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Histological assessment of cerebellar granule cell layer in postmortem brain; a useful marker of tissue integrity?

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Abstract

Tissue quality control measures are routinely performed in brain banks with the assessment of brain pH being the most common measure. In some brain banks the assessment of the RNA integrity number is also performed, although this requires access to specialised equipment and is more expensive. The aim of this study is to determine if there is a correlation between the visual assessment of cerebellar granule cell integrity and brain pH or RIN. One hundred and five consecutive cases from the NSW Tissue Resource Centre, Sydney, Australia were accessed. The cerebrum was hemisected and one hemisphere sliced parasagittally at approximately 1–2 cm intervals and frozen. The other hemisphere was fixed in 15% buffered formalin for 2–3 weeks. The contralateral cerebellar hemisphere was preserved in the same manner as the cerebral hemisphere. Samples of fixed tissue were embedded in paraffin, 7 μ m sections cut and stained routinely with hematoxylin and eosin. The granular cell layer (GCL) was assessed microscopically to determine the degree of autolytic degradation. Degradation was graded as nil, mild, moderate or severe. Brain tissue pH and RIN were measured using standardised protocols. This study showed that both brain pH and RIN significantly correlated with the severity of the degradation of the cerebellar granule cell layer. This additional screening tool can be performed during routine histological review of the cerebellar tissue to assess the suitability for further investigation of tissue quality.

Keywords

Postmortem; Brain pH; RIN

Introduction

Brain banks provide important support and enhance research into neurological and psychiatric disorders. Paramount to the management of these facilities is the adherence to standardised protocols for brain collection, dissection, classification and storage. Unlike animal studies premortem events are a major contributor to the variability in tissue quality. Recognised premortem factors such as co-existing pathology (Sheedy et al. 2008), hypoxia and coma effect the histological and molecular structure of the brain (Harrison et al. 1995). Exclusion criteria for donation can limit the effect of premortem factors to a large degree, however it may sometimes be difficult to determine this information at time of death.

In any bio-bank the operational costs are considerable to ensure that the collection, classification, integrity and long-term viability of each case are of the highest quality. The measurement of brain pH is a widely recognised marker of brain tissue quality that is utilised in most brain banks to assist in the allocation of research cohorts (Kingsbury et al. 1995; Monoranu et al. 2009). The positive correlation between low brain pH and degraded RNA has been reported previously and validates the use of pH as a marker of tissue integrity (Kingsbury et al. 1995; Monoranu et al. 2009; Schroeder et al. 2006; Webster 2006).

The RNA quality marker, as indicated by the RNA integrity number (Jolles et al. 1992), for molecular studies is recognised as more reliable predictor of RNA integrity than the previously employed ratio of 28S:18S ribosomal RNA (Schroeder et al. 2006; Weis et al. 2007). However, the measurement of mRNA, DNA and protein degradation requires access to specialised instruments and these can be expensive or unavailable to some banks.

A number of previous studies have shown that neurons of the cerebellar granule cell layer are sensitive to postmortem interval, agonal state and to the conditions of postmortem tissue storage (Albrechtsen 1977a, b; Ikuta et al. 1963; Ogata et al. 1986). A previous report of a pilot study from NSW Tissue Resource Centre (NSW TRC) showed a correlation between the severity of granule cell layer autolysis and a lower mean brain pH (Sheedy et al. 2008). The NSW TRC has a primary focus on the collection of neurological disorders associated with alcohol abuse; psychiatric disorders such as schizophrenia and bipolar disorder; neurodegenerative diseases such as motor neuron disease, and multiple sclerosis, as well as healthy controls. As the current NSW TRC quality control protocol incorporates the measurement of brain pH and RIN this study investigated the relationship between the visual assessment of the granule cell layer of the cerebellum and brain pH and RIN. Histological assessment of granule cells may provide an additional quality screening assessment tool to incorporate in routine diagnostic screening procedures.

Methods and materials

One hundred and five consecutive cases collected by the NSW TRC over a 20 month period were assessed. The NSW TRC has approval from the Human Research Ethics Committee of Sydney South West Area Health Service (X07-0074 and X08-0065) and The University of Sydney. Collection and dissection protocols have been previously reported (Sheedy et al. 2008). In brief, cases are donated through either a prospective donor program or a forensic institution. The NSW TRC protocols have exclusion criteria for donation to reduce the potential of tissue degradation. These include artificial ventilation with 24 h of death, coma, protracted agonal period, stroke, head trauma and postmortem interval greater than 72 h. At postmortem, the cerebellum and brainstem are removed from the cerebrum and hemisected. One cerebellar hemisphere is sliced parasagittal at approximately 1–2 cm intervals and frozen. The other hemisphere is fixed in 15% buffered formalin for 2–3 weeks. Routine samples of fixed tissue, including the lateral cerebellar hemisphere, were embedded in

paraffin, 7 μm sections cut and stained with hematoxylin and eosin for neuropathological assessment. Diagnosis was performed using standard protocols.

Brain tissue pH

A small segment (approximately 3 g) of the lateral cerebellar hemisphere was taken at the time of the autopsy and frozen at -80°C . Brain tissue pH was measured using one gram of tissue homogenized (IKA T10 basic Homogeniser; Crown Scientific) in 10 volumes of distilled water at neutral pH (Kingsbury et al. 1995). Measurements were made in triplicate using the Mettler Toledo MP220 temperature compensating pH meter and the mean value calculated. Two calibration points were used, pH 4 and pH 7.

RNA integrity

Assessment of the RIN, for assigning integrity values to RNA measurements, was performed as employed in other banks (Miller et al. 2004; Schroeder et al. 2006; Stan et al. 2006; Webster 2006). In brief, frozen cerebellum samples (30 mg) were homogenised using the IKA T10 basic Homogeniser (IKA®-Werke GmbH & Co, Cat. #3420000, Germany). Total RNA extraction was carried out using the Purelink RNA Mini Kit (Invitrogen by Life Technologies, Cat. #12183018A, USA), according to the manufacturer's instructions. Following extraction, RNA was eluted into 60 μl of supplied RNase/DNase free water. Aliquots of 2 μl were sampled for measurement of RNA concentration using the Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, USA). For quality assessment, 5 μl aliquots samples were electrophoresed according to the Agilent Bioanalyser 2100 (Agilent Technologies, USA) manufacturer's protocol (provided in the RNA Nano 6000 kit) for this analysis. The 2100 Bioanalyzer Expert software then automatically determines the RIN, a quantitation estimate and the 28S/18S ribosomal ratio. The RIN reflected the presence or absence of degradation products; where higher RNA degradation was assigned a lower RIN value.

Histological assessment of autolytic change in cerebellar granule cell layer

The granular cell layer of the cerebellum was assessed microscopically to determine the degree of autolytic degradation by one of the authors (AH) blinded to case information. Degradation was graded as nil, mild, moderate or severe as described by Ogata et al. (Albrechtsen 1977a, b; Ogata et al. 1986). Briefly, these categories are defined as; nil when no morphological abnormality was detected and granule cells nuclei were clearly visible; mild when there is a slight decrease in the number of the nuclei but remaining nuclei were clearly identified; moderate when there is a diffuse decrease in the number of nuclei but some individual nuclei are still clearly seen and severe when few or no nuclei are clearly seen and there is vacuolation of the granule cell layer (Fig. 1).

Clinical indices

Postmortem clinical characterisation is performed in accordance with Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) diagnosis. Cases that are unable to have a diagnosis confirmed are excluded from the main research cohorts. The mode of death or agonal duration was determined by assessment of the rapidity of death and related circumstances (Tomita et al. 2004). These were classified as: *rapid*- a terminal phase of less than 1 h; *intermediate*- a terminal phase of between one and 24 h and *long term*- longer than 1 day. Postmortem interval (death until freezing of tissue) was calculated in hours for all cases.

Statistical analysis

Group effects were tested using analysis of variance (ANOVA) and Fisher's protected least squares difference (PLSD) was used for *post hoc* analysis where appropriate. Standard linear regression was used to assess the relationship between postmortem interval; mode of death; granule cell layer classification and the tissue quality measures of brain pH and RIN (a P value of <0.05 was used for significance). Analysis was performed using JMP statistical software package (SAS Institute, Cary, NC).

Results

The cases were allocated to the following diagnostic groups: alcohol abuse disorders, controls, motor neuron disease (MND), multiple sclerosis (Moxley et al. 2006), psychiatric disorders, and "other" (cases with an unconfirmed clinical diagnosis). Psychiatric disorders group includes cases of DSM-IV diagnosis of schizophrenia, bipolar and major depression. Alcohol abuse disorders group includes cases DSM-IV diagnosis of harmful use, substance abuse (alcohol) and substance dependence (alcohol). Of the 105 cases, 26 were donated through the prospective donor program and the remainder from the forensic donation process. Case DSM-IV classification was not significantly correlated with either brain pH, RIN or GCL. Post mortem interval (PMI) ranged from 3 to 72.5 h.

There was a positive correlation between brain pH and RIN ($F_{1,101} = 69.11$, $P < 0.0001$). No significant correlation was seen with PMI and RIN ($P = 0.383$). A correlation with PMI and brain pH ($y = 6.35 + 0.0053x$, $r^2 = 0.10$, $P = 0.0013$) within the whole group was observed. The longer the PMI the brain pH increased. Further post hoc analysis of the diagnostic groups showed this observation was more prominent in the alcohol abuse disorders and MS groups though not highly significant, $P = 0.05$ and $P = 0.07$ respectively (Table 1).

Granule cell layer

Grading of the cerebellar granule cell layer determined there were 91 normal cases; 6 with mild changes; 5 with moderate change and only 3 with severe autolysis. The mean values for the quality markers brain pH and RIN for each category are shown in Table 2.

Brain pH

As shown in Fig. 2a there was a reduction in the mean of brain pH in relation to the severity of degradation of the granule cell layer. This is highlighted in the comparison of the mean values for the normal GCL grade 6.60 (± 0.23) to the severe grade 5.77 (± 0.15). Oneway ANOVA showed significance for brain pH ($F_{3,103} = 24.13$, $P < 0.0001$). Post-hoc analyses revealed the severity of the degradation of the GCL showed significance for brain pH when compared to the normal GCL; mild $P = 0.0004$, moderate and severe both $P < 0.0001$. The comparison between mild grade of autolysis to severe was significant $P = 0.005$ as well.

RIN

The mean RIN values as shown in Fig. 2b also decreases with the severity of the GCL degradation. The mean RIN of the normal GCL grade was 7.30 (± 0.90) as compared with the severe grade, mean 1.6 (± 2.77). Oneway ANOVA for the RIN ($F_{3,103} = 37.44$, $P < 0.0001$) displayed significance. Further post-hoc analyses found the RIN was affected by the severity of degradation. The comparison of normal, mild and moderate groups to the severe group showed to be highly significant ($P < 0.0001$). There was an effect between the normal and moderate groups $P = 0.0027$. No significant difference was seen for normal to mild or mild to moderate.

Mode of death

The mode of death in the total cohort comprised of the ratio; 45% rapid, 30% intermediate and 25% long term. Due to the characteristics of the disease, the cases with MND and MS had a larger proportion (62%) with a long agonal phase. Of the three severe GCL cases, only one had a long-term agonal phase, the other two had an intermediate phase. The effect of mode of death on GCL was not significant; Chi Sq $P > 0.367$.

Discussion

Brain banks endeavour to provide material of high quality for a range of research techniques. The adherence to standardised protocols assists in meeting this goal. Allocation of cases to study cohorts is also dependent on brain tissue quality markers; therefore having an additional screening tool assists in the assessment of case suitability.

The normal density of the cerebellar granule cells in humans is approximately 10^7 per mm^3 and during autolysis, cell lysis proceeds selectively leading to a lower pH as increased lysosomal enzyme release leads to increase loss of remaining cells (Albrechtsen 1977a, b; Averback 1980). Changes seen in the cerebellar granule cell layer without glial reaction have been shown to be a postmortem event (Albrechtsen 1977a, b). In previous pathological studies of human postmortem cerebellar cortex, the activity of the proteolytic enzyme, naphthylamidase (LNase), is consistently shown when the pH was at an optimum of 5.8 (Albrechtsen 1977a, b). This mainly occurred in the granule cell layer of the cerebellar cortex. Our study shows a similar finding in the cases with severe degrading of the GCL where the mean brain pH of was 5.77.

Brain tissue pH is an important factor in the interpretation of molecular data. Gene expression studies have shown strong correlations to brain pH (Li et al. 2004; Mexal et al. 2006; Tomita et al. 2004; Weickert et al. 2010). Expression patterns exhibited by samples with lower pH tend to involve energy metabolism and proteolytic activities. This increases the genes encoding stress related response proteins and transcription factors (Li et al. 2004).

The ability to provide study cohorts that have been reviewed for quality integrity is essential to postmortem research studies and the interpretation of their results. Even though there were only a small number of cases with moderate or severe changes to the granule cell layer the robust correlations with pH and RIN suggest that the visual assessment of GCL may be a useful and inexpensive screening tool. Review of the histopathological changes of the cerebellar granular cell layer can be performed during the routine neuropathology examination, without adding time or complexity to the screen process. Indeed, the visual assessment of the granule cell layer may reduce the necessity of performing expensive RNA integrity analyses in those cases that have severe cerebellar granule cell layer autolysis. Combined with the measurement of brain pH, the assessment of the cerebellar granule cell layer may assist banks that do not have facilities to perform the extraction for RNA integrity.

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Fig. 1. Grading scheme used to assess the increasing severity of autolysis of the cerebellar granule cell layer; **a** Normal, **b** Mild, **c** Moderate, **d** Severe. Sections are stained with haematoxylin and eosin and photographed at magnification $\times 400$

Fig. 2.

a Effect of granule cell layer integrity grade on brain pH. Individual values and means $\pm$ SD are shown for each histological grade. A significant difference was found between mild grade and normal pathology ($^+P = 0.004$); moderate grade and normal pathology ($^{\wedge}P < 0.0001$); severe grade and normal pathology ($^*P < 0.0001$) and severe and mild grades ($^{\#}P = 0.0050$). **b** Effect of granule cell layer integrity grade on the RNA Integrity Number (RIN). Individual values and mean $\pm$ SD are shown for each histological grade. A significant difference was found between moderate grade and normal pathology ($^+P = 0.0027$) and between severe grade and each of the other grades ($^*P < 0.0001$ vs. normal pathology; $^{\#}P = 0.0001$ vs. mild grade; $^{\wedge}P < 0.0001$ vs. moderate grade)

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Table 1
Demographic details and mean PMI, mean brain pH and mean RIN of clinical classification groups

Classification	(N)	Sex (N), Mean age (y)	PMI (sd)	Brain pH (sd)	RIN (sd)
Alcohol abuse disorders	22	F (7), 55 M (15), 56	36 (16)	6.50 (0.32)	6.80 (1.89)
Control	34	F (8), 73 M (26), 61	30 (15)	6.51 (0.30)	7.07 (1.55)
Motor neurone disease	14	F (5), 67 M (4), 68	16 (11)	6.59 (0.27)	7.22 (0.86)
Multiple sclerosis	9	F (5), 58 M (4), 56	18 (12)	6.27 (0.33)	6.37 (0.98)
Psychiatric disorders	12	F (5), 52 M (7), 57	41 (18)	6.65 (0.22)	7.33 (1.03)
Other	14	F (8), 57 M (6), 60	44 (17)	6.60 (0.23)	7.28 (0.68)

Other—unconfirmed clinical diagnosis, *PMI* post mortem interval, *RIN* RNA integrity number

Table 2

Mean brain pH, mean RIN, and mean PMI for granule cell layer grading groups

Granule cell layer grade	N	PMI, h (sd)	Brain pH (sd)	RIN (sd)
Normal	91	33 (18)	6.60 (0.23)	7.30 (0.90)
Mild	6	24 (17)	6.24 (0.27)	6.60 (0.74)
Moderate	5	23 (11)	6.05 (0.22)	5.80 (0.29)
Severe	3	18 (4)	5.77 (0.15)	1.60 (2.77)