Int J Legal Med (2001) 115 : 6–11

ORIGINAL ARTICLE

L. Quan · B.-L. Zhu · K. Ishida · S. Oritani
M. Taniguchi · M. Q. Fujita · H. Maeda

Intranuclear ubiquitin immunoreactivity of the pigmented neurons of the substantia nigra in fatal acute mechanical asphyxiation and drowning

Received: 7 August 2000 / Accepted: 21 November 2000

Abstract To evaluate the significance of immunohistochemical staining of ubiquitin (heat shock protein) in the midbrain for the medico-legal diagnosis of fatal asphyxiation and drowning, we investigated forensic autopsy cases of fatal mechanical asphyxia (n = 18), manual/ligature strangulation (n = 9), hanging (n = 4), aspiration/choking (n = 5) and drowning (n = 16). These were compared to control groups (n = 30) consisting of fatalities from brainstem injury (n = 12) and acute myocardial infarction (n = 18). Ubiquitin was clearly demonstrated in the nuclei of pigmented substantia nigra neurons, showing two intranuclear staining patterns: a type of inclusion (possibly Marinesco bodies) and a diffuse staining. The diffuse staining was significantly more frequently observed in cases of drowning. The percentage of total ubiquitin positive neurons was frequently higher in strangulation (5.1–28.4%, mean 17.0%), aspiration/choking (5.3–32.0%, mean 17.6%) and drowning (7.0–34.1%, mean 19.8%), but relatively low in hanging (5.1–12.7%, mean 8.6%), brainstem injury (0–10.4%, mean 5.0%) and acute myocardial infarction (1.5–16.9%, mean 8.3%). These observations suggest that intranuclear ubiquitin immunoreactivity of the pigmented substantia nigra neurons in the midbrain was induced by a fatal severe stress on the central nervous system in asphyxiation and drowning.

Keywords Ubiquitin · Immunohistochemistry · Pigmented substantia nigra neuron · Asphyxiation · Drowning

Introduction

Asphyxia in a forensic context is usually relevant to mechanical asphyxia due to various mechanisms including strangulation, hanging, smothering and choking or aspiration. The immediate cause of death involves not only systemic hypoxia due to airway obstruction but also cerebral ischemia and possible nerve effects in cases of pressure on the neck. Although conventional pathology usually greatly contributes to the diagnosis of asphyxial death in a typical case, more complicated cases are not rare and therefore, ancillary evidence may be required. In relation to fatal asphyxia, previous studies have mostly been undertaken to investigate pulmonary pathophysiology [1, 2, 3, 4, 5, 6, 7, 8, 9]. Recently, an increased immunoreactivity of an immediately early gene product, c-fos in the inferior olive of the human medulla oblongata in asphyxia was suggested [10].

Heat shock proteins have been investigated for postmortem markers of local and systemic responses to various traumas and stress [11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24]. Ubiquitin is a well-known heat shock protein that responds very rapidly to various types of stress [18, 19, 20, 21, 22, 23, 24, 25, 26, 27]. In our previous immunohistochemical investigation, a markedly clear ubiquitin staining was observed in the pigmented substantia nigra neurons in the midbrain and a significant increase in the intranuclear ubiquitin positivity was observed in fire fatalities [28].

In the present study, we examined forensic autopsy materials to evaluate the medico-legal significance of the immunohistochemical staining of ubiquitin in the midbrain for the diagnosis of fatal acute mechanical asphyxiation and drowning.

Materials and methods

Materials

Formalin-fixed paraffin-embedded midbrain tissue specimens (horizontal sections, sampled at the given post-mortem times) of forensic autopsy cases of fatal mechanical asphyxia and drowning (n = 34) at our institute were examined. The cases included manual or ligature strangulation (n = 9, 3 males and 6 females, 43–85 years of age, mean age 65.2 years, survival time < 30 min–1.5 h, 15–50 h post-mortem), hanging (n = 4, 3 males and 1 female,

L. Quan (✉) · B.-L. Zhu · K. Ishida · S. Oritani · M. Taniguchi
M. Q. Fujita · H. Maeda
Department of Legal Medicine, Osaka City University Medical School, Asahi-machi 1-4-3, Abeno, 545-8585 Osaka, Japan
Tel.: +81-6-66453767, Fax: +81-6-66343871

40–68 years of age, mean age 56.8 years, survival time < 30 min, 23.5–34 h post-mortem) and aspiration or choking (n = 5, all males, 27–75 years of age, mean age 52.4 years, survival time < 30 min, 7–20 h post-mortem) and drowning (n = 16, including 6 cases of possible suicide, 7 males and 9 females, 13–85 years of age, mean age 55.8 years, survival time < 30 min, 5–74 h post-mortem) in fresh water (n = 11) and salt water (n = 5). Control groups (n = 30) consisted of fatalities from brainstem injury (n = 12, 11 males and 1 female, 30–85 years of age, mean age 55.8 years, survival time < 5 min, 8–38 h post-mortem) and acute myocardial infarction (n = 18, 12 males and 6 females, 39–89 years of age, mean age 62.5 years, survival time < 0.5–15 h, 6–34 h post-mortem).

Methods

Tissue sections

Serial sections (4 µm thick) were prepared from the tissue specimens of the midbrain. The tissue sections were used for hematoxylin-eosin (H&E) and immunostaining.

Immunostaining

Polyclonal rabbit anti-ubiquitin serum (Dako A/S, Denmark) was used at a 100-fold dilution and a 3 h incubation at 37 °C with the Vectastain Universal Elite ABC kit (DAB) (Vector Laboratories, Burlingame, Calif.) according to the manufacturer's instructions (counterstaining with hematoxylin). Endogenous peroxidase was inactivated by incubation with 3% hydrogen peroxide for 5 min. For the control study to confirm the specificity of immunostaining, phosphate-buffered saline or normal rabbit serum was substituted for the primary antibody.

Quantitative analysis of Marinesco bodies and ubiquitin staining in the nuclei of pigmented neurons of the substantia nigra

Marinesco bodies were identified as eosinophilic nuclear inclusions in addition to the nucleolus in the pigmented neurons in the H&E sections. The neurons with nuclei in which Marinesco bodies were detected were quantitatively analysed, the number of total pigmented neurons and Marinesco body-containing neurons were counted in 10 fields under a 200 × magnification and the percentage was estimated.

Ubiquitin-positive pigmented neurons were quantitatively analysed in a similar manner: the number of neurons with nuclei in which ubiquitin immunoreactivity was detected were counted and the percentage of nuclear ubiquitin positivity (Ub-positive %) was estimated as described above.

Chemical analysis

Blood alcohol levels were determined by head-space gas chromatography/mass spectrometry [29]. Drug analyses were performed by gas chromatography/mass spectrometry.

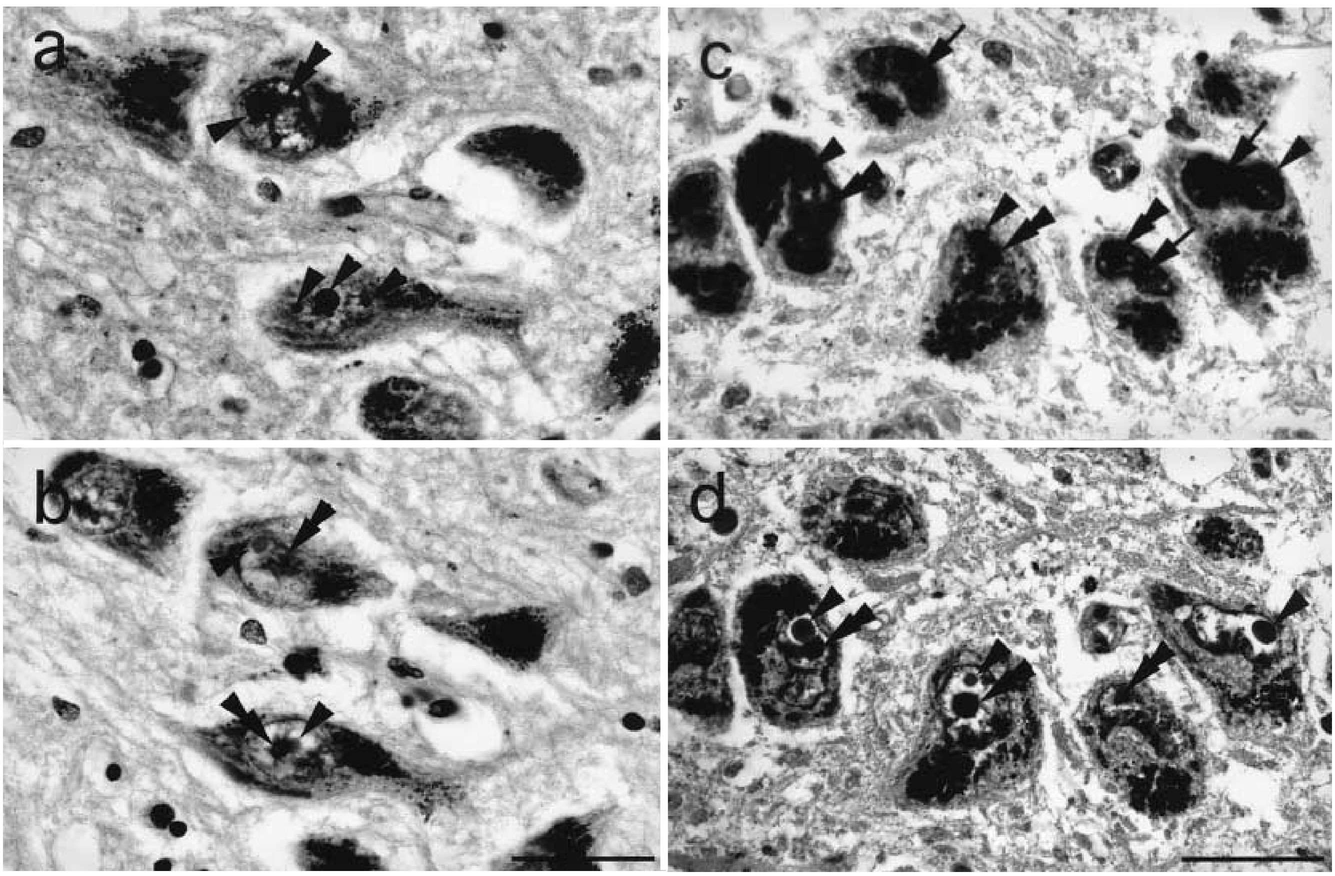


Fig. 1 a–d Intranuclear inclusions and ubiquitin immunoreactivity of the pigmented substantia nigra neurons in the midbrain (*Original magnification* × 1,000, *bar* 25 µm). **a** Intranuclear ubiquitin immunoreactivity *(arrowheads)* and **b** intranuclear inclusions (Marinesco bodies) by H&E staining *(arrowheads)* on serial sections in a case of fatal ligature strangulation (60-year-old female, 15 h post-mortem). **c** Intranuclear diffuse immunostaining of ubiquitin *(arrows)* and **d** the H&E finding in a case of fatal drowning (67-year-old male, 18 h post-mortem). (Nucleoli shown by *double arrowheads*)

Statistical analyses

A regression analysis was used to examine the relationship of Marinesco bodies and nuclear ubiquitin positivity with the age of victims. Comparisons between groups were performed using Student's *t*-test and the Mann-Whitney U-test. A logistic regression test was used in the multivariate analyses. A P value less than 0.05 was considered statistically significant.

Results

Immunohistochemical distribution of ubiquitin

Intense immunostaining of ubiquitin was usually observed in the cerebral peduncles and the ependymal cells of the cerebral aqueduct, irrespective of the cause of death. The microglial cells in the central grey matter were densely positive in drowning cases, whereas the positive staining was only scattered in some cases of the other types of fatality. In the pigmented substantia nigra neurons, intranuclear ubiquitin staining was very clear to identify, showing two patterns; a type of inclusion body and a diffuse staining (Fig. 1 a, c). The diffuse staining partially involved ubiquitinated inclusions. Typical intranuclear inclusions were eosinophilic in H&E staining of the adjacent sections and were identified as Marinesco bodies (Fig. 1 b, d). The ubiquitinated nuclei were markedly larger and less stained than the others in H&E sections.

Quantitative analysis of Marinesco bodies

The percentage of Marinesco body-containing pigmented neurons of the substantia nigra gradually increased depending on the age of victims in the total cases, whereas age-dependency was not significant in each individual cause of death (Fig. 2 a). The percentage of positive staining in fatal strangulation (2.3–24.6%, mean 12.6%) was significantly higher than in brainstem injury (0–10.9%, mean 4.1%) and in acute myocardial infarction (0–15.3%, mean 5.8%), whereas it was relatively low in hanging (2.3–10.8%, mean 6.4%) and drowning (0–7.9%, mean 4.4%) (Fig. 3 a). In a multivariate analysis using logistic regression tests (odds ratios and P-values in parenthesis), the frequency of Marinesco body-containing neurons showed a correlation with the age (0.92, $P = 0.026$) and was significantly high in strangulation cases (0.051, $P = 0.03$ and 0.039, $P = 0.007$) in comparison with the controls (brainstem injury and acute myocardial infarction, respectively), with no relationship to the gender, post-mortem time or survival time. An increasing tendency of Marinesco body-containing neurons was observed in aspiration/choking cases (0.063, $P = 0.04$), when compared with the combined control groups.

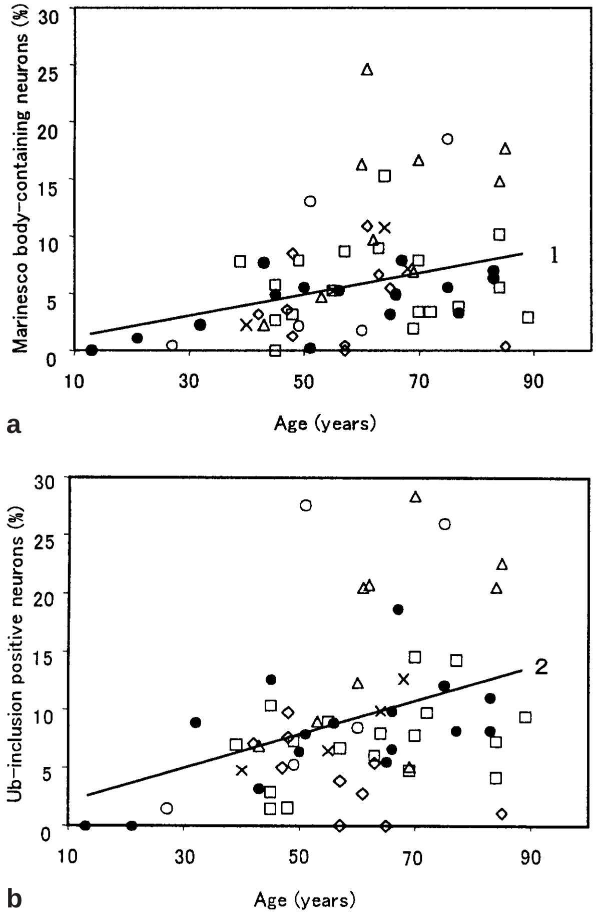
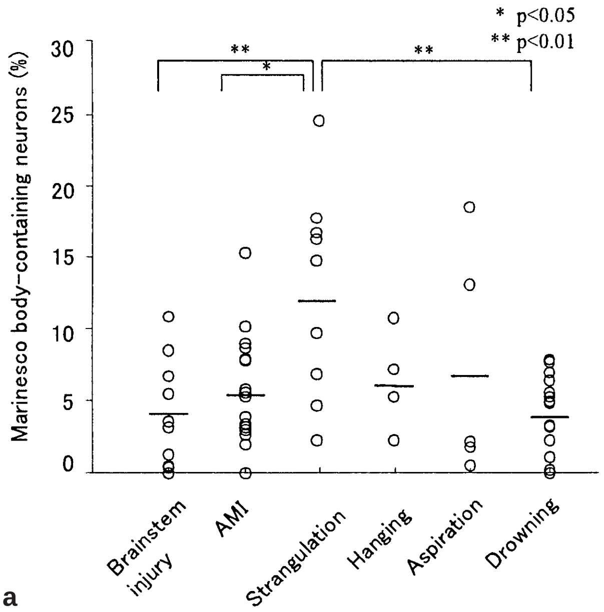


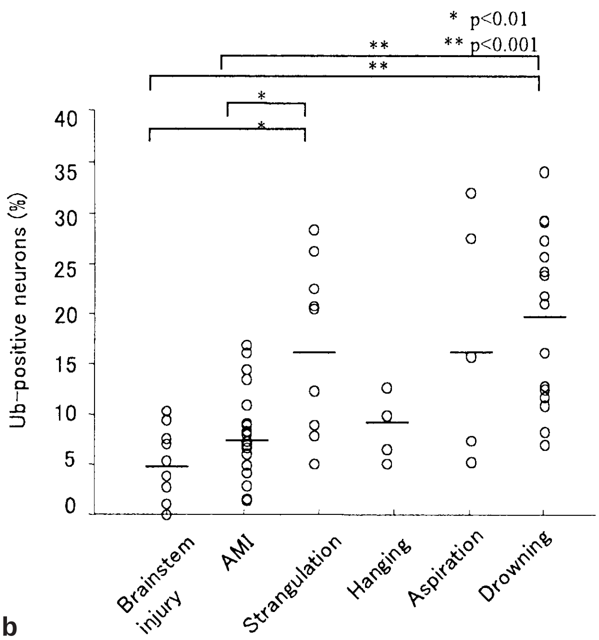
Fig. 2 Relationship of **a** Marinesco body and **b** the inclusion-type nuclear ubiquitin positivity frequency in the pigmented substantia nigra neurons with the age of victims (△ strangulation, × hanging, ○ aspiration/choking, ● drowning, ◆ brainstem injury, □ acute myocardial infarction, correlation equations: *1* Marinesco body in the total cases, $y = 0.11x - 0.37$, $n = 62$, $r = 0.36$; *2* inclusion-type ubiquitin positivity in the total cases, $y = 0.15x - 0.10$, $n = 62$, $r = 0.37$)

Quantitative analysis of nuclear ubiquitin positivity in the pigmented substantia nigra neurons

The inclusion-type Ub-positive % showed a correlation with the age of victims in total cases, whereas age-dependency was not significant in each individual cause of death (Fig. 2 b). There was no correlation between the total or diffuse type Ub-positive % and age. The total Ub-positive % in strangulation (5.1–28.4%, mean 17.0%) and drowning (7.0–34.1%, mean 19.8%), respectively, were significantly higher than those in the control groups of brainstem injury (0–10.4%, mean 5.0%) and acute myocardial infarction (1.5–16.9%, mean 8.3%), whereas no significant increase was observed in hanging (5.1–12.7%, mean 8.6%) (Fig. 3 b). Aspiration/choking cases (5.3–32.0%, mean 17.6%) showed an increasing tendency of the total Ub-positive %. The intranuclear diffuse ubiquitin staining was almost exclusively observed in fatal drowning (Fig. 3 c). The Ub-positive % in the total and that of



a



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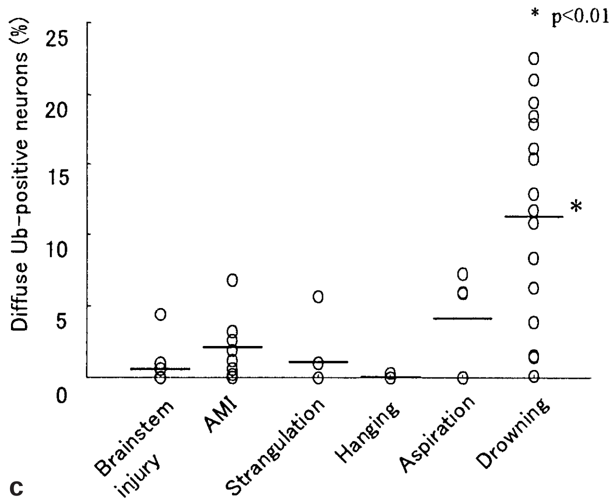

c

Fig. 3 Comparison of **a** Marinesco body, **b** the total nuclear ubiquitin positivity and **c** diffuse type nuclear ubiquitin positivity frequencies in the pigmented substantia nigra neurons between the victims of fatal asphyxiation, drowning, brainstem injury and acute myocardial infarction *(AMI)*. *P* values in Mann-Whitney U-test are shown

inclusion-type, correlated with the frequency of Marinesco body-containing neurons, usually showing a higher value (Fig. 4). In a multivariate analysis using a logistic regression test (odds ratios and *P*-values in parenthesis), the total Ub-positive % showed a significant increase in drowning cases (0.011, $P = 0.0007$ and 0.039, $P = 0.001$) in comparison with the controls (brainstem injury and acute myocardial infarction, respectively) and in cases of strangulation (0.054, $P = 0.022$) and aspiration/choking (0.051, $P = 0.036$) in comparison with the brainstem injury, with no relationship to the age, gender, post-mortem time or survival time. The diffuse type Ub-positive % was significantly higher in fatal drowning (0.019, $P = 0.002$ and 0.03, $P = 0.0004$) in comparison with the controls (brainstem injury and acute myocardial infarction, respectively) and a similar tendency was observed in aspiration/choking (0.082, $P = 0.024$), when compared with the combined control groups. There was no significant difference in the total Ub-positive % between the types of immersion medium (i.e. fresh and salt water) in drowning.

For the additional findings, strangulation and drowning cases of relatively low total Ub-positive % (< 15%, $n = 4$ and $n = 6$, respectively) included four out of six cases of possible suicidal drowning. In aspiration cases, the total Ub-positive % was low (5.3% and 7.4%) in two cases with blood alcohol at a potentially fatal or comatose level (4.59 mg/ml and 3.67 mg/ml, respectively). Sedative or hypnotic drugs were not detected.

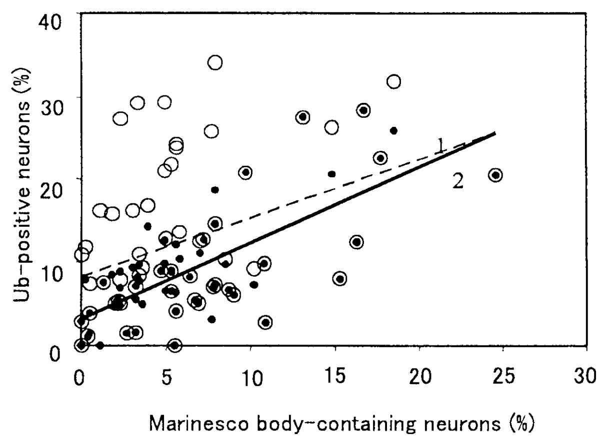

Fig. 4 Relationship between Marinesco body and nuclear ubiquitin positivity frequencies in the pigmented substantia nigra neurons (○ total ubiquitin-positive neurons *1*: $y = 0.71x + 8.23$, $n = 62$, $r = 0.41$, $P < 0.001$; ● inclusion-type ubiquitin-positive neurons *2*: $y = 0.92x + 3.13$, $n = 62$, $r = 0.72$, $P < 0.001$)

Discussion

Our previous study has shown that the intranuclear ubiquitin immunoreactivity of the pigmented substantia nigra neurons in the midbrain was induced by fatally severe stress in fires [28], possibly increased in older victims [30, 31, 32, 33, 34, 35, 36] and partially suppressed by cyanide poisoning, a factor which can lead to a reduction in survival time and physical activity. A possible explanation for the increased nuclear ubiquitin-positivity with inclusions may be neurodegeneration due to hyperactivity or overexcitation of the neurons (energy crisis or excitotoxicity) [37, 38, 39, 40], suggesting a possibility for evaluation of the stress on the CNS resulting from physical activity before death [28].

In the present study on fatal asphyxiation, although an age-dependent increase was observed in the frequency of neurons containing Marinesco bodies and inclusion-type nuclear ubiquitin-positivity in the total cases, such an age-dependency was not clear in the total nuclear ubiquitin-positivity, which usually showed a higher value than the Marinesco body positive value, as was previously reported [28]. In this analytical situation, a significant increase in Marinesco bodies was observed in strangulation and aspiration/choking cases showed a similar tendency. The total nuclear ubiquitin-positivity showed a significantly higher frequency also in fatalities from drowning. An explanation for the rapid appearance of the ubiquitin immunoreactivity in death from acute asphyxiation may be the possible activation and redistribution of ubiquitin to form larger aggregates rather than a de novo synthesis [41, 42]. The pigmented substantia nigra neurons in the midbrain are dopamine-neurons with a function related to skeletal muscle movements under control of the cerebral cortex [43]. A tentative speculation, in consideration of the function and cortical regulation of the neurons, may be a contribution of cortical stimulation to the neurodegeneration during alert consciousness, which is related to physical activity [28]. In this connection, it was notable that the nuclear ubiquitin-positivity of the pigmented neurons was relatively low in hanging cases, most cases of possible suicidal drowning and aspiration cases with blood alcohol at a potentially fatal or comatose level. Thus, it was suggested that intranuclear ubiquitin immunoreactivity of the pigmented substantia nigra neurons was induced by fatally severe stress in acute mechanical asphyxiation and drowning, possibly independently of cerebral hypoxia or ischemia [28]. Further investigations are necessary with respect to the age-dependency and the factors related to the physical activity before death, e.g. the effect of alcohol, sedative-hypnotic drugs and CNS stimulants. It also appeared interesting to investigate more cases of typical and atypical hanging in comparison with strangulation for differentiation between a suicide and a homicidal assault.

Additionally, there was a difference in the findings of drowning cases from the other fatalities: the diffuse nuclear ubiquitin-positivity pattern was significantly frequently observed in drowning and ubiquitin-positivity of the microglial cells was dense. The frequency of the Marinesco body-containing neurons was relatively low, although the total nuclear ubiquitin-positivity was as high as in mechanical asphyxiation. A similar finding was observed in aspiration/choking cases. Such a diffuse nuclear ubiquitin-positivity pattern has been noted in some fire victims, showing an inverse relationship to blood cyanide levels, which can be a factor to reduce the physical activity [28]. A possible explanation for these findings may be that the diffuse nuclear ubiquitin-positivity pattern, accompanied by a relatively low frequency of the Marinesco body-containing neurons, may be due to more advanced neurodegeneration related to a longer or more intense stress, although further investigations are required.

In conclusion, the present study suggests that intranuclear ubiquitin-immunoreactivity of pigmented substantia nigra neurons in the midbrain was induced by fatally severe stress in acute mechanical asphyxiation and drowning, possibly independently of cerebral hypoxia or ischemia, therefore suggesting a possibility for evaluation of the stress on the CNS resulting from physical activity before death.

Acknowledgement This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (Grant Nos. 11670425 and 12470109).

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