A specific ELISA for measuring neurofilament heavy chain phosphoforms

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Abstract

Neurofilaments (Nf) are the major constitute of the axoskeleton and Nf body fluid levels are an important tool for estimating axonal degeneration *in vivo*. A new sandwich ELISA which allows to quantify the NfH^{SMI35} phosphoform from the CSF, brain tissue and cell–culture homogenate is presented. The sensitivity of the NfH^{SMI35} ELISA calculates to 0.2 ng/mL with a recovery of 119% and a mean within- and between–batch precision of 10.6%, 23%, respectively. CSF NfH^{SMI35} was stable at 4°C, not influenced by freeze–thaw cycles and proteolysis present at room temperature could be prevented by adding protease inhibitors. Aggregate formation was observed for HPLC purified bovine NfH and could be resolved by sonication. CSF NfH was found to be significantly more resistant to protease digestion by alkaline phosphatase than NfL.

The upper reference value for CSF NfH^{SMI35} levels (0.73 ng/mL) was defined as the 95% cumulative frequency from 416 CSF samples. Based on this cut–off a significantly higher proportion of patients with amyotrophic lateral sclerosis, space–occupying lesions, disc–prolapse and subarachnoid haemorrhage had pathological elevated NfH^{SMI35} levels if compared to patients with cluster headache or demyelinating disease.

In order to facilitate for future work the comparison between ELISA, immunoblotting and immunocytochemistry, properties of the monoclonal capture antibodies have been reviewed and a new nomenclature is proposed.

Key words: neurofilament, nfh, nfl, nomenclature, elisa, csf, neurodegeneration, axonal injury, smi34, smi35, smi310, smi32, smi33, smi37, smi38, smi311, ne14

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Neurofilaments (Nf) are the principal component of the axoskeleton and released during axonal damage. Body fluid levels of Nf are an important candidate to be validated as a surrogate marker for axonal degeneration in neurological disorders. At present there are no commercial ELISA kits available for the quantification of any of the three Nf subunits. One group developed a sandwich ELISA technique for the detection of the Nf light chain (NfL) in the cerebrospinal fluid (CSF), based on in–house antibodies [1]. Confirmation of the results derived by this group is however hampered by the non–availability of the antibodies. Another group published an ELISA for NfL and two phosphoforms of the Nf heavy chain (NfH) in brain homogenate [2], but the reproducibility of this assay was not rigorous.

Nf are obligate heteropolymers composed of 3 subunits (NfL, NfM, NfH) and belong to the class IV–intermediate filaments [3]. Nf subunits share a α–helical rod domain which is required for the formation of oligomers. It has been suggested that the N–terminal head domain has regulatory functions of Nf assembly and that the C–terminal tail domain which is the major site of phosphorylation, plays a role in protein–protein and protein organelles interaction [4]. The length of the C–terminal tail domain differs considerably between the Nf subunits and accounts for the different molecular mass. The molecular mass of NfL is 68 kDa and of NfM 160 kDa in SDS gels [3]. NfH is coded on chromosome 22q12.2 and consists of 1020 amino acids [5], with a molecular mass of 111 kDa. Dependent on the degree of phosphorylation migration in sodium dodecyl sulfate (SDS) gels slows down, thus explaining the range of 190 to 210 kDa reported in the literature. The phosphorylated NfH has shown to be relatively resistant to protease activity [6], whilst NfL is not [7,8].

The aim of this study was to develop a sandwich ELISA method for the quantification of phosphorylated NfH in the CSF of neurological patients.

1 Materials and methods

1.1 Antibodies

Capture (first) antibodies: mouse monoclonal anti–NfH antibodies (SMI clones 32, 33, 34, 35, 37, 38, 310, 311) were purchased from Sternberger Monoclonals (Sternberger Monoclonals Incorporated, Utherville, USA) and Sigma (clone NE14, Sigma, UK). Mouse monoclonal anti–NfL was purchased from Sigma (clone NR4). The details of the capture antibodies are summarised in Table 1. The detector (second) antibody: rabbit polyclonal anti–NfH and anti-NfL was purchased from Affinity (Affiniti Research Products, Exeter, UK). The indicator (third) horse–radish peroxidase (HRP) labelled swine polyclonal anti–

rabbit antibody was purchased from DAKO (DAKO, Copenhagen, Denmark).

1.2 Chemicals

Sodium barbitone, barbitone, ethylene diamine tetra acetic disodium salt (EDTA), type III bacterial alkaline phosphatase (ALP, P4252), protease inhibitor cocktail (PI, P8340), calcium lactate, 30% hydrogen peroxide, $NaHCO_3$, Na_2CO_3, Tween 20, bovine serum albumin (BSA) were of analytical grade (Sigma). TMB was purchased from DAKO. Hydrochloric acid (HCl) was obtained from Merck (Darmstadt, Germany). Bovine HPLC purified GFAP and NfM were obtained from Affiniti Research Products. NUNC Maxisorb Microtitre plates were obtained from Life Technologies (Paisley, Scotland).

1.3 Standards

Bovine NfH and NfL were obtained from Affiniti Research Products. Bovine Nf subunits were chosen instead of Nf of human origin as the supply of proteins from human brains could not be guaranteed. A potential way around this, that may be explored in future work, would be the use of recombinant protein technology, however this approach has the disadvantage that post–transcriptional modifications such as phosphorylation and glycosylation are absent. The standard curve based used for the NfH assay was calculated from dilutions of 50 μg HPLC purified bovine NfH protein in barbitone buffer (pH 8.9) containing 0.1% bovine serum albumin, 6 mM EDTA and ranged from 0 to 2 ng/mL. The standards for the NfL assay were made from 50 μg HPLC purified bovine NfL protein similar to the procedure described for NfH and ranged from 0 to 0.5 ng/mL. The standards were in 1 mL aliquots at -20°C.

1.4 CSF samples

In total CSF samples from 638 neurological patients undergoing a routine diagnostic lumbar puncture were obtained. The selection was coded and randomised from the department's sample library. The CSF samples were centrifuged on receipt and stored at 4°C. Analysis was performed within 1 week. Pooled CSF was obtained by taking an aliquot from routine samples. Ten pools of 10 mL were obtained and stored at -70°C until further analysis. The CSF analysis was in accordance with the MRC guidelines for handling of biological specims and approved by the local ethics committee.

1.5 Preparation of cell culture homogenate

Primary cortical astrocytic, microglial and neuronal cell cultures were cultured and prepared from Wistar rats (1–2 days old, 7 days old and day 17 of gestation, respectively) as described [9,10,11]. Astrocytes were cultured for 13 days, removed from the flask by trypsin digestion and seeded onto tissue culture plates at a density of 10^6 cells/well (1500 cells/mm^2) [9,10]. Neurons were dissociated as above and seeded onto poly–ornithine–coated cell culture plates at a density of 2.5 $\times 10^6$ cells/well (3750 cells/mm^2) [9]. Microglia cells were plated at 6 $\times 10^4$ per 13-mm coverslip [11]. Culture medium was decanted, cells were washed with sample buffer, scratched off the plate/coverslip and snap frozen in 1 mL of sample buffer. Cells were sonicated for 1 minute on ice with a cover of argon gas, spun down at 20,000 g. The supernatant was stored at -70°C until further analysis.

1.6 Preparation of brain and spinal cord homogenate

Snap-frozen blocks of brain and spinal cord tissue from of between 0.5 and 1g wet weight, were cut and re-suspended at 1:5 g/mL in Tris-HCl buffer (100mM Tris, pH 8.1 with 1% Triton X-100). Samples were homogenised on ice by sonication, triturated 3 times through 19 and 21 gauge needles and spun at 20,000g. Myelin protein was extracted by adding di–iso–propyl ether (1:5,000), centrifugation at 20,000 g and removal of the lipid layer. The protease inhibitor cocktail was added in a dilution 1:100 to supernatant. After dilution into aliquots of 1:1,1000, 1:5,000, 1:10,000 and 1:100,000 the samples were stored at -70 °C until further analysis.

1.7 Analytical procedure

The microtitre plates were coated overnight with capturing antibody diluted 1:5000 in 0.05 M carbonate buffer, 100 μL, pH 9.5. The plate was washed with barbitone buffer containing 0.1% BSA and 0.05% Tween 20 (pH 8.9). The plate was blocked with 250 μL of barbitone buffer containing 1% of BSA. After washing, 50 μL of barbitone buffer, 6 mM EDTA, 0.1% BSA were added as sample diluent to each well. Fifty μL of standard, control or CSF sample were then added in duplicate to the plate. The plate was incubated at room temperature (RT) for 1 h. After washing, 100 μL of the appropriate second antibody diluted 1:1000 in barbitone buffer were added to each well and the plate was incubated for 1 h at RT. The microtitre plate was washed and HRP–labelled swine anti–rabbit antibody diluted 1:1000 was added and incubated for 1 h at RT. After a final wash, 100 μL of TMB substrate were added. The

plate was incubated for 20 minutes at RT in the dark, the reaction was stopped by adding 50 μL 1 M HCl and the absorbance was read at 450 nm with 750 nm as the reference wavelength on a Wallac Victor2.

1.8 Statistical evaluation

All statistical analyses and graphs were done using SAS software (version 8.2, SAS Institute, Inc., Cary, North Carolina, USA). Independent variables were compared using the non-parametric two-sample exact Wilcoxon rank-sum test or the unbalanced two-way ANOVA (general linear model) for more than two groups [12]. In order to adjust for small sample size significant results were checked by comparison of proportions with the one–tailed Fisher's exact test (α=0.05). The linear relationship between continuous variables was evaluated using the Spearman correlation coefficient (α=0.05). Linear regression analysis was performed using the least–squares method.

2 Results

2.1 NfH phosphoforms

2.1.1 Selection of the capture antibody

The standard curves for the NfH capture antibodies binding to phosphorylated NfH (NfH^{SMI34} NfH^{SMI35} NfH^{SMI310} NfH^{NE14}) and non–phosphorylated NfH (NfH^{SMI32} NfH^{SMI33} NfH^{SMI37} NfH^{SMI38} NfH^{SMI311}) are shown in Figure 1 A. The signal–to–noise ratio (OD of the lowest standard divided by the OD of the blank reading) for each standard curve was calculated (Figure 1 B). The highest signal–to–noise ratio and widest analytical range were obtained for SMI35 as capture antibody. Therefore SMI35 was chosen as capture antibody for further development of the assay.

2.2 Reproducibility of the standard curve

Reproducibility of the standard curve was determined by expressing the optical density (OD) obtained for each calibrant as a percentage of the optical density. Twenty consecutive standard curves were normalised and the results averaged. The resulting regression for NfH^{SMI35} is shown in Figure 2 A and for NfL^{NR4} in Figure 2 B.

2.3 Precision

CSF samples (neat and spiked with either NfH or NfL) were used to calculate the within- and between–batch precision and recovery.

NfH^{SMI35} ELISA: the within–batch and between–batch precision was determined for high and low concentrations of 20 consecutive assays. The within–batch precision was 5.6% for 2 ng/mL, 6.6% for 0.4 ng/mL, 19.8% for 0.2 ng/mL, averaging to 10.6%. The between–batch precision was 15% for 1.3 ng/mL, 31% for 0.16 ng/mL, averaging to 23%. The detection limit was 0.1 ng/mL. The sensitivity was calculated as the mean + 3 standard deviations of the blank signal from 20 assays. The sensitivity was 0.2 ng/mL. The recovery of NfH^{SMI35} in CSF was 121% for pool 1 and 116% for pool 2, averaging at 119%.

NfL^{NR4} ELISA: the within–batch precision was 27% for 0.25 ng/mL. The between–batch precision was over 50%. The sensitivity was 0.25 ng/mL. The recovery of NfH in CSF was 60% for pool 1 and 66% for pool 2, averaging at 63%.

2.4 Stability and proteolytic susceptibility of NfH and NfL

Stability and susceptibility to proteolysis of NfH^{SMI35} and NfL^{NR4} was tested at room temperature (RT), 4°C and -70°C. Five sets of pooled CSF samples were spiked with NfH or NfL. The CSF pools were then divided into aliquots of 1 mL and frozen at -70°C. The aliquots were thawed on day 0, 2, 5 and 7. After thawing the aliquots were stored at 4°C or RT until analysis. The measured OD for NfH of the samples was normalised to the OD of day 0 (Figure 3 A). There was a statistical significant difference between the groups ($F(11,51)=2.56$, $p<0.05$). The subgroup analysis revealed that at RT a significant decrease of OD was present only between day 0 and day 7 (mean OD=1 versus 0.76, $p<0.01$), which could be prevented by adding proteinase inhibitors. For NfL the SD was to wide to make an accurate statement, some samples showed an increase and others an exponential decline of CSF NfL concentrations (data not shown).

In order to measure the succeptibility of NfH and NfL to proteolysis by ALP aliquots were taken from 2 CSF pools and 10 mU ALP were added. There was a significant difference between the Nf subunits over the 7 day observation period ($F(7,16)=3.7$, $p<0.001$). The subgroup analysis revealed lower NfH levels on day 7 compared to day 0 (OD=1 versus OD=0.76, $p=0.05$). NfL levels increased from day 1 (OD=1) to day 2 (OD=1.26, $p<0.05$). However NfL levels on day 5 (OD=0.12) and day 7 (OD=0.10) were decreased compared

to day 1 ($p<0.001$, $p<0.001$, respectively).

Stability of NfH to repetitive freezing and thawing was tested in 3 pools of CSF of which 2 aliquots were made. One aliquot was stored at -70°C, whilst the other underwent 5 freeze–thaw cycles prior to analysis. The OD of the latter was normalised to the former. The mean OD of the samples undergoing 5 freeze–thaw cycles was decreased by about 8% (±13%), which remained within the range of the within–batch precision and did not reach statistical significance.

2.5 Parallelism

Parallelism between calibrant and CSF was studied by quantifying reciprocal dilutions of buffer spiked with NfH^{SMI35} (10 ng/mL, 15 ng/mL and 20 ng/ml) and CSF samples (11.7 ng/mL, 17.3 ng/mL and 22.4 ng/mL). The obtained OD for each series of dilutions was normalised to the highest value within this series (100%). Because of the vertical displacement the top value is not shown. The parallel relationship is demonstrated in Figure 4. This suggested the absence of endogenous binding between CSF NfH^{SMI35} and other CSF substrates. The NfL^{NR4} assay was not sensitive enough to allow for accurate assessment of parallelism between CSF and standards.

2.6 Aggregate formation of purified neurofilaments

The bovine neurofilament heavy chain aggregated in spiked CSF at 4°C, -20°C and -70°C. Eight pooled samples of spiked CSF were measured without and after sonication. Samples were sonicated for 1 minute on ice with a cover of argon gas. Aggregates could be broken up by sonication (Figure 5 A). The measured amount of NfH for frozen samples after sonication was about twice as high if compared to samples which were not sonicated. The error-bars (SD) between the groups overlapped suggesting the presence of aggregates at all temperatures. Spontaneous disassembly of neurofilament protein aggregates was tested at RT and 4°C. In spiked CSF samples spontaneously disassembly was observed about 3 days after thawing (Figure 5 B). The large errorbars indicate considerable heterogeneity over this period, probably caused by simultaneous presence of aggregate dissolution and proteolysis.

2.7 Stability of reagents

No problems were found with stability of antibodies. To minimise any problems with cumulative contamination, the antibodies were stored in 100 μL aliquots and used within 4 weeks. All buffers were stored at 4°C and used within 1 week.

2.8 Cross reactivity

Measuring double dilutions of a known amount of protein, the cross–reactivity of the NfH^{SMI35} ELISA with bovine NfM was 7.8%, with bovine NfL 6.5% and with bovine GFAP 0.06%. There was no cross–reactivity with red–cells, white cells or haemolysed blood.

2.9 Cell–culture and human CNS tissue homogenate

About 25 to 57–fold higher NfH^{SMI35} levels were found in neuronal (0.68±0.04 ng/mg protein; mean±SD) cell cultures in contrast to microglial (0.027±0.028 ng/mg protein) or astrocytic (0.012±.01 ng/mg protein) cell cultures (Figure 6 A).

The highest NfH^{SMI35} levels were found in tissue homogenate from human spinal cord white matter (WM) (213.6±187.5 ng/mg protein), followed by cortical WM (52.3±32.9) and cortical grey matter (GM, 12.47±11.5 ng/mg protein, Figure 6 B).

2.10 Reference population

Out of 561 CSF samples 463 were assayed for NfH^{SMI35} and 127 for NfL. In 29 patients enough CSF was available to assay for NfH^{SMI35} and NfL^{NR4} .

CSF NfH^{SMI35} : the distribution of the NfH^{SMI35} CSF levels was non–Gaussian (Figure 7 A). In order to obtain a biological representative population the top 10% (NfH > 1.119 ng/mL) have been removed, leaving 416 patients. The mean age of the so determined reference population was 42.4±17.9 years, with a median of 41.9 (IQR 31.2-55.8) years. There was no correlation between CSF NfH^{SMI35} and age (R=0.065, p=0.2). Fifty–eight percent (242/416) were female, 39.9% (166/416) male, and in 8 (1.9%) patients no gender information

was available. There was no significant difference in CSF NfH^{SMI35} levels between the 2 genders.

The relative frequency distribution of the reference–population was then subjected to non–parametric definition of the upper reference value of the 95 percentile [13]. The upper reference value corresponds to 0.73 ng/mL (Figure 7 A, reference line). The mean value of CSF NfH^{SMI35} was 0.25±0.23 ng/mL with a median of 0.21 ng/mL and an interquartile range of 0.07 to 0.35 ng/mL. The data were skewed towards zero by 1.33. The kurtosis was 2.15 indicating a high slim peak with more values in the tails than expected by the Gaussian type. This was due to 18% of samples with non detectable CSF NfH^{SMI35} levels.

CSF NfL^{NR4} : the distribution of the CSF NfL^{NR4} levels was non–Gaussian (Figure 7 B). In order to obtain a biological representative population the top 10% (NfL$^{NR4} > 0.24$ ng/mL) have been removed, leaving 113 patients. The 95 percentile corresponds to 0.14 ng/mL (Figure 7 B, reference line). The mean value of CSF NfL^{NR4} was 0.024±0.06 ng/mL with a median of 0.00 ng/mL and an interquartile range of 0.00 to 0.013 ng/mL. The data were skewed towards zero by 2.9. The kurtosis was 7.4 indicating a high slim peak with more values in the tails than expected by the Gaussian type. This was due to 71% samples having no detectable NfL^{NR4} .

The mean age of the reference population was 46.3±18.2 years, with a median of 44.7 (IQR 34.7–60.9) years. There was no correlation between CSF NfL and age (R=0.089, p=0.39). Fifty–two percent (59/113) were female and 42% (47/113) male, in 7 (6%) patients no gender information was available. There was no significant difference of CSF NfL levels between the 2 genders.

There was no correlation between CSF NfL^{NR4} and CSF NfH^{SMI35} (R=-0.1, p=0.6) in those 29 samples were both proteins were measured.

2.11 CSF NfH^{SMI35} in neurological disorders

After determination of the upper reference limit for the NfH^{SMI35} assay, a second population of patients was selected according to the clinical diagnosis: Nine patients had cluster headache (HD) with no other evidence for organic pathology in the central nervous system. Two had a space–occupying lesion (SO) due to one cyst of the right lateral ventricle and one non Hodgkin lymphoma. Three had amyotrophic lateral sclerosis (ALS), 3 a lumber disc prolapse (DP), 36 laboratory supported (isolated intrathecal IgG synthesis) evidence for demyelinating disease (DM), 14 had Guillain–Barré syndrome (GBS) and 5 had a subarachnoid haemorrhage (SAH). All CSF was taken by routine lumbar puncture, except for SAH where the CSF was taken for routine infectious screening 2 days after placement of an extra-ventricular drain. The

values for the SAH therefore represent ventricular CSF.

Because of the small patient numbers statistical significance was checked on a categorical level by the Fisher's exact test comparing proportions of patients with CSF NfH^{SMI35} levels above the previously calculated upper reference value (cut–off) of 0.73 ng/mL. A significantly higher number of patients with SAH had CSF NfH^{SMI35} levels above cut–off if compared to HD ($p<0.001$) or DM ($p<0.001$). A significantly higher number of patients with SO had CSF NfH^{SMI35} levels above cut–off if compared to HD ($p<0.05$) or DM ($p<0.01$) and a significantly higher number of patients with ALS or DP had CSF NfH^{SMI35} levels above cut–off if compared to DM ($p<0.05$, $p<0.05$, respectively).

3 Discussion

The novel ELISA method presented here is straight forward and based entirely on commercially available antibodies. The NfH^{SMI35} protein was stable over a 1–week period if samples were stored at 4°C. For longer storage either freezing at at least -20°C or addition of a protease inhibitor cocktail is recommended. The detection limit of the NfH^{SMI35} ELISA was 0.01 ng/mL and the sensitivity 0.02 ng/mL with a reasonable precision profile (5.6% – 31%). In comparison the sensitivity of the NfL ELISA was 0.25 ng/mL with a relative poor precision (CV>50%).

The upper reference value of 0.73 ng/mL NfH^{SMI35} has to be regarded as conservative. It applies to a hospital population of a tertiary referral centre biased towards neurological diseases. In a general hospital population a more suitable upper reference value would need to be established, which is likely to be lower.

Because this ELISA is the first one reported to measure NfH in human CSF, no cross–validation with another NfH assay was possible. An ELISA for measuring NfH in brain homogenate has been reported, but the reproducibility was not rigorous [2]. An ELISA for quantifying the light chain (NfL) has already been developed [1]. A stability profile of NfL in CSF was not presented, but the authors mentioned the high susceptibility of NfL to proteolysis and refer to previous work [7,8]. This finding is confirmed by the present results. However the sensitivity of the present NfL^{NR4} assay only allowed for an approximative estimate of the kinetics of NfL proteolysis in teh CSF. The strategy chosen by Rosengren *et al.* to prevent protease digestion of NfL was to snap–freeze samples directly after lumbar puncture and store at -70°C [1]. Samples were only thawed directly prior to analysis [1].

Repeated freeze–thaw cycles (n=5) did not significantly affect NfH^{SMI35} stability

in native CSF. However, for CSF spiked with HPLC purified bovine NfH it was shown that freezing caused the formation of aggregates. Once aggregates have formed they could be broken up by sonication. The finding of temperature and time–dependent increasing NfH^{SMI35} levels after aggregate formation suggests the presence of enzymatic breakdown mechanisms. This would also explain why an initial increase (day 2) of bovine NfL levels was observed after ALP incubation. After enzymatic release from aggregates NfL would become accessible to proteolytic enzymes. On the basis that NfL is able to assemble on its own, whilst NfM and NfH cannot [14] it could be hypothesised that aggregate formation might be of some relevance for the measurement of NfL.

Rosengren *et al.* (1996) report a cross–reactivity of their assay with NfH of 15% [1]. The amount of CSF–NfH contributing to the CSF–NfL levels in a series of studies [1,15,16,17,18] is not known, but could be considerable if the in–vivo half–life of NfL in the CSF is shorter than that of NfH as suggested by the present results and others [7,8]. In comparison the cross-reactivity of the present NfH^{SMI35} ELISA with NfM (7.8%) or NfL (6.5%) below the ones reporte by Rosengren *et al.*. This might be explained by the use of a capture antibody against an epitope on the C–terminal NfH tail domain which is unlikely to share homology with NfL or NfM. The possibility of contamination of the HPLC purified NfL used in this study by some residual NfH should also be taken into account. This hypothesis should be addressed by the use of recombinant Nf proteins in future work.

The NfH^{SMI35} ELISA recognises with high specificity NfH present in primary neuronal cell cultures, compared to primary astrocytic or microglial cultures. It is of note that under normal conditions the perikaryal content of phosphorylated NfH in neurons is much lower than in axons [3,19]. Therefore it was not surprising that the highest levels of NfH^{SMI35} were measured in homogenate from spinal cord white matter, where axons are packed with high density. Whilst the ELISA has been optimised for the measurement of NfH^{SMI35} from CSF, we found that the range of the assay allows to extend the standard curve considerable, which was more suitable for measurement of NfH^{SMI35} from CNS tissue homogenate.

An important caveat of the present NfH^{SMI35} ELISA is that the phosphorylation and dephosphorylation of NfH is a highly dynamic process [20] likely to influence the levels of CSF NfH^{SMI35} . This particularly applies to pathological conditions of the central nervous system [21,22,3,19]. It would be desirable to develop ELISA techniques which would allow to quantify a profile of different NfH phosphoforms. Unfortunately, none of the capture antibodies against non–phosphorylated NfH epitopes in non–phosphorylated NfH (SMI32, SMI37, SMI38, SMI311) or against a non–phosphorylated epitope in phosphorylated NfH (SMI33) provided a satisfactory sensitivity with the present detector system.

In neurological diseases the highest levels of CSF NfH^{SMI35} were observed in the ventricular CSF of patients with SAH, followed by lumbar CSF from patients with amyotrophic lateral sclerosis, space–occupying lesions and disc prolapses. The comparison of ventricular with lumbar CSF has to be done with caution as nothing is known about the NfH concentration across the CSF compartment. Interestingly, the distribution for CSF NfH^{SMI35} was bimodal in GBS patients. This finding warrants further investigation.

In order to accommodate for the emerging need to differentiate between NfH phosphoforms with immunological methods a new nomenclature has been proposed in Table 1. This nomenclature was based on the name of the capture antibody and would facilitate the comparison between immunocytochemistry, immunoblot and ELISA using these antibodies. This nomenclature would also take into account that the exact binding epitopes are not always known and that epitope recognition might be influenced by post–translational modifications. The proposed nomenclature could easily be expanded to incorporate other capture antibodies which might contribute to distinguish different NfH phosphoforms.

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Table 1: *The proposed protein nomenclature for the quantified NfH phosphoforms, the immunocytochemical staining pattern, the antibody binding epitope, the antibody type, the previously published and commercial (Sternberger Monoclonals Incorporated) notation for the mouse monoclonal antibodies used in this study are summarised. AD = Alzheimer's disease, MS = multiple sclerosis.*

posed				**Published**	**Commercial**
nenclature	**Immunocytochemistry**	**Epitope**	**Antibody**	**Notation**	**Notation**
NR4	perikaryal, axons (unselective) [23]	unknown	IgG1	—	NR4
SMI32	Purkinje cell bodies and dendrites, nucleus ruber cell bodies, thick axons, cerebellar white matter axons and some basket cell fibres [24,25,26], axon-terminal spheroids in MS [27]	binds to a non–phosphorylated epitope which can be masked by phosphorylation [24,6]	IgG1	02–135	SMI32
SMI33	perikaryon, dendrites, some thick axons, cerebellar white matter axons and some basket cell fibres, some macrophages [24,25,26]	binds to a non–phosphorylated epitope in phosphorylated and non–phosphorylated NfH [28,6]	IgM	02–40	SMI33
SMI34	thick and thin axons, white matter axons, basket cell dendrites, in some pathological conditions also the perikaryon, intraneuronal tangles [28,25,29,30]	an extensively phosphorylated NfH [24,28,6]	IgG1	07–5	SMI34
SMI35	axons (unselective),cerebellar white matter axons, early changes in neuronal cell body staining, basket cell fibres [28,25,26,29,30]	high (200 kDa, pI 5.1) to low (170 kDa, pI 6.2) phosphorylated NfH phosphoforms [28,6]	IgG1	03–44	SMI35
SMI37	a subpopulation of neuronal cell bodies, dendrites, some thick axons [24]	non–phosphorylated epitope [24]	IgM	06–32	SMI37
SMI38	neuronal cell bodies, dendrites, some thick axons [24]	a non–phosphorylated epitope [24]	IgG1	10–1	SMI38
SMI310	cerebellar white matter axons, extranneurofibrillary tangles in AD [31]	a phosphorylated epitope in extensively phosphorylated NfH [28,6,24]	IgG1	04–7	SMI310
SMI311	neurons (unselective) [32]	against non-phosphorylated NfH epitopes [28,6,24]	antibody cocktail	—	SMI311
NE14	axons, somata of type II spiral ganglion neurons [33]	a phosphorylated epitope [34]	IgG1	—	NE14

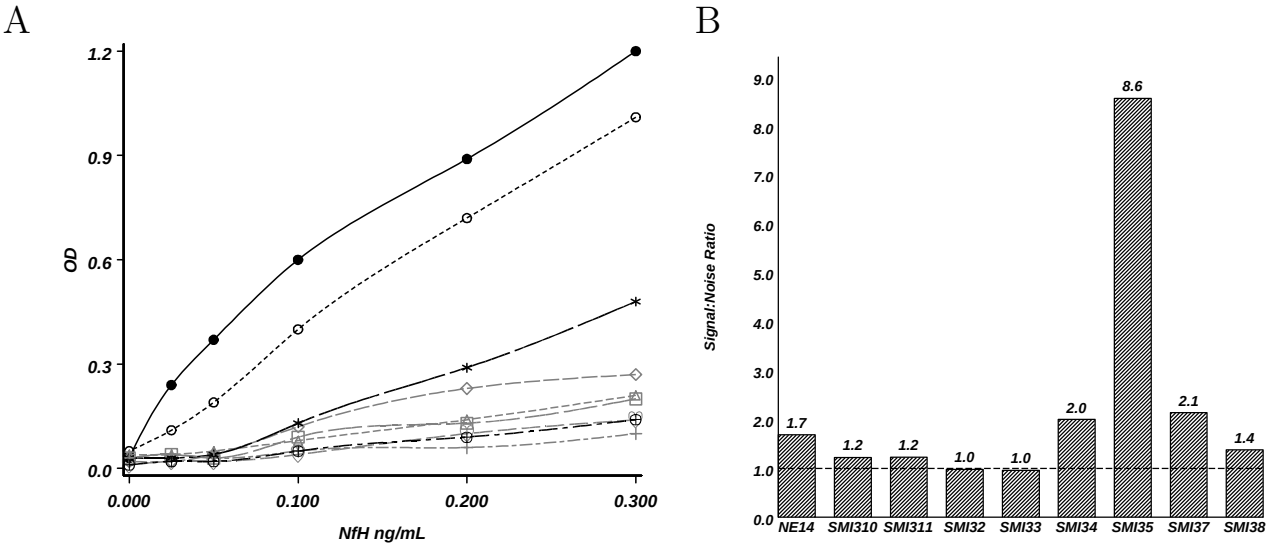


Figure 1. *(A) NfH phosphoforms. The standard curves for different capture antibodies against phosphorylated ($NfH^{SMI34} = \circ$, $NfH^{SMI35} = \bullet$, $NfH^{SMI310} = *$, $NfH^{NE14} = \oplus$, lines in black) and non–phosphorylated epitopes ($NfH^{SMI32} = \triangle$, $NfH^{SMI33} = \diamond$, $NfH^{SMI37} = \heartsuit$, $NfH^{SMI38} = \square$, $NfH^{SMI311} = +$, lines in grey) are shown. (B) The signal–to–noise ratio (OD of the lowest standard divided by the blank OD) for different capture antibodies. A signal–to–noise ratio of greater than 1 (reference line) is mandatory.*

A

B

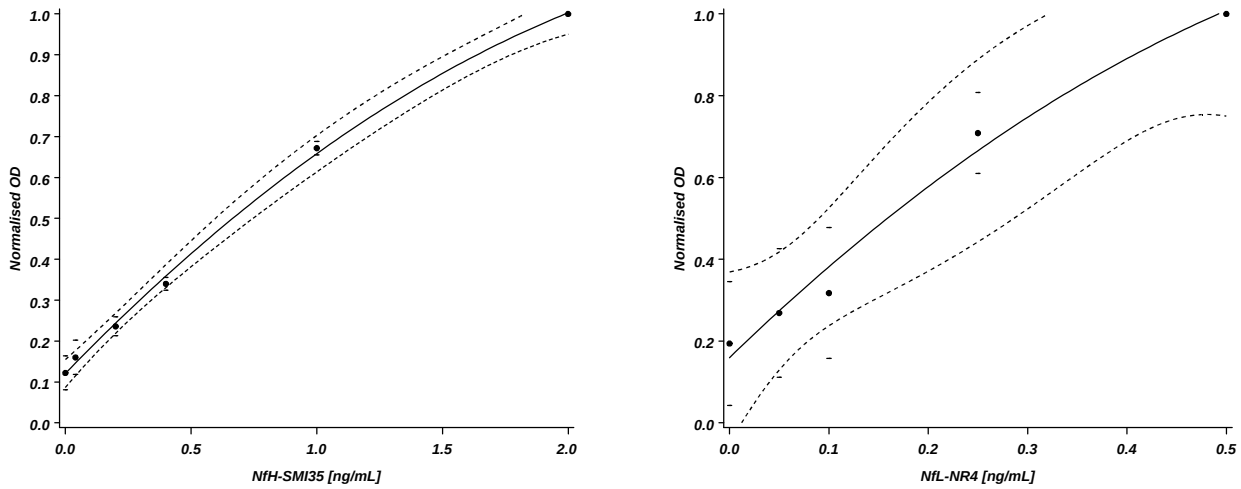


Figure 2. *Standard–curve for (A) the NfH^{SMI35} assay and (B) the NfL^{NR4} assay. Reproducibility of twenty consecutive normalised calibration lines. The graph shows the mean values (dots) ± standard deviation (bars), quadratic regression line, 5% and 95% confidence interval curves.*

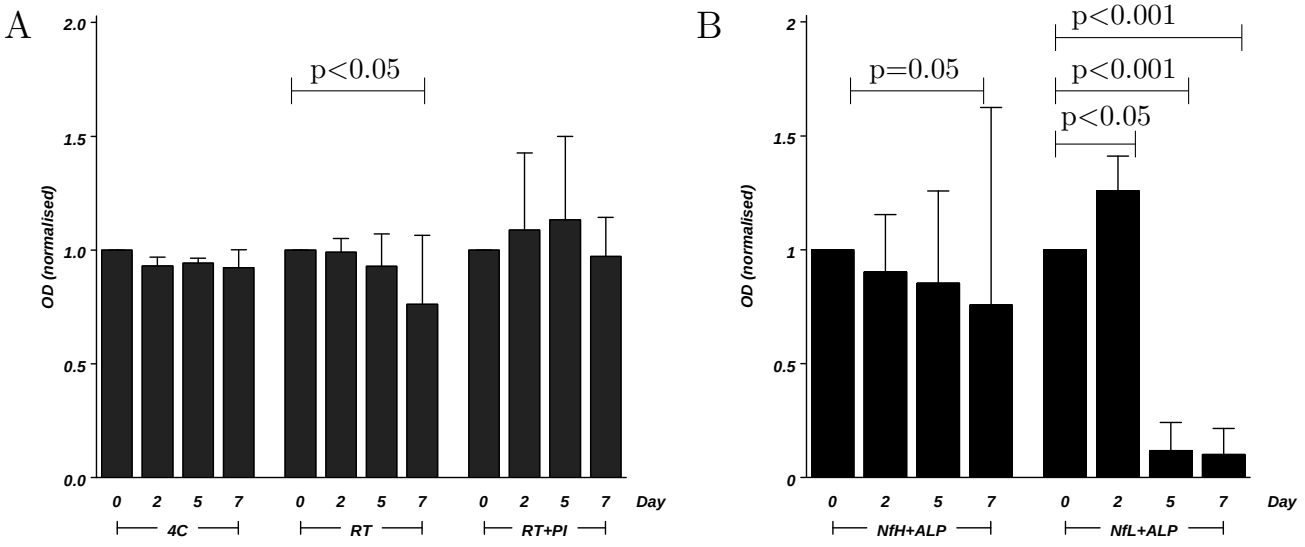


Figure 3. *Stability profile of (A) NfH^{SMI35} in native CSF (n=6) over 7 days at room temperature (RT) or 4°C in percentage OD to a sample stored at -70°C. PI indicates samples with added protease inhibitors stored at RT. (B) Proteolytic susceptibility to ALP of NfH^{SMI35} and NfL^{NR4} in spiked CSF at room temperature over 7 days. The mean±SD are shown.*

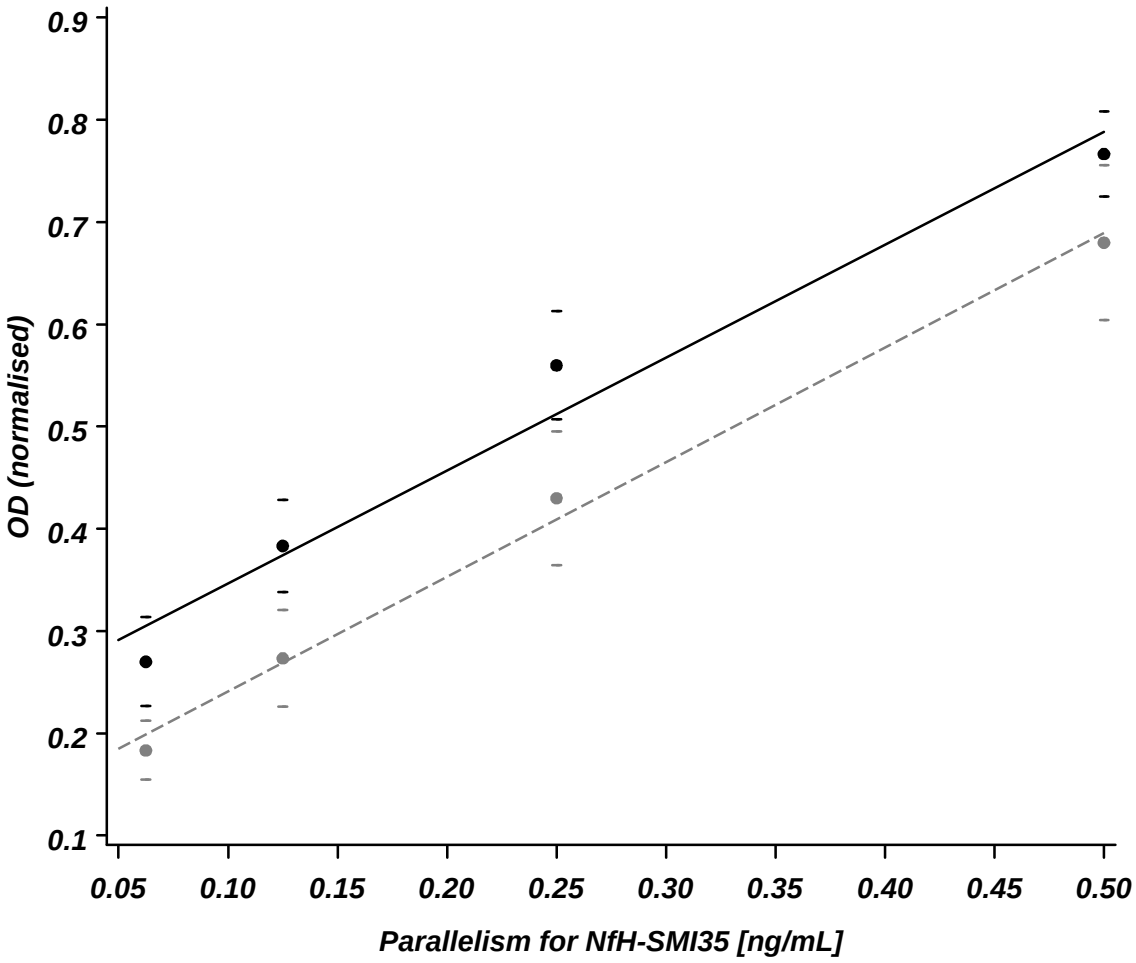


Figure 4. *Parallelism for NfH^{SMI35} between calibrant (open, grey circles) and CSF (closed, black circles). The linear regression, mean (circles) and standard deviation (horizontal bars) are shown.*

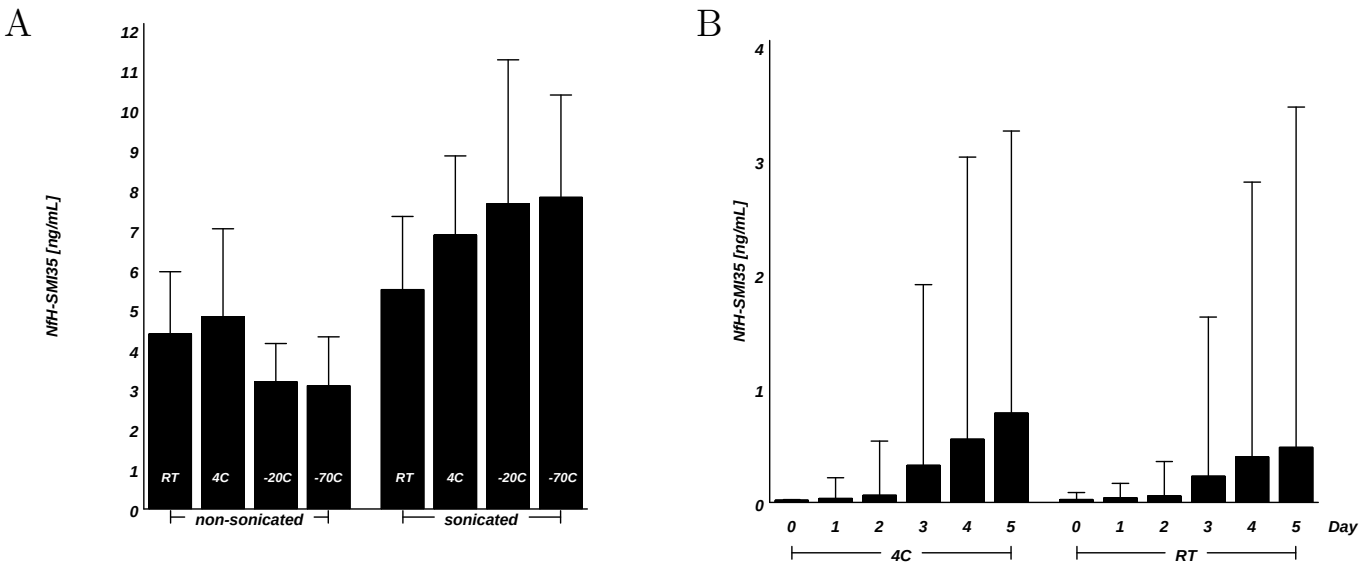
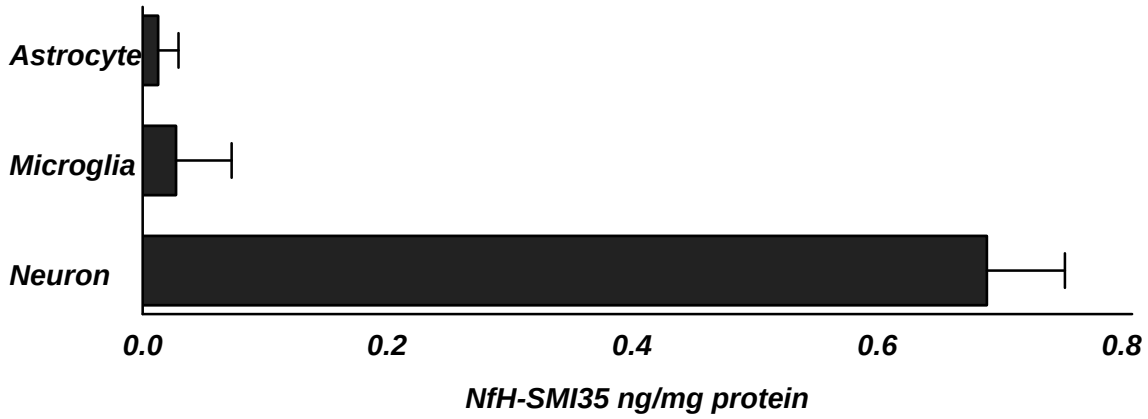


Figure 5. *(A) NfH^{SMI35} aggregate formation in spiked CSF (mean, SD). Non–sonicated (left) and sonicated (right) samples are shown for 1 week storage at room temperature (RT), 4°C, -20°C and -70°C. (B) Spontaneous disassembly of CSF NfH^{SMI35} aggregates of spiked CSF samples at RT and 4°C over a 5 day observation period. The mean±SD are shown.*

A



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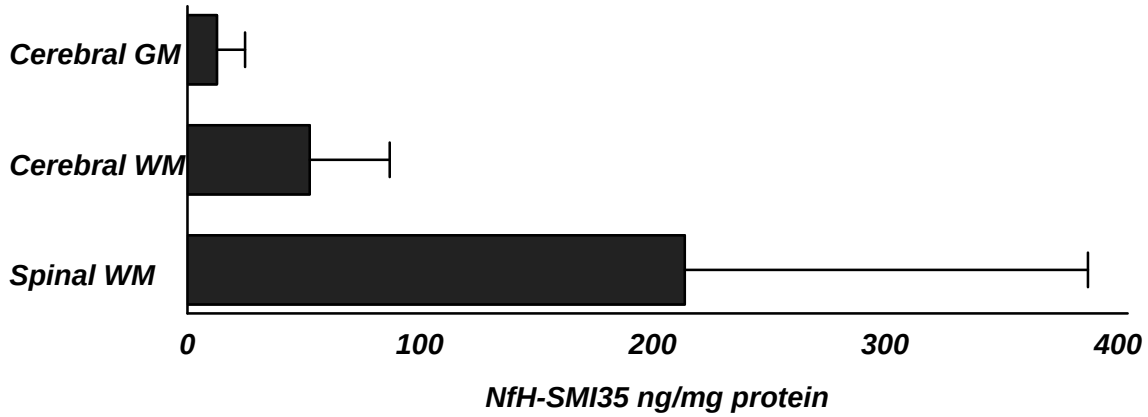

Figure 6. *Levels of NfH^{SMI35} ng / mg protein in (A) homogenised neurons, astrocytes and microglia, (B) in tissue homogenate from cerebral grey matter (GM), white matter (WM) and spinal cord white matter (mean±SD).*

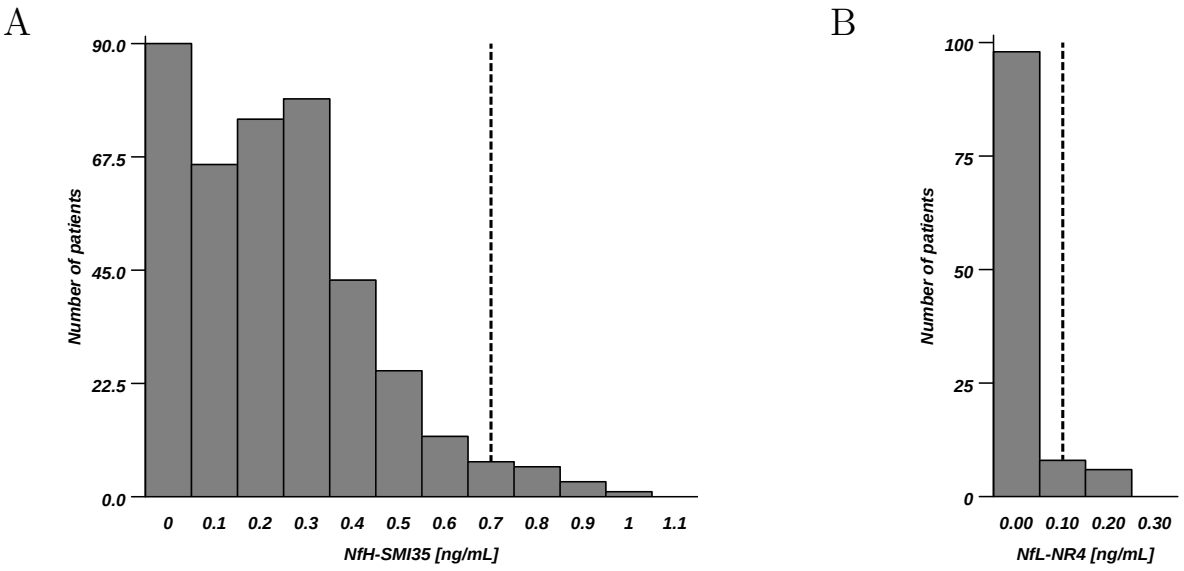

Figure 7. *Frequency histogram for the reference population for (A) CSF NfH^{SMI35} (n=416), the upper reference value of 0.73 ng/mL is shown (dotted line). (B) CSF NfL^{NR4} (n=113), the upper reference value of 0.14 ng/mL is shown (dotted line).*

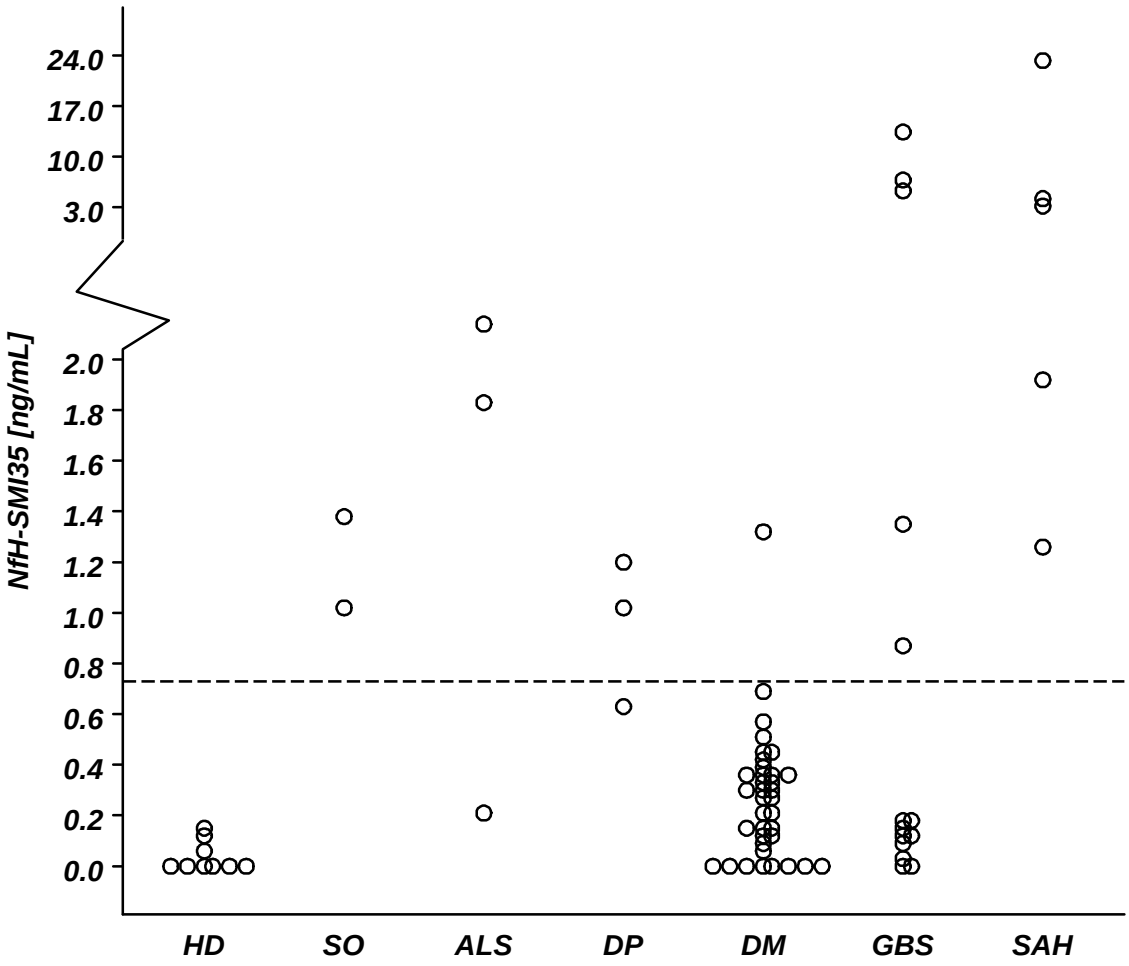

Figure 8. *Scatter plot of CSF NfH^{SMI35} levels in patients with cluster headache (HD), space–occupying lesions (SO), amyotrophic lateral sclerosis (ALS), disc prolapse (DP, demyelinating disease (DM), Guillain–Barré syndrome (GBS) and subarachnoid haemorrhage (SAH). The horizontal reference line (dotted) represents the cut–off (0.73 ng/mL) derived from the reference population, the y–axis is split at 2 ng/mL.*