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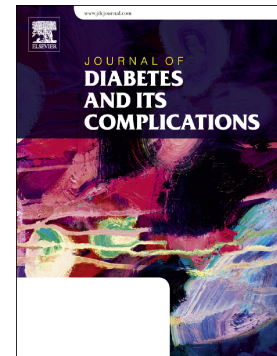
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*Original Article***Brain spectroscopy reveals that N-acetylaspartate is associated to peripheral sensorimotor neuropathy in type 1 diabetes**

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## Abstract

### Aims

Emerging evidence shows, that distal symmetric peripheral neuropathy (DSPN) also involves alterations in the central nervous system. Hence, the aims were to investigate brain metabolites in white matter of adults with diabetes and DSPN, and to compare any cerebral disparities with peripheral nerve characteristics.

### Methods

In type 1 diabetes, brain metabolites of 47 adults with confirmed DSPN, were compared with 28 matched healthy controls using proton magnetic resonance spectroscopy (H-MRS) in the parietal region including the sensorimotor fiber tracts.

### Results

Adults with diabetes had 9.3% lower ratio of N-acetylaspartate/creatine (NAA/cre) in comparison to healthy ( $p<0.001$ ). Lower NAA/cre was associated with lower sural ( $p=0.01$ ) and tibial ( $p=0.04$ ) nerve amplitudes, longer diabetes duration ( $p=0.03$ ) and higher age ( $p=0.03$ ). In addition, NAA/cre was significantly lower in the subgroup with proliferative retinopathy as compared to the subgroup with non-proliferative retinopathy ( $p=0.02$ ).

### Conclusions

The association to peripheral nerve dysfunction, indicates concomitant presence of DSPN and central neuropathies, supporting the increasing recognition of diabetic neuropathy being, at least partly, a disease leading to polyneuropathy. Decreased NAA, is a potential promising biomarker of central neuronal dysfunction or loss, and thus may be useful to measure progression of neuropathy in diabetes or other neurodegenerative diseases.

Keywords: Diabetes neuropathy: Magnetic Resonance Spectroscopy: Metabolites: Central nervous system: Peripheral Neuropathy: N-acetylaspartate

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## 1. Introduction

Type 1 diabetes is associated with microvascular complications, which leads to distal symmetric peripheral neuropathy (DSPN) in up to 50% of the cases<sup>1</sup>. DSPN affects quality of life and increases morbidity and mortality considerably, but the pathophysiological processes are not fully elucidated<sup>2</sup>. The traditional concept is that DSPN predominantly affects the peripheral (somatic and autonomic) nervous system. However, accumulating evidence indicates that diabetes concomitantly involves alterations in the central nervous system (CNS)<sup>1,3-6</sup>. Hence, it is plausible that poor glycemic control and disease duration may cause concomitantly systemic neuronal damage, which also involve neurons in the CNS<sup>1</sup>. Hitherto, magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS) have provided valuable insights into changes in the CNS. Especially, cognitive and functional impairment<sup>7,8</sup>, structural changes<sup>9,10</sup>, and altered cerebral metabolism<sup>11-14</sup> have been shown.

In type 1 diabetes, the CNS alterations occur diffusely throughout the brain<sup>15</sup> and occurrence of white matter alterations have been shown in type 1 diabetes<sup>16,17</sup>. As the white matter rich parietal region also involves major sensorimotor fiber tracts, it is of special interest to investigate metabolic alterations in this region and its relation to peripheral neuropathy changes. Previously, proton MRS in white matter parietal region of adults with diabetes have been investigated<sup>16,18</sup>. To address whether alterations in white matter occur concomitantly with peripheral neuronal damage, we used MRS, which is a non-invasive method to estimate brain metabolites. Associations to DSPN have previously been demonstrated in thalamus<sup>14</sup>. We focused on N-acetylaspartate (NAA), which is a recognized marker of neuronal functionality and density; creatine (cre), which is involved in neuronal energy metabolism; glutamate (glu), which is a neurotransmitter, myo-inositol (mI), which is a glial marker and choline containing compounds, which are associated to membrane turnover<sup>19</sup>, and thus present potential indicators or biomarkers of neurodegeneration.

We hypothesized that adults with type 1 diabetes and confirmed DSPN, would have abnormal brain metabolite concentrations in the white matter parietal region and that these alterations were associated to severity of the DSPN. Specifically, we aimed to 1) identify differences in NAA/cre as the primary outcome, 2) explore other brain metabolite concentrations between adults with type 1 diabetes and confirmed DSPN and healthy controls and 3) to explore the association between metabolite concentrations, clinical characteristics and neurophysiological assessments of peripheral nerve function.

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## 2. Material and methods

### 2.1. Study population

Forty-eight adults with type 1 diabetes and confirmed DSPN and 28 healthy controls were included in this study.

Data from adults with diabetes were collected as baseline data in a double-blind randomized controlled trial where the neuroprotective effects of liraglutide for treatment of diabetes neuropathy were investigated (EUDRA CT 2013-004375-12). Subjects were recruited at the Department of Endocrinology, Aalborg University Hospital, Denmark. Subjects were only included in the study if they had confirmed DSPN according to the Toronto Diabetic Neuropathy Expert Group<sup>20</sup>, based on a neurophysiological assessment of nerve conduction velocity and amplitudes of the larger axons from the extremities, conducted and interpreted by a specialist at the Department of Clinical Neurophysiology, Aalborg University Hospital, Denmark. Photographs of the right retina were used to assess severity of diabetic retinopathy and were graded by a specialist at the Department of Ophthalmology, Aalborg University Hospital, Denmark. MRI scans were carried out at Department of Radiology, Aalborg University Hospital, Denmark not longer than 1 month apart from the neurophysiological testing. Inclusion criteria were age over 18 years, verified diagnosis of type 1 diabetes for a minimum of 2 years ( $\text{HbA}_{1c} \geq 48 \text{ mmol/mol}$  ( $\geq 6.5\%$ )), stable treatment, confirmed DSPN as outlined above, and body mass index (BMI) equal or above  $22 \text{ kg/m}^2$ .

An established dataset (N-20090008 approved by The North Denmark Region Committee on Health Research Ethics) of healthy controls obtained from Department of Radiology, Aalborg University Hospital, Denmark were age-matched to the dataset in diabetes. All controls were clinically screened to confirm a healthy status without any relevant CNS related diseases, medication or diabetes.

Approval from the local ethics committee was obtained and all participants gave written, informed consent and were free to withdraw from the study at any time.

## 2.2. Magnetic Resonance Spectroscopy

We used a 3T GE scanner (GE Signa HDxt, General Electric, Milwaukee, WI, USA) with a standard eight-channel head coil. The head was fixed using foam pads. Single voxel PRESS (Point RESolved Spectroscopy) MRS were acquired (TR/TE = 2,000/30 ms). The scan time was 5 minutes and total number of scans was 128. The voxel of interest (VOI) was positioned in the parietal cortex (15 x 15 x 50 mm) contralateral to the side of the dominant hand. The VOI was positioned on a high resolution axial T1-weighted structural scan and placed to cover as much white matter as possible, see example in Figure 1. To monitor potential scanner drift, PRESS MRS was acquired from a phantom on each day. Axial FLAIR and 3D T2-weighted structural scans were evaluated by a radiologist for the presence of white matter hyperintensities (WMH) and other relevant pathology. Post-processing analyses were performed in LCModel (Version 6.3)<sup>21</sup>. Water scaling and eddy-current correction were applied and metabolites were fitted in the chemical shift range 0.1-4.0 ppm. Cre, NAA, glu, mI, GPC (glycerophosphocholine), NAA/cre, glu/cre, mI/cre and GPC/cre were analyzed. Metabolite concentrations are often expressed as ratios as partial volume effects can be avoided. Cre is frequently used as the reference and assumed to be constant. However, alterations can be observed for instance in pathological conditions<sup>22</sup>. Thus, we included both concentrations and ratios. All metabolites had sufficient quality with Cramér-Rao bounds <20%.

## 2.3. Neurophysiological measurements

To determine the severity of peripheral neuronal function of the extremities, amplitude and nerve conduction velocity measurements from an electroneurography test performed on the sural nerve (sensory), the tibial nerve (motor), median nerve (motor) and radial nerve (sensory) were used. Nerve action potentials were recorded antidromic using surface electrodes. Skin temperature was maintained above 32°C during testing. In case of an unmeasurable value, the value prior to lowest detected value was assigned.

#### *2.4. Statistical analysis*

All statistical analyses were performed in IBM SPSS Statistics (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.). Differences between healthy and diabetes were assessed using appropriate independent-sample t test or chi-squared test. The primary outcome was NAA/cre. Furthermore, brain metabolites and clinical measures of neuropathy are reported. Data are presented as mean  $\pm$  standard deviation and  $p < 0.05$  was considered significant. To compensate for the multiple testing of metabolite levels a Bonferroni correction was used and  $p < 0.006$  was considered significant for comparisons of metabolite levels. Pearson's correlations were calculated to explore the association between significant metabolite changes, clinical characteristics and nerve conduction measurements.

### 3. Results

#### 3.1. Demographics and data

47/48 adults with diabetes (38 men, mean age  $50.0 \pm 8.5$  years) and 28 healthy controls (17 men, mean age  $49.9 \pm 11.9$  years) underwent the MRI scan. One subject suffered from claustrophobia and did not undergo MRI. Baseline demographics are provided in Table 1 (some parameters have previously been published in a paper focusing on gastrointestinal transit<sup>23</sup>). The control group was age ( $p=0.98$ ) and gender ( $p=0.08$ ) matched, however a significant difference in BMI ( $p<0.001$ ) was observed. Due to high Cramér-Rao ( $>46\%$ ), glu, mI and GPC measurements were not measurable for one adult with diabetes and the GPC measurement was not measurable for one healthy controls. Sural nerve amplitude and nerve conduction velocity were measurable for 30 adults with diabetes (18 values were assigned to  $0.79\mu\text{V}$  and  $31.0\text{m/s}$ , respectively) and tibial nerve amplitude and nerve conduction velocity were measurable for 41 and 43 adults with diabetes, respectively (7 values were assigned to  $0.19\text{mV}$  and 5 values were assigned to  $20.0\text{m/s}$ ). For radial nerve amplitude and nerve conduction velocity 2 values were not measurable and thus assigned to  $1.19\mu\text{V}$  and  $39.0\text{m/s}$ , respectively. Three subjects in the diabetes group had amputations at the toe level and MRS was obtained ipsilateral to the side of amputations. No large WMH were observed within the MRS voxels. There was no difference in the number of WMHs between adults with diabetes and healthy controls (diabetes: no WMH: 70.2%, 1-5 WMH: 19.1%,  $>5$  WMH: 10.6%; controls: no WMH: 64.3%, 1-5 WMH: 25.0%,  $>5$  WMH: 10.7%,  $p=0.83$ ) No other relevant pathology was observed in the spectroscopy voxels.

### 3.2. Magnetic Resonance Spectroscopy

Metabolite concentrations are provided in Table 1. Adults with diabetes had in average 9.3% lower levels of NAA/cre as compared to healthy controls ( $p<0.001$ ). No significant differences between other metabolites were shown (all  $p>0.01$ ). No scanner drift was observed on MRS phantom scans. No significant difference was found in signal-to-noise ratio between spectra from the diabetes and control groups ( $22.6\pm 5.3$  and  $21.0\pm 6.3$ , respectively ( $p=0.23$ )).

### 3.3. Metabolic correlations to neurophysiological assessments and clinical characteristics

There was a positive association between NAA/cre and sural nerve amplitude ( $r=0.36$ ,  $p=0.01$ ) and tibial nerve amplitude ( $r=0.30$ ,  $p=0.04$ ) (Figure 2). No associations were found for sural ( $r=0.20$ ,  $p=0.18$ ) or tibial ( $r=-0.01$ ,  $p=0.95$ ) nerve conduction velocities or for median nerve amplitude ( $r=0.06$ ,  $p=0.69$ ), conduction velocity ( $r=0.003$ ,  $p=0.99$ ), radial nerve amplitude ( $r=0.12$ ,  $p=0.43$ ) and conduction velocity ( $r=0.03$ ,  $p=0.84$ ). Furthermore, there was a negative association between NAA/cre and diabetes duration ( $r=-0.31$ ,  $p=0.03$ ) and age ( $r=-0.32$ ,  $p=0.03$ ). No association was found between NAA/cre and BMI ( $r=0.80$ ,  $p=0.59$ ). Thus, lower NAA/cre levels were associated with lower sural and tibial nerve amplitudes, longer diabetes duration and higher age. Additionally, the NAA/cre levels were lowest in the group of adults with type 1 diabetes and DSPN and proliferative retinopathy ( $1.39\pm 0.14$ ) as compared to adults with type 1 diabetes, DSPN and non-proliferative retinopathy ( $1.49\pm 0.14$ ) ( $p=0.02$ ). Figure 3 illustrates the NAA/cre levels in the two diabetes groups and in healthy controls.

#### 4. Discussion

This study investigated involvement of the CNS in adults with type 1 diabetes and confirmed DSPN. The study showed alterations in brain metabolites in white matter rich parietal regions evident as lower levels of NAA/cre in the diabetes group. Lower NAA/cre levels were associated with lower nerve amplitudes of the lower extremities, and with proliferative retinopathy. This altogether supports the increased recognition of diabetic neuropathy being, at least partly, a disease that also affects the central nervous system.

NAA is one of the most abundant brain metabolites, mainly synthesized from acetyl-CoA and aspartate and NAA is believed to be a marker of neuronal functionality and density<sup>22,24</sup>. Cre is involved in neuronal energy metabolism<sup>19</sup>. We demonstrated lower NAA/cre levels in adults with type 1 diabetes and confirmed DSPN. The data supports previous findings, where decreased levels of NAA/cre have been identified in thalamus in adults with type 1 diabetes<sup>14</sup>, and in pons and left posterior parietal white matter in children with poorly glyceemic control<sup>25</sup>. Furthermore, a similar reduction in NAA was reported in the dorsolateral prefrontal cortex in adults with type 1 and type 2 diabetes and neuropathy<sup>12</sup>. In addition reduction in NAA has been shown in the occipital and parieto-occipital lobe in adults with type 1 diabetes during a hyperglycemic clamp<sup>26</sup>. Lower levels of NAA in the thalamus has also been reported in non-diabetic patients with neuropathic pain<sup>27</sup>, indicating that similar metabolic changes could be induced by both diabetic nerve damage and neuropathic pain mechanisms. Thus, our results may support that the pathomechanisms underlying neuropathy, despite origin, includes neurodegeneration caused by e.g. oxidative stress. Three subjects in the diabetes group had amputations at the toe level. Amputations could potentially lower levels of NAA which has been reported in upper limb amputees contralateral to the missing hand together with unaltered ipsilateral NAA levels<sup>28</sup>. MRS in our study was acquired ipsilateral to the

side of amputations. Furthermore, potentially decreased NAA/cre may also reflect more severe neuropathy, as amputations can result from diabetes neuropathy<sup>29</sup>.

Previously Zhang et al, showed that alterations of the sensory nerve amplitudes was the most sensitive and reliable predictor of DSPN<sup>30</sup>. We demonstrated an association between NAA/cre and sural (sensory) and tibial (motor) nerve amplitudes, interpreted as an association between the severity of peripheral nerve damage (lower nerve amplitudes) and the degree of loss of central neuronal functionality/density (lower NAA/cre). No correlations were found to nerve conduction velocities, which may indicate axonal loss rather than demyelination of the peripheral nerves to be associated to CNS alterations. Moreover, the associations were only demonstrated for the lower extremities, implying the longest nerve fibers to be most affected. On the other hand, measurements from the upper extremities could be more affected by other conditions such as carpal tunnel syndrome or traumatic nerve damage. Thus, the association between NAA/cre and lower nerve amplitudes of the lower extremities implies primarily an axonal loss and that the pathogenesis of loss of density and function from sensory and motor nerves are not limited to the periphery, but supports the emerging recognition of diabetic neuropathy being, at least partly, a disease that affects the entire nervous system. A similar finding has been demonstrated in the thalamus<sup>14</sup>, indicating an affection of both the thalamic relay station and cortical projections of sensory fibers. Additionally, the lower levels of NAA/cre were associated to age and diabetes duration. There was a significant difference in BMI between the two groups, however no association was found to NAA/cre. Finally, as retinopathy is a marker of general microangiopathy and advanced retinopathy is associated to structural changes in the brain<sup>31,32</sup> and to microbleeds<sup>33</sup>, we explored the levels of NAA/cre in the groups of subjects with non-proliferative and proliferative retinopathy. NAA/cre was significant lower in the group with proliferative retinopathy. This has also been shown in type 2 diabetes<sup>34</sup> and supports that NAA is a relevant neuronal marker of the diabetic brain. The result of NAA/cre being

associated to peripheral nerve dysfunction supports a shared pathogenesis in the periphery and brain, sustaining that central neuronal dysfunction or loss also occur concomitantly. These findings, however, cannot distinguish whether central findings are a consequence of altered upstream activation or peripheral symptoms are caused by altered central processing. No difference in numbers of WMH was present between the diabetes group and healthy controls, which imply the NAA changes observed at likely related to neuropathic process and not microvascular complications. In order to definitively conclude whether changes are due to diabetes itself or to diabetic neuropathy, a diabetes group without neuropathy could be included in future studies. Finally, subgrouping diabetic neuropathy into painful and nonpainful neuropathy may explain the mechanisms involved even further. More research is needed to establish these causalities.

We observed a trend to higher levels of cre in the group with diabetes as compared to healthy controls, however not reaching statistical significance. A similar trend was also observed by Manga et al., who reported an increase of 3% in white matter rich parieto-occipital region in adults with type 1 diabetes<sup>26</sup>. Increased levels of cre are thought to reflect impaired mitochondrial bioenergetics and reduced oxidative phosphorylation<sup>22</sup>, and a slight increase in cre could speculatively be related to enhanced oxidative stress, systemic neurodegeneration or general metabolic alteration in energy demanding metabolism due to diabetes. Even though the voxel of interest was carefully selected to avoid partial volume effect, another explanation could be that the parietal voxel in general contained less white matter compared to the healthy controls due to brain atrophy, enlarged ventricles or small infarcts which are more frequent in diabetes. The concentration of cre is higher in gray matter than white matter<sup>35,36</sup> which potentially also can explain slightly higher levels of cre. However, bias from partial volume effect would systematically affect all metabolite concentrations. In order to further elucidate the relationship between concentrations of cre and oxidative stress studies simultaneously assessing cre and mitochondrial function need to be conducted.

## 5. Conclusions

In conclusion, neuronal metabolic alterations in the central nervous system were observed in type 1 diabetes. NAA/cre levels were associated to peripheral neuropathy severity, grade of retinopathy and disease duration indicating that the parietal white matter is also involved in diabetes neuropathy. Thus, MRS can be useful to increase our understanding of the involvement of CNS in diabetic neuropathy and potentially be implemented as a tool for diagnosing and classifying diabetic neuropathy. Together with knowledge of structural and functional alterations of the brain, the central mechanisms involved in diabetic neuropathy may be better understood. This may support the development of preventative and therapeutic management of diabetes complications. Taken together, decreased NAA, is a potential promising biomarker of central neuronal dysfunction or loss, and thus may be useful to measure progression of neuropathy in diabetes or other neurodegenerative diseases.

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## Tables

**Table 1** - Overview of demographical data, clinical data and metabolite concentrations.

	<b>Adults with type 1 diabetes and DSPN n=48</b>	<b>Healthy controls n=28</b>	<b>p value</b>
Sex (M/F)	38/10	17/11	0.08
Age (years)	50.0 ± 8.5	49.9 ± 11.9	0.98
BMI (kg/m <sup>2</sup> )	28.3 ± 4.4	24.9 ± 2.6	<b>&lt;0.001*</b>
Weight (kg)	90.0 ± 16.0	79.1 ± 12.9	<b>0.003*</b>
Handedness (L/R)	7/41	4/24	0.97
Duration of diabetes (years)	32.2 ± 9.5		
HbA <sub>1c</sub> (mmol/mol)	65.8 ± 10.2		
HbA <sub>1c</sub> (%)	8.2 ± 0.9		
Non-proliferative DR, n (%)	33 (69)		
Proliferative DR, n (%)	15 (31)		
Sural amplitude (µV)	2.6 ± 2.3		
Sural velocity (m/s)	38.2 ± 7.3		
Tibial amplitude (mV)	2.6 ± 2.3		
Tibial velocity (m/s)	37.3 ± 8.9		
Median amplitude (mV)	8.2 ± 2.2		
Median velocity (m/s)	49.7 ± 4.9		
Radial amplitude (µV)	14.0 ± 7.9		
Radial velocity (m/s)	54.6 ± 7.6		
<b>Magnetic resonance spectroscopy</b>			
	<b>n=47</b>	<b>n=28</b>	
Cre (mM)	5.82 ± 0.41	5.55 ± 0.52	0.02
NAA (mM)	8.46 ± 0.72	8.85 ± 0.57	0.02
Glu (mM)	4.91 ± 0.69 <sup>a</sup>	4.79 ± 0.99	0.52
mI (mM)	4.80 ± 0.78 <sup>a</sup>	4.82 ± 1.21	0.94
GPC (mM)	2.01 ± 0.33 <sup>a</sup>	1.91 ± 0.42 <sup>b</sup>	0.13
NAA/cre	1.46 ± 0.15	1.61 ± 0.21	<b>&lt;0.001**</b>
Glu/cre	0.84 ± 0.12 <sup>a</sup>	0.87 ± 0.18	0.53
mI/cre	0.82 ± 0.13 <sup>a</sup>	0.87 ± 0.21	0.26
GPC/cre	0.35 ± 0.06 <sup>a</sup>	0.34 ± 0.07 <sup>b</sup>	0.49

**Notes:** Data are expressed as mean ± standard deviations unless otherwise stated. \* indicates significant findings, \*\* indicates significant findings (Bonferroni correction), <sup>a</sup> n=46, <sup>b</sup> n=27.

**Abbreviations:** DSPN: diabetes symmetric peripheral neuropathy; DR: diabetes retinopathy M: males; F: females; BMI: body mass index; L: left; R: right; cre: creatine; NAA: N-acetylaspartate; glu: glutamate; mI: myo-inositol; GPC: glycerophosphocholine.

**Figure legends**

Figure 1: The position of the voxel of interest in the parietal cortex (15 x 15 x 50 mm).

Figure 2: The association between NAA/cre and sural ( $r=0.36$ ,  $p=0.01$ ) (a) and tibial ( $r=0.30$ ,  $p=0.04$ ) (b) nerve amplitudes.

Figure 3: The NAA/cre levels (mean and standard deviation) for healthy controls ( $n=28$ ), adults with diabetes, confirmed DSPN and non-proliferative retinopathy (NPDR) ( $n=32$ ) and adults with diabetes, confirmed DSPN and proliferative retinopathy (PDR) ( $n=15$ ).

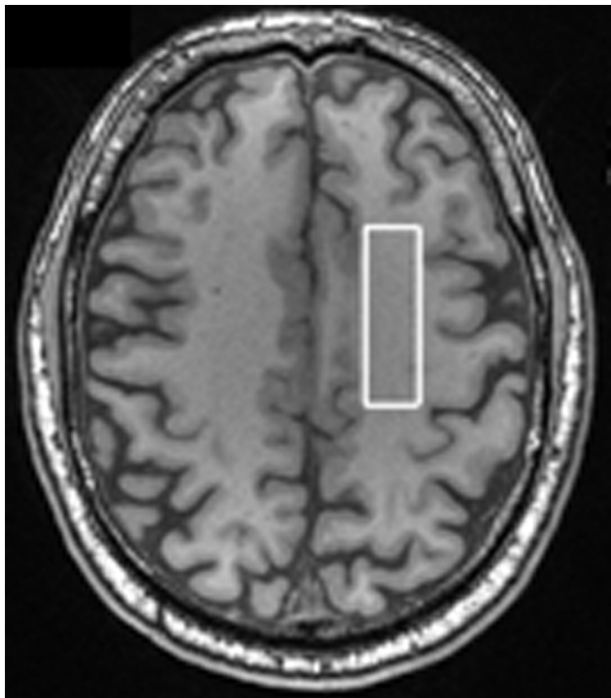


Figure 1

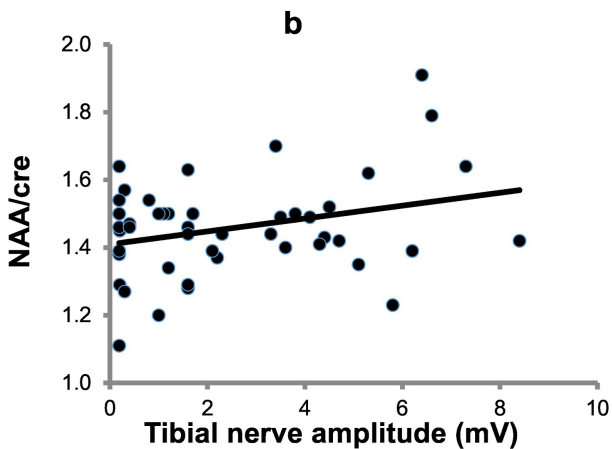
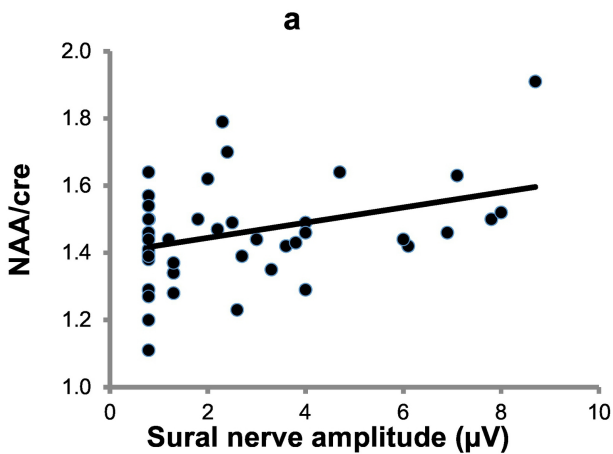


Figure 2

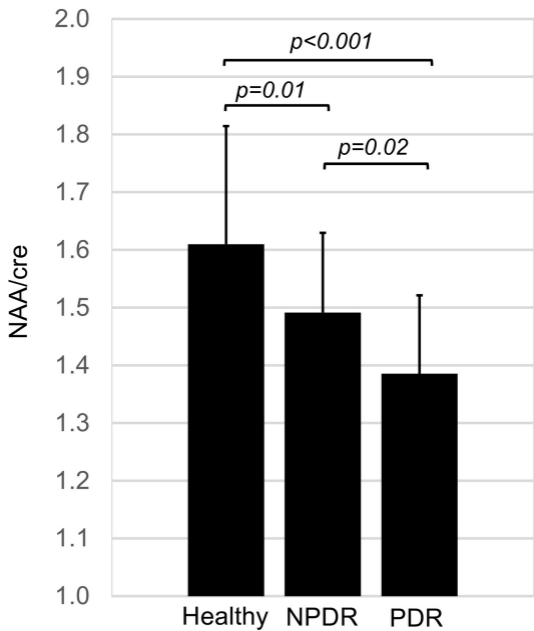


Figure 3