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Postmortem Interval Estimation by Evaluating Saposin D Levels and Morphological Alterations in Hippocampal Neurons

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Abstract

Background: The correct estimation of the time of death is critical in the field of forensics and legal medicine, and often determines the outcomes of criminal investigations. Current postmortem interval (PMI) estimation methods involve the evaluation of physical, chemical, biological, and histological postmortem changes; however, their accuracy is limited.

Aims, Study design and Methods: In this study, we used rat brains to characterize postmortem alterations in the levels of prosaposin (PSAP) and its degradation product saposin D, as well as morphological changes in the hippocampus. Furthermore, we used fluorescent microscopy and observed profound morphological alterations in hippocampal pyramidal neurons after death.

Results: We found that PSAP levels decreased after death, whereas saposin D levels increased. Morphological alterations prolonged in hippocampal pyramidal neurons up to 7 days after death. Conclusion: These findings suggest that morphological alterations in the hippocampus and the PSAP and saposin D levels may improve the accuracy of PMI estimation.

Key words: Postmortem interval, MAP2, Prosaposin, Immunohistochemistry, Western blotting

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INTRODUCTION

The accurate estimation of the postmortem interval (PMI) is crucial in forensic sciences, given the importance of the PMI in determining the circumstances of death. Many PMI estimation approaches are based on physical, chemical, or biological cues. Algor mortis, livor mortis, rigor mortis, and supravital activity are among the most commonly used early PMI estimates, whereas physical changes (e.g., arthropod activity) and physiological changes (e.g., typical signs of decomposition) are used as late PMI estimates (1, 2). The decrease in body temperature is common PMI estimation method(3, 4). However, the temperature method can only be used up to 36 hours postmortem depending on the environmental conditions. Additionally, its usefulness is limited in certain death cases, such as burns and severe trauma. Similarly, livor mortis provides limited accuracy in cases of severe blood loss and severe trauma. Although forensic entomology can also provide viable information in later stages of death, this approach is restricted to local fauna and insect accessibility to the dead body. Each of these methods provides only a rough estimate of PMI, and often different methods yield contradicting results. Thus, novel, more accurate PMI estimation methods are required.

Postmortem decomposition of biomolecules has gained increasing attention over the last years. Several studies have investigated postmortem alterations of RNA, DNA, or proteins (5-10). We have used rat models to investigate postmortem changes in brain biomolecules, which are protected from environmental insults (e.g., bacterial infections) by the skull. Specifically, we have analyzed changes in protein levels and histological changes in rat brains at two different time points after death. Increasing evidence suggests that the levels of RNA, DNA, proteins, and other biomolecules decrease over time after death. Nevertheless, it remains unclear if the levels of some biomolecules increase after death. Prosaposin (PSAP) is a ~66-kDa glycoprotein precursor of four sphingolipid activator proteins, saposins A, B, C, and D. Saposins are abundantly found in the brain, where they act as neurotrophic factors (11-15). Importantly, the structure of PSAP is highly conserved between rats and humans. We hypothesized that due to decomposition, PSAP levels decrease and saposin levels increase after death.

Organs decay over time. In particular, the abdominal organs are strongly affected by autolysis and bacterial putrefaction, leading to specific alteration patterns (16). Tomita et al. (17) comprehensively analyzed the alterations in abdominal organs postmortem. However, the postmortem histological changes that occur in the brain remain unknown (18). In this study, we investigated the usefulness of the PSAP and saposin levels and histological changes in the brain to accurately estimate the PMI.

MATERIALS AND METHODS

Animals and tissue preparation

Nine Wistar rats (8 weeks old) were purchased from CLEA Japan (Kyoto). Rats were housed at a constant temperature (22°C) under a 12/12-h light/dark cycle and were given food and water ad libitum. All experiments were conducted in accordance with the ARRIVE guidelines and the Guide for Animal Experimentation of the Ehime University School of Medicine, Japan. Animal protocols were approved by the Animal Care Committee of Ehime University (permit number 05A261). Animals were euthanized by carbon dioxide inhalation and stored in a 21°C thermostatic chamber for 0, 3, 6, or 12 h, or 1, 2, 3, 5, or 7 d postmortem (n = 1 at each time point). Brains were collected and divided into left and right parts. Brain tissues were placed into Eppendorf tubes (1.5 mL) on ice and stored at −80°C until homogenization.

Anti-rat PSAP antibody

The anti-rat PSAP antibody (PSAP-Ab: IM-1) was prepared as previously described (19). Briefly, the amino acid sequence of rat PSAP was identified (20), and a synthetic oligopeptide corresponding to the proteolyzed portion of PSAP (409-PKEPAPPKQPEEPKQSALRAHVPPQK-434; Fig. 1) was used to generate a rabbit polyclonal antibody against rat PSAP. The generated antibody reacted with PSAP but not with saposins.

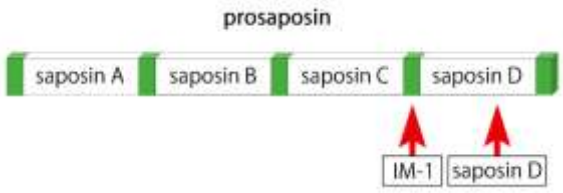

Figure 1. Structure of prosaposin (PSAP). PSAP is the precursor of four saposins. Anti-PSAP antibody (IM-1) was generated using a synthetic oligopeptide corresponding to the proteolysis site located between saposin C and saposin D. Because this sequence is absent from mature saposins, the antibody does not react with saposins; it only reacts with PSAP. The anti-saposin D antibody reacts with both PSAP and saposin D.

Immunohistochemical analysis

Rat brains were fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer), embedded in paraffin, sectioned, and deparaffinized. The sections were washed for 15 min in PBS plus Tween 20 (PBS-T) and blocked overnight in PBS (0.1 M) containing 5% bovine serum albumin, 5% normal swine serum, and 0.1% NaN3. Subsequently, samples were probed with the primary antibodies rabbit polyclonal anti-PSAP IgG (1:5,000) and mouse monoclonal anti-MAP2 IgG (1:5,000). The sections were washed twice for 15 min in PBS and incubated with the secondary antibody Cy3 anti-rabbit IgG (1:250) or FITC-conjugated anti-mouse IgG or IgM (1:500). Cell nuclei were counterstained with DAPI for 2 h. Then, the sections were washed twice for 15 min in PBS, mounted in PermaFluor Aqueous Mounting medium (Thermo Shandon; Thermo Scientific, West Palm Beach, FL, USA), and examined under a Nikon A1 confocal microscope (Nikon, Tokyo, Japan). The

hippocampal CA1 regions were examined to assess pyramidal cell body alignment. Pyramidal cell bodies are aligned in rows, extending long apical dendrites in the same direction. Tissues were compared for these characteristic histological features.

Nomenclature of hippocampal CA1 neurons

The hippocampal CA1 region is divided into five layers: alveus, stratum (st.) oriens, st. pyramidale (pyramidal neuron layer), st. radiatum, and st. lacunosum-moleculare (Fig. 3)(21). Typically, one or two primary apical dendrites emerge from the neuronal cell body of CA1 pyramidal neurons, and then they are divided within the st. radiatum. Some basal dendrites originate from the CA1 cell body, bifurcating two or three times before terminating near the alveus (22-24).

Western blotting

Tissue samples were homogenized using a homogenizer (Tietech, Japan) in 50 mM Tris-HCl buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl) supplemented with 1× complete protease inhibitor cocktail (1%, Nacalai Tesque, Inc., Kyoto, Japan). Homogenates were centrifuged for 15 min at 12,000 × g at 4°C, and the supernatants were collected, aliquoted, frozen in liquid nitrogen, and stored at −80°C until further use.

Equal amounts (21 μg) of protein were loaded onto NuPAGE Bis-Tris gels following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). After electrophoresis, proteins were transferred onto 0.45-μm polyvinyl difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% BSA in 1× Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and incubated at 4°C overnight with one of the following primary antibodies: anti-saposin D (1:1,000), anti-PSAP (1:1,000), anti-α-tubulin (1:1,000), anti-β-catenin (1: 500), or anti-GAPDH (1:500). Subsequently, membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (1:5,000, Dako, Denmark). Protein signals were developed using ECL Prime Western blotting detection reagent (GE Healthcare, Buckinghamshire, UK) and imaged on an ImageQuant LAS 4000 imaging system (GE Healthcare, Marlborough, MA, USA).

RESULTS

Postmortem changes in PSAP and saposin levels

We extracted crude brain proteins at different time points (up to 7 d) after death to investigate changes in the levels of each proteins. The levels of α-tubulin and β-catenin, which are abundantly expressed in most tissues, decreased over time early postmortem (Fig. 2. a, b). No significant changes were observed in GAPDH levels until 5 d after death, when its levels began to decrease (data not shown). We also found that the PSAP levels (single band at ~65 kDa) decreased over time (Fig. 2c, d). In contrast, the saposin D levels (single band at ~25 kDa) gradually increased after death (Fig. 2e, f).

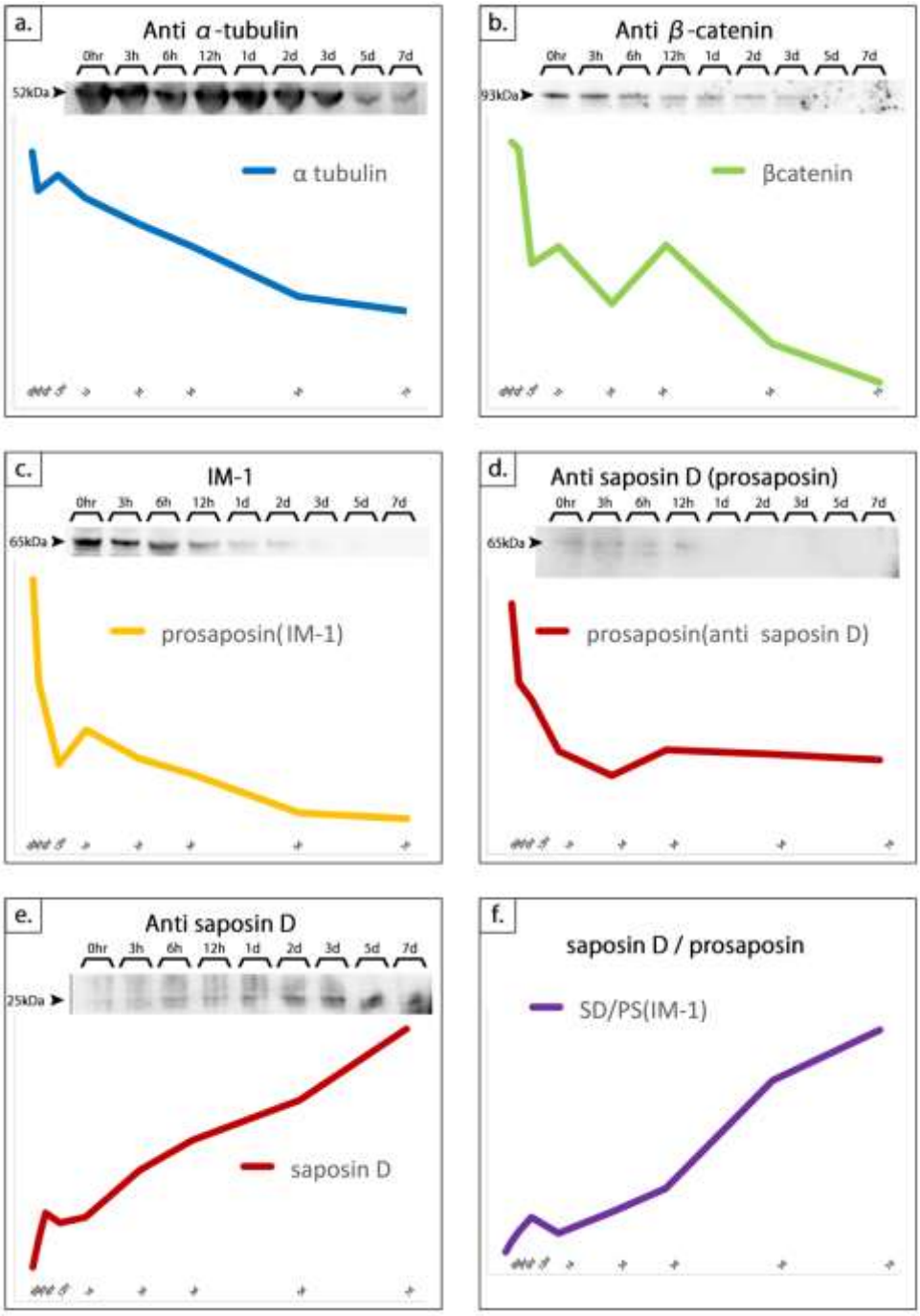

Figure 2. Crude hippocampal extracts obtained at different time points (from 0 h to 7 d) after death were stained with anti-α-tubulin (a), anti-β-catenin (b), anti-PSAP (c), or anti-saposin D (d, e). The intensity of the single band at 65 kDa (possibly corresponding to PSAP) decreased over time. (e, f) The intensity of the single band at 25 kDa (possibly corresponding to saposin D) increased over time. (f) The ratio of saposin D to PSAP gradually increased during the postmortem period (f).

Histological postmortem changes in CA1 pyramidal cells

We also compared the histological characteristics of CA1 pyramidal cells between the time of death (0 h; Fig. 3) and at different time points after death (Fig. 4). Pyramidal cells consist of the soma (containing the nucleus), axons, apical dendrites, and basal dendrites. The apical dendrite forms oblique branches terminating in a narrow tuft. PSAP is predominantly localized in the cytoplasm around the nuclei and large dendrites. The structures of the hippocampal CA1 region and pyramidal neurons were assessed by immunostaining with anti-MAP2, anti-PSAP, and DAPI (Fig. 3). The hippocampal CA1 region is divided into five layers. The dendrites of pyramidal neurons stained strongly with anti-MAP2. Primary apical dendrites were evident at the neuronal cell body and branched within the st. radiatum. Some basal dendrites originated from the CA1 cell body and bifurcated into secondary basal dendrites. PSAP was localized mainly in the cytoplasm of the CA1 cell body and in some dendrites (Fig. 3, 4).

We found almost no morphological changes in the CA1 pyramidal at cells 3 h after death (Fig. 4a1-4). The density of basal dendrites started to decrease by 6 h after death (Fig. 4b4). At this time point, the apical dendrite width was decreased but the length and number were maintained; some of the secondary branches had disappeared 6 h after death (Fig. 4b1, b2). The morphology of the cell soma, as well as the expression levels and distribution pattern of PSAP, were unchanged at 6 h after death (Fig. 4b3). The density of basal dendrites was markedly reduced by 12 h after death (Fig. 4c4). Additionally, the distal part of apical dendrites and the side branches had almost disappeared at 12 h after death (Fig. 4c2), and the cell nuclei had begun to shrink (Fig. 4c3). We observed no basal or apical primary dendrites at 1 d after death, and only the stems of pyramidal neurons were maintained (Fig. 4d1, d2). The nuclei had shrunk further, and the cell bodies became narrower (Fig. 4d3). The PSAP levels were further reduced extracellularly at 1 d after death, although PSAP levels were maintained intracellularly (Fig. 4d3, d4).

Basal primary dendrites had disappeared completely at 2 d after death, and the stems of pyramidal neurons had started breaking down (Fig. 4e2). PSAP immunostaining was prominent in the nuclei of CA1 neurons at 2 d after death (Fig. 4e3). Almost no apical dendrites were observed at 3 d after death (Fig. 4f2), and nuclei shrinkage and deformation were even more evident. Furthermore, MAP2 was almost undetectable around the cell nuclei (Fig. 4f3). All dendrites and cytoplasmatic extensions had disappeared at 5 d after death (Fig. 4g1), and MAP2 immunoreactivity was observed only as fragments (Fig. 4g3). Seven days after death, the hippocampal CA1 region was fully deformed (Fig. 4h1) except for some remaining nuclei. The structural changes in hippocampal pyramidal neurons and the alterations in PSAP expression patterns are illustrated in Figure 5. Gradual changes were observed in dendrites up to 3 d after

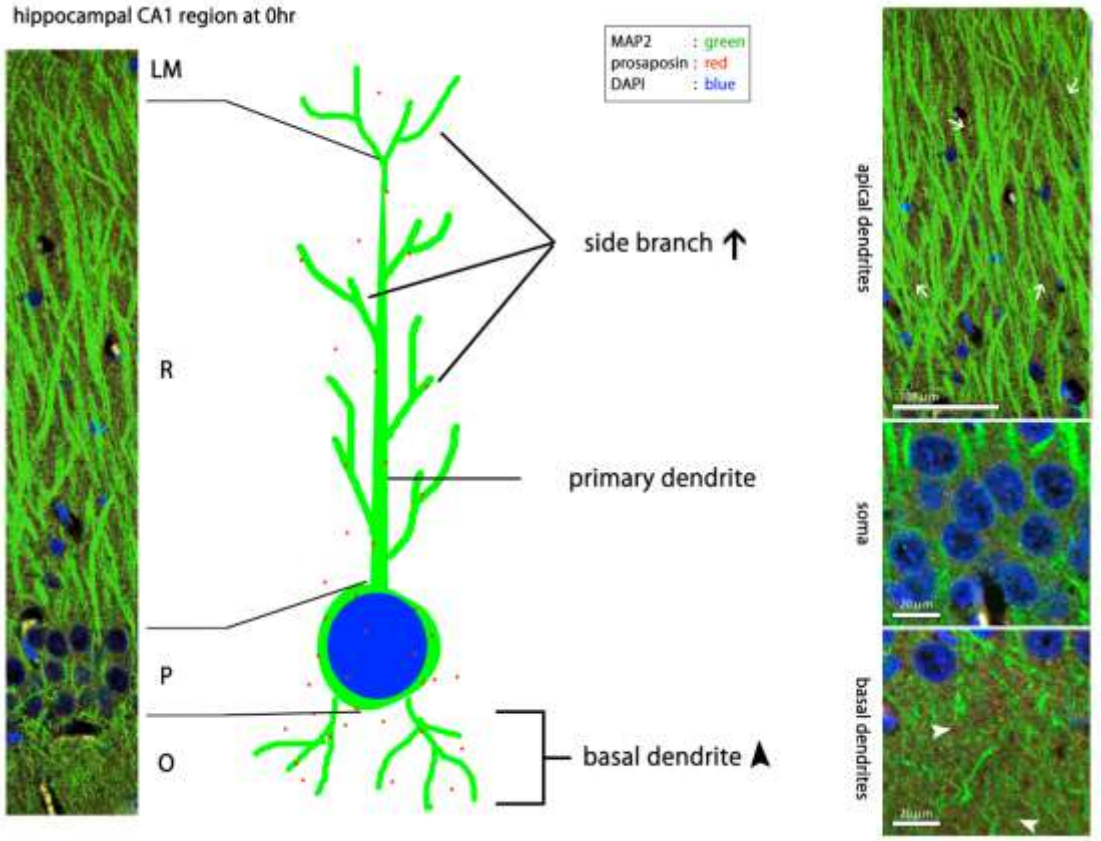

Figure 3. Immunofluorescence micrographs at 0hr. Structure of the hippocampal CA1 region and pyramidal neurons after immunostaining with anti-MAP2 (green) or anti-PSAP (red) antibodies or DAPI (blue). The hippocampal CA1 region is divided into five layers: alveus, st. oriens (O), st. pyramidale (P), st. radiatum (R), and st. lacunosum-moleculare (L-M). Primary apical dendrites emerge from the neuronal cell body and branch into the st. radiatum. Some basal dendrites emerge from CA1 cell bodies and bifurcate into secondary basal dendrites. PSAP was predominantly localized in the cytoplasm or dendrites of CA1 neurons.

death, and cell bodies were entirely fragmented at 7 d after death. Nuclear translocation of PSAP was observed mainly between 2 and 5 d after death. The structural changes in CA1 pyramidal neurons (apical and basal dendrites and neuronal somata), as well as the alterations in PSAP levels and its distribution during the postmortem period, are detailed in Fig. 5b.

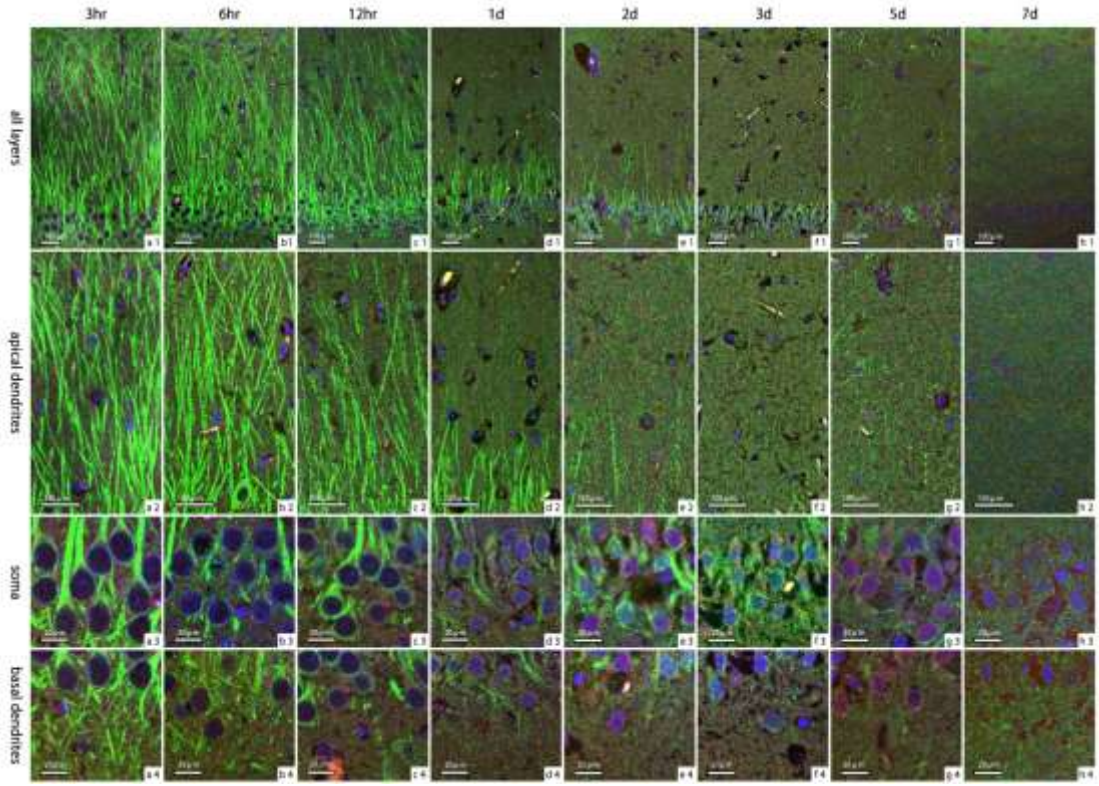

Figure 4. Representative immunofluorescence micrographs showing the characteristic structural changes in CA1 pyramidal neurons and the distribution of PSAP. Immunostaining was performed as in Fig. 3. At 3 h after death (a), almost no changes were observed. At 6 h after death, the density of basal dendrites was profoundly reduced (4b4). At the same time, the width of apical dendrites and the density of secondary branches were decreased, although the length and number of primary apical dendrites were unchanged (4b1, b2). Twelve hours after death (c), no basal primary dendrites, distal parts of apical dendrites, or secondary branches were evident. At 1 d after death, primary basal and apical dendrites had disappeared, and only the stems of pyramidal neurons were observed (d1, d2). The nuclei had shrunken further, and the cell bodies had become thinner (d3). The PSAP levels were further reduced extracellularly, but were maintained intracellularly (4d3, d4). Basal primary dendrites had completely disappeared by 2 days after death (e), and the stems of pyramidal neurons had begun breaking down. PSAP signals were prominent in the nuclei of CA1 neurons at 2 d after death. (f) Almost no apical dendrites were observed at 3 d after death, nuclear shrinkage and deformation were even more evident (f2, f3), and MAP2 signals were almost undetectable around the cell nuclei (f3). At 5 d after death (g), all dendrites and cytoplasmatic extensions had disappeared (g1), and MAP2 signals were only detected in cellular fragments. At 7 d after death (h), the hippocampal CA1 region was fully deformed (Fig. 4h1) except for some remaining nuclei.

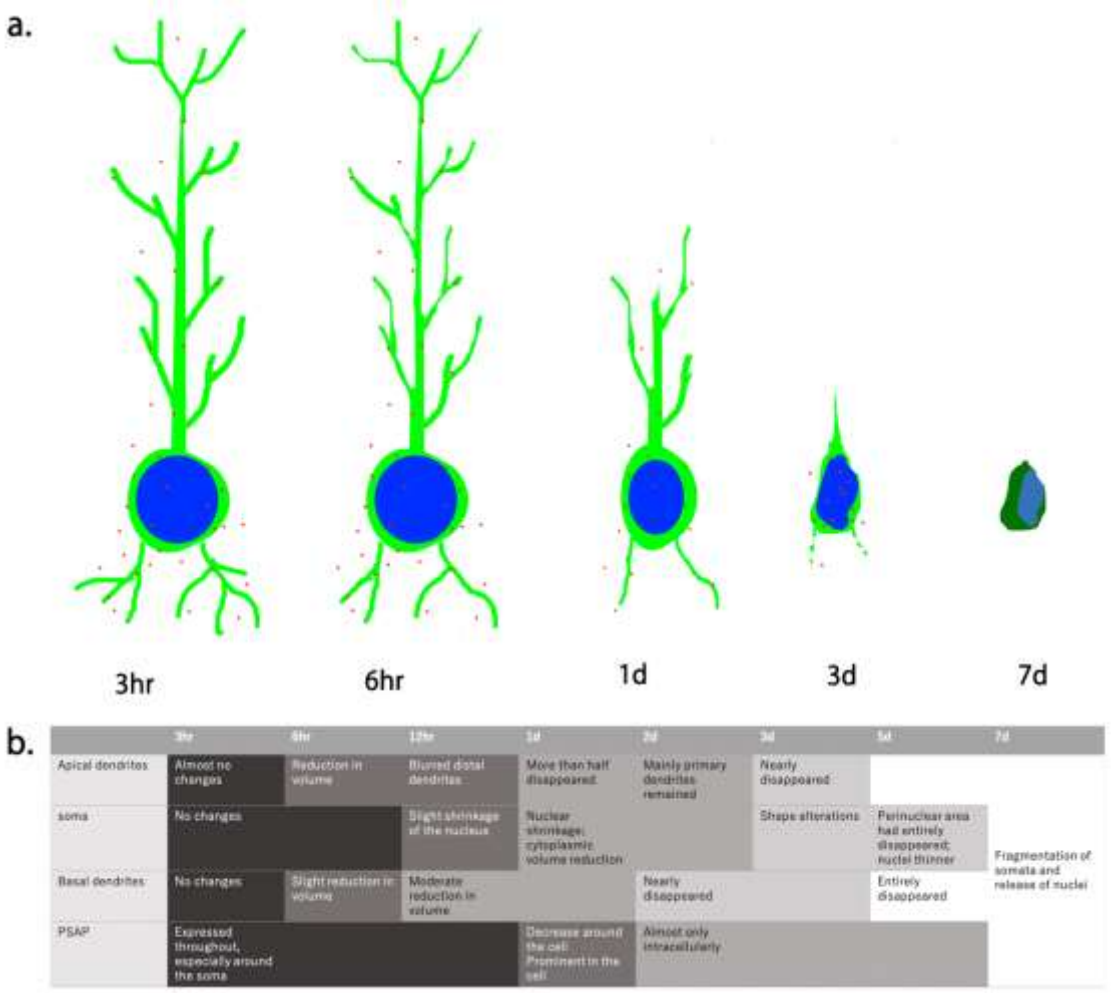

Figure 5.

a. Schematic presentation of the structural changes in CA1 pyramidal neurons and the distribution of PSAP during the postmortem period. At 3 h after death, no apparent changes were observed. At 6 h after death, secondary dendrites of both apical and basal neurons were fragmented. At 1 d after death, primary dendrites of basal and apical neurons had disappeared, only the stems of pyramidal neurons were observed, and PSAP immunostaining was observed in the cytoplasm around the nuclei of CA1 neurons. Neuronal bodies had disappeared at 3 d after death, and cell bodies were entirely fragmented by 7 d after death.

b. Detailed postmortem alterations in CA1 pyramidal cell morphology (apical and basal dendrites, neuronal somata) and PSAP levels and distribution.

DISCUSSION

In forensics, PMI estimation is predominantly based on corpse characteristics (e.g., temperature reduction, livid mortis, rigor mortis, and putrefaction) and forensic entomology. Nevertheless, these methods are strongly affected by premortem conditions and environmental factors, limiting their accuracy. Therefore, novel, more accurate, and objective PMI estimates are required. Current forensic research efforts are

focused on postmortem changes in the levels of different biomolecules (25-27). In this study, we characterized the postmortem alterations in rat brains. We identified characteristic postmortem changes in the levels and distribution of PSAP and saposin D (Fig. 2), as well as profound morphological changes in pyramidal neurons of the hippocampal CA1 region (Fig. 3, 4, 5).

Histological postmortem changes in the hippocampal CA1 region

Here, we focused on morphological alterations in the hippocampus, a structure located in the center of the brain. The hippocampal region has been extensively studied in the context of ischemia and memory. The morphology and structure of the hippocampal CA1 and CA3 regions have been comprehensively characterized (22, 23). In this study, we found that pyramidal cells and their long apical dendrites were aligned in rows (Fig. 3). Due to its well-defined structure, the hippocampus is the most suitable region to characterize morphological changes during the postmortem period. In this region, we performed immunohistochemistry for MAP2, PSAP, and DAPI, which allowed us to observe the overall hippocampal structure, as well as the morphology of neuronal somata, apical dendrites, and basal dendrites (Fig. 3-5). Density reduction of basal dendrites was the first morphological change in the hippocampal CA1 region postmortem, followed by characteristic changes in apical dendrites, which became narrower and shorter. Subsequently, cell nuclei shrunk, and the neuronal bodies eventually disappeared (Fig. 5).

Although numerous studies have assessed the usefulness of morphological changes to estimate PMI, the characterization of postmortem morphological alterations has proved challenging. Janssen et al. (28) failed to identify postmortem changes and antemortem degeneration by microscopical examination. Autolysis in a dead body can be distinguished from cell death in a living body by the fact that the former is diffuse rather than focal and does not invoke inflammatory responses (29). Additionally, postmortem structural changes do not involve the formation of cellular structures (e.g., autophagic vacuoles) or the induction of hydrolytic enzymes, and cell death does not coincide with organismal death. Excessive cell death frequently accompanies pathological processes and is a natural process occurring throughout life (30). Hence, cell death or tissue degeneration alone cannot be used as a PMI estimate, and comprehensive morphological characterization of relatively large tissue specimens is needed. To this end, we conducted fluorescence microscopy of the hippocampus; confocal microscopy may further increase imaging resolution. The morphological evaluation of the hippocampus after immunofluorescent staining enabled the identification of postmortem structural changes in pyramidal neurons at the tissue level that might not have been evident at the cellular level.

Currently, the most precise method to determine PMI is temperature measurements, as the body temperature decreases drastically after death.

However, this method is only useful in the early postmortem stage (0-36 h). Nunley et al. (30) and Tomita et al. (17) reported about morphological postmortem changes had mainly occurred by 24 h after death. In contrast to these findings, using fluorescence microscopy, we observed morphological changes in hippocampal pyramidal neurons up to 7 d after death, suggesting that morphological postmortem alterations can be used to estimate PMI for more extended periods.

Furthermore, immunostaining of the hippocampal CA1 region for PSAP revealed that PSAP gradually accumulated in the nucleus after death. Although PSAP has been previously reported to translocate into the nucleus in cell culture (31) and during development (32), this is the first study to show the nuclear accumulation of PSAP after death. However, the role of the potential neurotrophic effects of PSAP in dying pyramidal cells remains elusive. The clinical implementation of PSAP nuclear accumulation in estimations of PMI requires further investigation.

Postmortem changes in the levels of PSAP and saposin D

Numerous studies have demonstrated the postmortem degradation of proteins. In contrast to these previous approaches, we focused on saposin D, the levels of which increase after death. We found that although the PSAP levels were decreased after death, the levels of saposin D, a degradation product of PSAP, were increased postmortem. The levels of α-tubulin and β-catenin, which are abundantly expressed throughout the body, gradually decreased after death. The GAPDH levels only started decreasing by 3 d after death. Importantly, the use of two different antibodies (IM-1 and anti-saposin D antibodies) demonstrated that the PSAP levels gradually decrease after death. When the IM-1 antibody was used, the PSAP levels were already reduced by 50% at 3 h after death, and PSAP was almost undetectable at 3 d after death. In contrast, the saposin D levels increased after death due to the rapid degradation of PSAP. Therefore, the saposin D to PSAP ratio sharply increased after death, suggesting that it is a promising PMI estimate.

In conclusion, we identified characteristic postmortem alterations in the levels of PSAP and saposin D in the rat brain, in addition to extensive morphological postmortem changes in the hippocampal region. The implementation of these methods may improve the accuracy of PMI estimation when used up to 7 days after death. Considering that the rate of postmortem protein degradation is reduced at low temperatures or other unfavorable environments (7), future studies are warranted to further optimize the measurement of PSAP and saposin D levels to estimate the PMI.

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