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Pattern recognition analysis of proton nuclear magnetic resonance spectra of postmortem cerebrospinal fluid from rats with drug-induced seizure or coma

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ABSTRACT

Cerebrospinal fluid (CSF) is routinely subjected to gross evaluation in postmortem investigations; however, its use in chemical evaluations has not been fully realized. Analysis of nuclear magnetic resonance (NMR) spectra with pattern recognition methods was applied to CSF samples. Rats were treated with pentylenetetrazol (PTZ) to induce seizure or pentobarbital (PB) to induce coma, and postmortem CSF was collected after CO_2 gas euthanization. Pattern recognition analysis of the NMR data was performed on individual postmortem CSF samples. The aim of this study was to determine if pattern recognition analysis of NMR data could be used to classify the rats according to their drug treatment. The applicability of NMR data with pattern recognition analysis using postmortem CSF was also assessed. Partial Least Squares-Discriminant Analysis (PLS-DA) score plots indicated that the PTZ, PB, and NS (control) groups were clustered and clearly separated. PLS-DA correlation loading plots showed respective spectral and category variances of 41% and 42% for factor 1, and 17% and 27% for factor 2. Thus, factors 1 and 2 together described 58% (41% + 17%) and 69% (42% + 27%) of the variation, respectively. NMR study of postmortem CSF has the potential to be utilized as both a novel forensic neurochemistry method and in the clinical setting.

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1. Introduction

Cerebrospinal fluid (CSF) is known to reflect the condition of the central nervous system [1]. Thus, examination of CSF has been widely used in clinical medicine as a general diagnostic tool for infectious diseases [2–4] and other neurological disorders [5,6]. In the forensic literature, researchers have studied postmortem CSF for toxicological analysis [7–9], estimation of the postmortem interval [10,11], comparisons of individuals with and without mental illness and by cause of death [12], and for examination of the neurochemistry of life-threatening stress prior to death [13]. However, while the gross appearance of CSF is used to diagnose subarachnoid hemorrhage in postmortem examination [14], postmortem chemical analytical tests using CSF, especially those that use nuclear magnetic resonance (NMR) [15], are seldom carried out. Accurate diagnosis of brain diseases is considered challenging using postmortem CSF testing due to the absence of obvious metabolomic changes. In addition, it is not clear whether the antemortem metabolomic status of the brain remains in postmortem CSF.

NMR measurement with spectral pattern recognition analysis has been used to discriminate between normal and abnormal groups of biological samples and to classify abnormal groups without identifying individual metabolites in cases where a reliable diagnostic evaluation has been conducted in advance [16–18].

Recently, Asano et al. applied pattern recognition analysis to NMR data of CSF obtained from 22 pediatric patients with convulsions [19]. The authors were able to visualize the unique characteristics of CSF from patients with acute encephalopathy vs. those with simple and complex febrile seizures, which are frequently difficult to clinically differentiate. The study suggested that a differential diagnosis can be made without the determination of individual metabolites as biomarkers using CSF samples, as the metabolic status of each seizure disease is reflected in CSF. Although postmortem examination can reveal seizure pathology, it is unable to determine whether there were seizure symptoms just before death.

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Therefore, we focused on whether these analytical techniques can be applied to postmortem CSF and whether they are able to reveal the antemortem activity status of neurons after death, as antemortem seizures cannot be demonstrated in postmortem examination. In this study, rats were administered one of two antagonistic drugs, pentylenetetrazol (PTZ) to induce seizures [20] or pentobarbital (PB) as a hypnotic/sedative [21]. Animals were then euthanized with CO_2 gas and postmortem CSF was collected. Next, pattern recognition analysis of the NMR data obtained from the postmortem CSF of each rat was performed. The aim of this study was to identify any relationships between the pattern recognition analysis of the NMR data and the groups of rats with drug-induced seizures or coma, eliminating the contribution of the administrated drugs. We also attempted to verify the applicability of the NMR data with pattern recognition analysis from postmortem CSF.

2. Materials and methods

2.1. Animals

All experiments were performed with the approval of the Nippon Medical School Ethics Committee (26-135, 27-162). A total of 37 male, 7-week-old Sprague-Dawley rats weighing 209–244 g (Sankyo Labo Service Co., Inc., Tokyo, Japan) were group-housed under controlled laboratory conditions at room temperature and in an artificial 10-h dark/14-h light cycle (lights-off phase between 06:00 and 20:00). Animals had free access to commercial rat pellets, MF (Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan) and tap water.

2.2. Drug administration, observation, and scoring

After habituation for 1 week, the animals were assigned to the following three groups: the PTZ (Sigma-Aldrich Co., LLC., Tokyo, Japan) group (n = 13), which received a single intraperitoneal (*i. p.*) injection of PTZ (70 mg/kg body weight), the PB (Somnopentyle®, PB 64.8 mg contained in 1 mL; Kyoritsu Seiyaku Co., Tokyo, Japan) group (n = 13), which received a single *i.p.* injection of PB (90 mg/kg body weight), and the control group (NS) (n = 9), which received a single *i.p.* injection of 0.6 ml 0.9% saline. All behaviors and responses were observed for 5 min in individual cages. The responses of the PTZ group were recorded according to the revised Racine's scale [22]. Because the PB group was observed to assess the anesthesia effect [23], in this study, the scale of behaviors and responses to PB was defined operationally, according to the degree of the anesthesia effect. Lastly, rats exhibited exploratory behavior in the NS group and those before presentation of the drug administration effects in the PTZ and PB groups. Generally, rats will scout a new environment, sniffing and touching the surroundings, and occasionally rearing up to explore higher areas. This behavior will continue for a few minutes and gradually decrease thereafter; the rats then display grooming behavior and are calm [24]. Therefore, we also operationally defined the scale of the exploratory behavior in the study. The scale is shown in Table 1. The behavior of animals was recorded with a single video camera (HC-V620M; Panasonic Co., Osaka, Japan) from injection to euthanasia. Scored behaviors and responses were represented as an ethogram, which is a catalog describing species-specific behaviors that form a basic behavioral repertoire [25]. In the ethogram constructed for this study, time and behavioral scores are displayed in the *x*- and *y*-axes, respectively. Following the 5-min observation time, the animals were sacrificed using CO_2 gas. After confirmation of death, rats were placed in the prone position, and the occipital skin was incised. CSF samples were extracted from the cisterna magna using a 27-gauge butterfly needle connected to a 1-mL syringe through the subcutaneous soft tissue [26].

Table 1
Scale of behaviors and drug-induced symptoms.

Scale	Behavior and drug-induced symptoms
PTZ-induced symptoms	
6	Convulsions including clonic and/or tonic–clonic seizures while lying on the side and/or wild jumping
5	Convulsions including clonic and/or tonic–clonic seizures while lying on the belly and/or pure tonic seizures
4	Clonic seizure in a sitting position
3	Neck jerks
2	Facial jerking with muzzle or muzzle and eye
1	Sudden behavioral arrest and/or motionless staring
Exploratory behaviors	
0	Normal rest
−1	Grooming
−2	Exploratory behavior (sniffing, scanning)
−3	Exploratory behavior (rearing, wall-seeking)
PB-induced symptoms	
a4	Coma
a3	Loss of righting reflex
a2	Decrease in locomotor activity
a1	Staggering when walking or after rearing

2.3. Sample preparation

All samples were centrifuged at 2200*g* for 5 min at 25 °C to remove cells, and stored at −80 °C until further analysis. CSF (50 μL) was mixed with 130 μL of deuterium oxide (D_2O; Isotec, St. Louis, MO, USA) and then pipetted into 3-mm NMR tubes (Wilmad-LabGlass, Vineland, NJ, USA). The tubes were placed in 5-mm NMR tubes (Wilmad-LabGlass) containing 300 μL of D_2O with 10 μM sodium (3-trimethylsilyl) tetradeuteriopropionate-2,2,3,3-d4 (TMSP) (MSD Isotopes, Montreal, Canada) for subsequent NMR measurements. D_2O provided a deuterium field frequency lock for the NMR spectrometer, while TMSP provided an internal chemical shift reference (δ = 0.00).

2.4. Proton nuclear magnetic resonance data acquisition

Solution-state ^1H NMR spectroscopy was performed at a proton resonance frequency of 300 MHz using an ECX-type NMR spectrometer interfaced with a TH5 probe, equipped with an automatic 16-position sample changer and Delta NMR processing and control software ver. 4.3.2 (all from Jeol Resonance, Tokyo, Japan). ^1H NMR spectra were acquired automatically at a probe temperature of 23 °C using the macro program in the Delta system for automatic measurement. The observation range of the NMR signal was 5580 Hz. The water resonance was suppressed using a conventional presaturation pulse sequence for the water (HDO) proton signal suppression based on homo-gated irradiation and DANTE pulse sequence (presaturation time = 2 s, DANTE pulse = 8 μs, DANTE interval = 0.1 ms, DANTE loop = 185, DANTE attenuator = 24 dB). Carr-Purcell-Meiboom-Gill spin-echo spectra were measured using a spin-echo loop time of 19.2 ms, a relaxation delay of 2.0 s, and 4000 transients.

2.5. Data processing and reduction

The acquired spectra were processed using Alice2 (ver. 5.5; Jeol Resonance). Free induction decays were subjected to an exponential weighing function of 0.2 Hz, Fourier transformed from the time to the frequency domain, and then phased manually, followed by linear baseline correction and referencing to the TMSP singlet at 0.00 ppm.

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