, in agreement with prior MRS studies of cerebellar hemispheres and pons in SCA1.^{9, 10} The decrease in the cerebellar hemispheres and pons was more substantial than that in the vermis, consistent with a more substantial pathological involvement and atrophy in these regions.⁸ In fact, the vermis has not been previously studied by MRS in SCA1 as it was thought to be relatively spared.¹⁰ In addition, glutamate was decreased and glutamine increased in the vermis of patients (Fig. 2). Glutamate is primarily localized to neurons, with the highest levels in glutamatergic neurons, while glutamine is preferentially localized in glial cells.³² Glutamine levels may increase with gliosis,^{21, 33} however, *myo*-inositol, also primarily localized to glial cells³⁴ and typically considered a gliosis marker,^{21, 33, 35, 36} only showed a trend to increase in the vermis in SCA1. Therefore, the Glu and Gln changes in the vermis are likely indicative of disruptions in glutamate-glutamine cycling, which could result from loss of glutamatergic synapses in the cerebellar cortex, the principal excitatory input to Purkinje cells.

In the cerebellar hemispheres, the decreased tNAA and Glu levels were likely due to the known loss of pontocerebellar and Purkinje cell axons, as well as loss of neurons in the dentate nucleus.³⁷ Astrocytosis is extensive in cerebellar white matter in SCA1³⁷ and was indicated by the increased *myo*-inositol and potentially also by increased creatine³⁸ and glutamine levels. Summed spectra also indicated lower GABA concentrations in SCA1 which may be an indicator of loss of the axons of GABAergic Purkinje cells that contain high levels of GABA.³⁹ The increased creatine levels may also indicate changes in cellular energy metabolism. Decreased cerebellar glucose metabolism in SCA1 was indeed demonstrated using [¹⁸F]fluorodeoxy-glucose-PET⁴⁰ and increased tCr levels were also observed in a mouse model of Huntington's disease, another CAG triplet repeat disease that

involves alterations in energy metabolism.⁴¹ A previous MRS study that used VOIs very similar to ours did not find a difference in creatine levels in SCA1⁹. In this study, metabolite levels were not corrected for CSF contribution since the researchers attempted to avoid CSF spaces by careful volume selection. However, we found that CSF contribution to the cerebellar hemisphere VOI was higher in patients than controls, even though this was not apparent on images during volume selection. Therefore metabolite concentrations may have been underestimated in patients in the previous study, at least partly explaining the discrepancy in the creatine findings.

Consistent with extensive neuronal loss and astrogliosis in the pons in SCA1,³⁷ we observed decreased NAA, glutamate and increased *myo*-inositol. The levels of these metabolites in the pons and cerebellar hemispheres enabled robust separation of patients from controls (Fig. 3). In general, changes in neurochemical levels greater than twice the CRLB can be detected in individual spectra with 95% confidence.²⁸ The mean CRLB of NAA, *myo*-Ins and Glu in the control spectra were 4, 5–7 and 10–11%, respectively, in the cerebellar hemispheres and pons with differences in patients of up to 37, 77 and 40% relative to controls, substantiating our ability to detect these changes in spectra from individuals (Fig. 1). These metabolites also correlated with the ataxia score (Fig. 4) indicating they are reliable markers of disease progression. This finding is consistent with a similar correlation observed between NAA levels in pons and a different ataxia score in SCA1⁹ and extends the number of neurochemicals that can potentially serve as biomarkers in SCA1.

Other changes observed in summed spectra, e.g. decreased GABA, need to be substantiated further to be utilized as reliable biomarkers, since these analyses do not permit a statistical comparison. Also note that the specificity and sensitivity levels reported here, e.g. 100% for the tNAA/*myo*-Ins ratio, may be somewhat lower with a larger sample. The SCAs present the ideal test case for the specificity/sensitivity assessment due to the availability of genetic testing as the gold standard for diagnosis. Clearly, MRS would not be utilized diagnostically in SCAs; however, this analysis demonstrates the robustness of these biomarkers. While the current study was performed at a magnetic field not immediately accessible in the clinic, we expect that most of our results will be applicable to 3T, an increasingly available field strength. Two limitations of our study are a small sample size and its cross-sectional nature. To demonstrate that MRS can be used to monitor disease progression and serve as a surrogate marker in clinical trials, longitudinal studies with larger sample sizes are needed.

In conclusion, these data demonstrate the potential for high field MRS to detect neurochemical alterations in SCA1 with high sensitivity. These non-invasive biomarkers are expected to enable monitoring of disease progression and treatment efficacy on an individual patient basis because they provide a more direct assessment of neuronal and glial status than peripheral and even CSF biomarkers.

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Figure 1. Localized proton MR spectra obtained from the vermis (A), cerebellar hemispheres (B) and pons (C)

Left: healthy volunteer (50 year old male); right: patient with SCA1 (55 year old female).

The alterations in total NAA, glutamate, *myo*-inositol and total creatine visible in the spectra of the patient are shown.

Figure 2. Neurochemical profiles of control subjects and patients with SCA1

Only metabolites that were quantified with CRLB $\leq 50\%$ in most subjects in a group are plotted. Error bars are $2 \times \text{S.E. (95\% C.I.)}$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Abbreviations are in Methods.

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PONS

Figure 3. Separation of patients with SCA1 from controls based on total NAA, *myo*-inositol, glutamate and total creatine levels

The patient with the highest ataxia score (14.5) had the lowest tNAA and highest *myo*-inositol levels (shown with arrows).

Figure 4. Correlation of neurochemical levels and clinical status
Concentrations of total NAA, *myo*-inositol and glutamate in the cerebellar hemispheres and total NAA and *myo*-inositol in the pons are significantly correlated with the SARA score. Correlation coefficients and significance levels are shown.

Table 1

Demographic and clinical characteristics of subjects, CSF levels of F₂-isoprostanes, GFAP, glucose and lactate in 5 patients and 5 controls and spectral quality measures (Values given as counts or as mean $\pm$ SD, as appropriate).

	SCA1 (N=11)	Controls (N=15)	P-values
Gender (Female/Male)	4/7	8/7	
Age (years)	55 $\pm$ 6	52 $\pm$ 8	0.23
Age of onset (years)	44 $\pm$ 11	N / A	
Disease duration (years)	11 $\pm$ 9	N / A	
# of CAG triplet repeats in the long allele of ataxin-1 gene	44 $\pm$ 3 (range 40–50)	N / A	
SARA score	9 $\pm$ 4 (N = 11)	0 $\pm$ 0 (N = 10)	< 0.01
F ₂ -Isoprostanes (pg/mL)	27 $\pm$ 12	32 $\pm$ 11	0.57
GFAP (ng/mL)	1.1 $\pm$ 0.6	0.8 $\pm$ 0.1	0.26
Glucose (mg/dL)	61 $\pm$ 6	57 $\pm$ 8	0.34
Lactate (mM)	1.6 $\pm$ 0.1	1.9 $\pm$ 0.2	0.02
SNR [*] , vermis	16 $\pm$ 3	17 $\pm$ 2	0.27
SNR, cerebellar hemispheres	11 $\pm$ 2	13 $\pm$ 1	0.02
SNR, pons	12 $\pm$ 2	12 $\pm$ 3	0.62
Linewidth, vermis (Hz)	6 $\pm$ 1	6 $\pm$ 1	0.53
Linewidth, cerebellar hemispheres (Hz)	8 $\pm$ 1	7 $\pm$ 1	0.09
Linewidth, pons (Hz)	7 $\pm$ 2	8 $\pm$ 2	0.46
% CSF, vermis	33 $\pm$ 10%	12 $\pm$ 5%	< 0.01
% CSF, cerebellar hemispheres	7 $\pm$ 6%	2 $\pm$ 1%	0.02
% CSF, pons	4 $\pm$ 3%	3 $\pm$ 1%	0.36

* SNR: signal-to-noise ratio