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Astrocytic Clasmatodendrosis in Hippocampal Organ Culture

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Abstract

Mechanisms by which astrocytes are irreversibly injured from ischemic brain injury remain incompletely defined. More than 90 years ago Alzheimer showed that astrocytes lose their distal processes (i.e., undergo “clasmatodendrosis”) when irreversibly injured by a reduction in blood flow, a process shown by Friede and van Houten (1961) to be due to energy failure and acidosis. Such alterations in astrocytic morphology can relate directly to changes in cell function. However, astrocytic clasmatodendrosis has largely been lost to the modern literature, perhaps because of an inability to study it under controlled conditions. In the present study, novel four-dimensional (4D) and digital deblurring imaging of glial fibrillary acidic protein (GFAP) immunostaining changes in hippocampal organ cultures (HOTCs) were used to establish an in vitro model of astrocytic clasmatodendrosis. Also, astrocytes in primary culture were transfected with green fluorescent protein (GFP) to show the occurrence of clasmatodendrosis via a parallel and separate means. In HOTCs, a significant reduction in astrocytic process length occurred 15 min (and remained for 60 min) after exposure to acidic Ringer’s and mitochondrial inhibition in the pyramidal cell body layer. Time-lapsed images of primary cultures showed thinning of cell processes within 15 min of exposure to acidic Ringer’s and mitochondrial inhibition. Distal processes subsequently broke away but retained their fluorescence for minutes before disintegrating along with their parent cell bodies. This report shows the spatiotemporal occurrence of clasmatodendrosis in astrocytes of HOTCs closely parallels that seen in vivo. Thus, HOTCs, where microenvironmental conditions can be controlled and single, identified cells can be followed in space and time, can be applied to study the interrelations between energy metabolism and pH that result in clasmatodendrosis.

Keywords

digital deconvolution; point spread function; volume rendering; ischemia; cell death

INTRODUCTION

Histologic characterization of neural cell changes from brain ischemia is a fundamental standard for determining the intensity and extent of anatomic evidence for ischemic neuronal injury (Graham, 1992). For example, routine hematoxylin and eosin (H&E) staining can be used to document stereotypic and progressive neuronal changes after irreversible injury from ischemia. First, neurons show evidence of microvacuolization where the vacuoles are mostly dilated mitochondria (Duchen, 1992). Later, so-called “established ischemic cell change” becomes progressively evident. This consists of neurons that are shrunken and eosinophilic.

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Next, the ischemic process shows evidence of “incrustations” that are dark-staining invaginations of neuronal cytoplasm. Finally, so-called “homogenizing cell change” becomes evident as neurons enter their last stage of ischemic degeneration. Here, no structure is seen in the neuronal cytoplasm and the nucleus appears shrunken or fragmented.

Astrocytes do not show such specific and progressive changes via routine staining with H&E when they are irreversibly injured (Duchen, 1992). With irreversible astrocytic injury, these cells simply begin to show dissolution of the plasma membrane and nucleus (Duchen, 1992), offering little opportunity to gain insight to the development of relevant pathophysiologic mechanisms.

By contrast, another fundamental change of irreversibly injured astrocytes might begin to offer considerable insight to pathomechanisms. Alzheimer first noted in 1910 that irreversible injury of astrocytes from ischemia causes a loss of their distal processes (Penfield, 1928), a phenomenon termed “clasmotodendrosis” by Cajal (Duchen, 1992) (Fig. 1). This unique and dramatic alteration in astrocytic shape has largely been lost to the modern literature except work beginning with the discovery by Friede and van Houten (1961). These workers showed that astrocytic clasmotodendrosis in acute brain slices can be triggered by acidosis coupled to mitochondrial inhibition. Almost 20 years later, Kraig and Chesler (1990) showed that clasmotodendrosis occurs in astrocytes made severely acidotic from hyperglycemic and complete ischemia, conditions that result in astrocytic death and infarction if reperfusion occurs. In spite of the now recognized importance of astrocytes to brain function (e.g., for review, see Vernadakis, 1996; Hertz et al., 2000); the importance of acidosis to the evolution of ischemic brain injury (Siesjö et al., 1996; Swanson et al., 1997; Kraig et al., 1998); and the direct evidence provided by intracellular injections of horseradish peroxidase (HRP) to confirm astrocytic clasmotodendrosis (Kraig and Chesler, 1990), recognition, and integration of this fundamental change remains a rarity in the modern literature. Accordingly, the spatiotemporal pattern of astrocytic process changes were quantified after mitochondrial inhibition and acidosis in a novel in vitro preparation, the hippocampal organ culture. Computer-based four-dimensional (4D) imaging of astrocytic process length from glial fibrillary acidic protein (GFAP) immunostaining and cells transfected with green fluorescent protein (GFP) were used to show these cells progressively lost a significant extent of their distal processes. The coupling of acidity to the inhibition of mitochondrial ATP production for initiation of astrocytic clasmotodendrosis may stem from the teleologic preference of these cells for ATP derived from glycolysis (Erecinska and Silver, 1989). If so, clasmotodendrosis may reflect a specific biological, biochemical and biophysical sequelae of excessive acid production, a fact that has clear relevance to the pathogenesis of brain infarction from ischemia (Kraig et al., 1998). This work has appeared in preliminary form (Kraig et al., 1996a, 1996b).

MATERIALS AND METHODS

Preparation of Cultures

Hippocampal organ cultures (HOTCs) were prepared and maintained as previously described (Stoppini et al., 1991; Kunkler and Kraig, 1997). Neocortical polygonal astrocytes in primary culture were prepared according to the methods of McCarthy and DeVellis (1980), as described previously (Lascola and Kraig, 1996).

Induction of Astrocytic Clasmotodendrosis

Astrocytic clasmotodendrosis was induced in HOTCs (n = 25) and astrocytes in primary culture (n = 10 coverslips) by exposure to an “ischemic Ringer’s” solution that paralleled the ionic constituents of interstitial space found during global ischemia in rodent brain (Hansen, 1985). Furthermore, the pH of this solution was adjusted to the interstitial pH value that causes

astrocytic death in vivo after 20-min acid injections (Kraig et al., 1987) and in vitro (Norenberg et al., 1987; Goldman et al., 1991), i.e., 5.2–5.3. Furthermore, this pH value is also seen within astrocytes during hyperglycemic and complete ischemia (Kraig and Chesler, 1990) when interstitial pH reaches a level of 6.2 (Kraig et al., 1986). Here, interstitial pH of HOTCs was “clamped” to 5.3 with acidic Ringer’s solution to ensure that astrocytic pH reached a level associated with clasmatodendrosis in vivo. Ischemic Ringer’s solution contained (in mM): 46 sodium lactate, 75 KCl, 26 NaHCO₃, 0.1 MgCl₂, 0.1 CaCl₂, 1.5 KH₂PO₄, 5 Hepes, and 1 µg/ml antimycin. The latter allowed glycolysis to continue from endogenous glucose stores but blocked aerobic respiration (Harold and Walz, 1992). Other HOTCs were left in normal media (n = 5; normals) or exposed to normal Ringer’s solution (n = 5; sham controls). Normal Ringer’s solution contained (in mM): 124 NaCl, 2 KCl, 26 NaHCO₃, 1 MgCl₂, 2.5 CaCl₂, 1.2 KH₂PO₄, and 6 glucose, adjusted to 7.4 pH with 5% CO₂/balance air. All cultures were maintained at 37°C during exposure to the ischemic Ringer’s (15, 30, 45, and 60 min with n = 5 per group), normal Ringer’s (60 min), and normal media (60 min) and then processed as described below.

Culture Staining Procedures

HOTCs were processed for GFAP immunostaining as previously described (Kunkler and Kraig, 1997). Selected cultures were processed for H&E staining or combined GFAP and toluidine blue staining using standard procedures. Finally, astrocytes in primary culture were transfected with GFP, using reagents from Gene Therapy Systems (San Diego, CA) according to the manufacturer’s directions.

Data Acquisition and Processing

Images of GFAP immunostaining in the CA3 pyramidal cell layer of HOTCs were viewed with a 50× oil immersion objective (numerical aperture, 1.0) on a compound microscope (Laborlux D; Leica Mikroskopie und Systeme GmbH, Wetzlar, Germany) using Kohler illumination. Differential interference contrast (DIC) and conventional brightfield illumination techniques were used. To function most accurately, digital deconvolution requires linear variation in light signals (Shaw, 1994). However, DIC optics improve contrast of images although non-linearly (Inoué and Spring, 1997). Accordingly, three-dimensional (3D) images derived from DIC and conventional brightfield techniques were acquired, deconvolved and process lengths measured to discover whether these two illumination methods showed similar changes in astrocytic morphology. Images were acquired using a digital image processing system. A 12-bit cooled charged coupled camera (CCD) (CH250 with KAF1400 chip; Roper Scientific, Trenton, NJ) was used with a z-stage controller (LUDL Electronic Products, Ltd, Hawthorne, NY) to obtain optical sections (Agard and Sedat, 1983; Rizzuto et al., 1998). The z-stage controller and CCD were controlled using VolumeScan (version 3.0; Vaytek, Fairfield, IA). Serial sections (volume) were sampled at 0.2-µm intervals and transferred to an optical hard drive (Sierra; Pinnacle Micro, Irvine, CA) for subsequent processing. Volumes were composed of images (1024 × 1024 pixels; each pixel 6.8 µm²). Images were acquired so that they displayed both a full dynamic range (0–4,095 grayscale levels) and a mean pixel value of 2000. Range and mean pixel values were achieved by varying exposure rates for each volume acquisition. The camera, z-stage, z-stage controlling software, and optical hard drive were controlled by a 486 DX50 AST computer (AST Research, Irvine, CA). A flat-field correction technique from previous digital image processing protocols was used (Caggiano and Kraig, 1996).

All images from a specific volume set were digitally deconvolved (MicroTome version 6.0; Vaytek), using a constrained iterative algorithm. The latter was based on an empirically sampled point spread function (PSF) for the 50x oil immersion objective. It was derived from a z-axis image stack of 1-µm microspheres (Mango Red; Molecular Probes, Eugene, OR)

embedded in 3% agar/150 mM NaCl with the same lighting conditions (intensity, interference contrast/no interference contrast, and Kohler illumination) used for experimental samples. The microsphere image stack approximated the vertical extent of the experimental data from HOTCs (e.g., 20–30 μm in thickness) so that light path aberrations were comparable. The resultant PSF was then used to estimate the out-of-focus light produced from GFAP images. Predicted out-of-focus light was then “removed” from each image of z-axis volume stack. The number of iterations used for each volume stack was determined by calculating the expected volume of a microsphere and varying the iterations until the correct value was achieved. The volume of the deconvolved sphere was obtained using a volume-rendering program and compared with its expected value (Voxblast version 1.3.2; Vaytek).

Three-dimensional GFAP images were reconstructed using the Voxblast software package. Each volume set was obtained by focusing to the top and bottom of the GFAP immunostaining within HOTCs and selecting the center most 20–30 μm .

GFP-transfected cells were visualized using time-lapsed digital (intensified PentaMax camera; Roper Scientific Instruments) imaging on an inverted microscope (DMIRBE; Leica Mikroskopie und Systeme GmbH, Wetzlar Germany) with a 20 $\times$ air objective (numerical aperture, 0.30). Glass coverslips containing cells were placed in a Leiden coverslip dish and mounted into an open perfusion micro incubator (PMI-II; Medical Systems, Greenvale, NY) with control of temperature (37°C) and pH (i.e., aeration with air/5% CO_2). Cells were excited (490 nm) with a scanning monochromator (Polychrome II, Applied Scientific Instruments, Eugene OR). Dibutyl cyclic adenosine monophosphate (DcAMP; 2 mM) was added to cells 2 h before image acquisition (while still in normal media) to induce process formation (Lascola and Kraig, 1996) similar to that seen in vivo. Images were acquired every 5 min for 1 h after exposure to normal Ringer’s or ischemic Ringer’s solution. After acquisitions, images were digitally deconvolved (MicroTome, Vaytek) with a theoretical PSF generated from information known about the objective used (numerical aperture, wavelength of light, refractive index of medium (air), spatial characteristics, and z-sampling rate). Images were then used to create a surface plot that conveyed intensity information in the z-axis (Image Pro Plus for Windows v 4.0, Media Cybernetics, Silver Springs, MD).

Data Analysis

Astrocytic process length was quantified from rendered three-dimensional images after several processing steps using Image Pro Plus software. First, an image was binarized using a spatial filter set to exclude background noise and isolated cellular elements (Kunkler and Kraig, 1997). Such features were easily differentiated from intact cells. Second, the resulting opaque image of cells was subjected to a morphological filter (erosion, 2×2 kernel, 2 passes) to further deemphasize any remaining background material and smaller noncontiguous components of the cells. Third, a mask was created from this final image and was inverted (e.g., cells bright) to ease visual inspection for whole cell measurements. Fourth, primary process length (defined as a contiguous, tapering length from the cell body) of all cells within a given image were measured manually. A stage micrometer was used to convert image Dx/Dy pixel values into microns (μm). Primary process length was determined from the combined distance of the fewest number of segments that remained within the axial boundaries of the process and extended from the cell body edge to the process tip. If a process could not be seen to its end because of overlying processes, the distal tip was taken to be that point at which progressive tapering of the primary process ceased tapering from the cell body. All cellular primary processes were measured. A mean process length was expressed for all measured processes of individual cells and a mean cellular process length expressed for each experimental condition. All measurements were done with the operator blinded to experimental conditions.

Measurements were saved to a spreadsheet (Excel 97; Microsoft Corporation, Redmond, WA) and grouped according to coded experimental group labels to calculate descriptive statistics using Sigma Stat (version 2.0; SPSS, Chicago, IL). Comparisons between groups were made using analysis of variance (ANOVA) in a pair wise comparison (Tukey's) to assess all experimental groups both within experiments and across acquisition method. Tukey's pair wise comparison was chosen for its conservative estimate of significant probabilities (Zar, 1984). After statistical analyses, data were decoded and results expressed as mean $\pm$ SEM.

Figure Preparation

All figures were composed in Corel Draw version 8 (Corel Corporation, Orem, UT) and edited using Photoshop (version 5.02, Adobe Systems, San Jose, CA) consistent with Anderson (1994). Enhancement (non data altering) of images was performed in Image Pro Plus as follows. First, reference images were subtracted to even image lighting. Second, images were refocused using a high gaussian filter (7×7 pixel matrix at 50% strength). Third, image brightness was raised and background noise lowered by applying brightness, contrast, and γ function values of 76, 50, and 0.6 to all images. Fourth, color was added to final images.

RESULTS

Astrocytes within HOTCs develop clasmatodendrosis within minutes after exposure to acidic Ringer's solution and mitochondrial inhibition. Initial characterization of astrocytic morphological changes ($n = 5$ HOTCs per group) was done using DIC illumination, as it appeared to enhance visualization of detail. Using DIC illumination GFAP⁺ cells exposed to normal media ($n = 182$) showed a process length of 9.79 ± 0.45 , while those exposed to normal (60 min) Ringer's ($n = 197$) a mean process length of 9.31 ± 0.35 . Exposure to acidic Ringer's and mitochondrial inhibition reduced process length at 15, 30, 45, and 60 min to 4.19 ± 0.16 ($n = 171$), 4.80 ± 0.26 ($n = 147$), 4.40 ± 0.13 ($n = 231$), and 4.86 ± 0.14 ($n = 280$), respectively. Comparison of the GFAP⁺ cells within normal HOTCs to cells from experimental groups (15-, 30-, 45-, and 60-min exposure) showed a significant decrease in mean process length difference ($P < 0.001$) with the power of this test α of $0.05 = 1.0$. Similarly, comparison of cells from normal Ringer's exposure (e.g., sham) to all experimental groups also showed a significant decrease in process length ($P < 0.001$) with a similar alpha value. No significant difference was seen between GFAP⁺ cells from normal media and normal Ringer's.

Brightfield illumination showed statistically similar changes in astrocytes but theoretically should provide more accurate reflection of process length changes than that afforded by DIC illumination. The following conclusion stems from the fact that digital deconvolution functions most accurately with light that varies linearly (e.g., brightfield) as opposed to illumination that varies nonlinearly (e.g., DIC) (Shaw, 1994). Figure 2 shows the sequential changes of typical astrocytic process length after exposure to normal Ringer's solution and acidic Ringer's plus mitochondrial inhibition as imaged with DIC to show finer process detail. Again, no significant change in astrocytic process length was seen in HOTCs exposed to normal media or normal Ringer's solution. Similarly, significant ($P < 0.001$) changes were seen between either cells from normal media or normal Ringer's and the experimental groups exposed to acidic Ringer's solution and mitochondrial inhibition but no significant changes were seen between these latter groups. The power of these tests were all 1.0 with an α value of 0.05. Brightfield illumination did, as expected, reduce mean process length of each group examined. However, this reduction in mean process length between DIC and brightfield illumination only reached a significant level ($P < 0.005$) at 60 min of exposure to acidic Ringer's and mitochondrial inhibition. Brightfield illuminated cells exposed to only normal media ($n = 60$) showed a process length of 9.22 ± 0.58 , while those exposed to normal Ringer's ($n = 98$) a mean process length of 9.31 ± 0.43 . Exposure to acidic Ringer's and mitochondrial inhibition reduced process length at 15,

30, 45, and 60 min to 3.99 ± 0.18 ($n = 108$), 3.62 ± 0.14 ($n = 108$), 3.99 ± 0.14 ($n = 66$) and 3.56 ± 0.12 ($n = 159$), respectively. Thus, while brightfield illumination theoretically should produce more accurate imaging of actual process length than DIC illumination, results here show these methods of illumination to produce essentially analogous results.

Paraffin-embedded and stained HOTCs demonstrated the heterogeneous distribution of irreversible astrocytic changes in space and time (Fig. 3). H&E staining confirmed typical eosinophilic changes of irreversibly injured neurons and showed astrocytes of variable size. GFAP immunostaining showed that astrocytic clasmatodendrosis was most evident at the pyramidal cell body layer. This is consistent with the findings of Friede and van Houten (1961). These workers found that astrocytic clasmatodendrosis was concentrated at the Purkinje cell body layer in acute slices of cerebellar cortex exposed to acidic Ringer's and mitochondrial inhibition. Finally, exposure to acidic Ringer's solution and mitochondrial inhibition increased the number of astrocytes positive for GFAP immunostaining (Fig. 3D; note number of cells counted per experimental group generally increased with time). This change may be due to the affect of acidity on GFAP immunogenicity, an effect reminiscent of fixative-dependent alterations in GFAP immunostaining (Petito et al., 1990).

GFP transfection of astrocytes in primary culture was used as a separate and parallel means to confirm that acidic Ringer's and mitochondrial inhibition was sufficient to induce astrocytic clasmatodendrosis (Fig. 4). GFP-transfected cells display the brightly fluorescent protein throughout the cytoplasm and distal dendritic processes, thus enabling dynamic imaging of irreversible cell injury and clasmatodendrosis. Figure 4 shows two GFP-transfected astrocytes undergoing progressive injury during a 30-min exposure to acidic Ringer's and mitochondrial inhibition. Before exposure to acid Ringer's, GFP is present throughout the many fine processes of the cells extending from the cell body to the distal terminal. Shortly after exposure to acid Ringer's, however, many of the fine processes linking the cell body to the distal most processes began to decrease in diameter and eventually disappeared, leaving behind fluorescent 'islands' surrounding the cell body. In some cases the processes appeared to be slowly stretched before quickly snapping, leaving behind enlarged distal processes. Minutes after the loss of distal processes, remaining GFP fluorescence decreased throughout both the cell body and residual distal processes, suggestive of an eventual loss in membrane integrity due to irreversible cell injury (e.g., see Fig. 4, lower right hand image). This conclusion was supported by the fact that transfected cells exposed to normal Ringer's displayed only a slight diminution of fluorescence intensity during an similar exposure period.

DISCUSSION

The present work shows that the morphological change of astrocytic clasmatodendrosis from acidosis and energy failure occurs in the model systems of HOTCs and primary cell cultures. Furthermore, it shows that astrocytes undergo clasmatodendrosis at a pH and time course consistent with astrocytic death (i.e., brain infarction) and astrocytic pH (i.e., 5.2–5.3) seen in vivo from hyperglycemic and complete ischemia (Kraig and Chesler, 1990). Also, for the first time, it shows the spatiotemporal dynamics of this fundamental reflection of irreversible astrocytic injury using four-dimensional and quantitative imaging strategies. Specifically, astrocytic process length determined from GFAP immunostaining became significantly shorter within 15 min of the onset of energy failure and acidosis in HOTCs and remained so for at least 60 min. Furthermore, astrocytic clasmatodendrosis was localized at the pyramidal cell body layer in HOTCs. Finally, GFP-transfected astrocytes in primary culture showed similar changes consistent with the clasmatodendrosis seen in HOTCs.

Commonly, alterations in cell form are related directly to changes in cell function. Thus, deciphering the mechanisms responsible for the morphological changes of astrocytic

clasmotodendrosis may reveal important insights to the role of astrocytes in ischemic brain injury. For example, the work of Friede and van Houten (1961) first showed that acidosis and energy failure were essential for astrocytic clasmotodendrosis. Thirty years later work from this laboratory showed (using direct microelectrode measurements of intracellular pH and injections of HRP) that astrocytic clasmotodendrosis occurred during hyperglycemic and complete ischemia when astrocytes became severely acidotic (Kraig and Chesler, 1990). Most recently, Tomimoto et al. (1996, 1997) used GFAP immunostaining to show that patients with brain ischemic lesions and those with Alzheimer's disease have GFAP⁺ cells in white matter that show clasmotodendrosis. One caveat should be mentioned here regarding GFAP immunostaining and astrocytic clasmotodendrosis. Astrocytes swell rapidly with reperfusion from ischemia presumably from uptake (or generation) of various osmolytes (Aschner, 1998). Schmidt-Kastner et al. (1993) suggest that this swelling causes alterations in GFAP intermediate filaments with resultant loss of GFAP immunostaining. Petito and Halaby (1993) similarly suggest that astrocytic swelling may account for the delay in detection of newly synthesized GFAP by immunostaining associated with ischemic injury. However, it is unlikely that cellular swelling could account for the loss of distal astrocytic processes of clasmotodendrosis seen in this work, since no biological or physicochemical mechanism can be imagined that would retain water to restricted aspects of astrocytic processes (e.g., disrupting GFAP-immunostaining at one site but maintaining it only microns away). Furthermore, the observation that GFP transfected astrocytes in primary culture underwent clasmotodendrosis similar to that seen with GFAP immunostaining in HOTCs supports the validity of the GFAP-based measurements in HOTCs.

Astrocytes are the likely source for the acidity of brain ischemia necessary for clasmotodendrosis. They contain comparatively large amounts of glycogen (Koizumi, 1974; Phelps, 1975; Rosenberg and Dichter, 1985; Cataldo and Broadwell, 1986; Swanson and Choi, 1993), which can be converted for use in glycolysis (Hochachka and Mommsen, 1983). Furthermore, astrocytes are powerful ion transporting cells (Walz, 1989) that preferentially use ATP derived from glycolysis for interstitial ion homeostasis. For example, glutamate rises in the interstitial space after the onset of ischemia (Benveniste et al., 1984; Caragine et al., 1998) via increased formation of glutamate from glutamine and subsequent release from injured neurons (Goldberg et al., 1988; Huang and Hertz, 1994; Newcomb et al., 1997; Hertz et al., 2000). Astrocytes then begin to take up interstitial glutamate, triggering sodium-potassium ATPase in these cells (Pellerin and Magistretti, 1996, 1997). The latter occurs with the extracellular release of lactate (Pellerin and Magistretti, 1994). Furthermore, proton generation from glycolysis stems from associated ATP hydrolysis (Hochachka and Mommsen, 1983). Thus, increased acid production is likely to occur mainly in astrocytes via neuronal glutamate release in response to brain activation and ischemia (Kraig et al., 1998). A similar scenario is likely to occur for other ions that are transported into astrocytes after accumulation in the interstitial space (e.g., potassium) (Raffin et al., 1992). This suggests that astrocytes, in an attempt to regulate the interstitial concentration of glutamate (and likely, other ions), can become so acidotic that they are lethally injured and undergo clasmotodendrosis.

Use of easily prepared HOTCs (Stoppini et al., 1991) that closely parallel changes of their in vivo counterparts (e.g., see Kunkler and Kraig, 1997, 1998) coupled to measurement of key anatomical and functional variables using 4D imaging strategies may clarify the interrelations between anatomy and function that account for the phenomenon of astrocytic clasmotodendrosis first defined more than 90 years ago.

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Fig. 1.

Initial evidence for astrocytic clasmatodendrosis in brain ischemic injury. The initial demonstration that irreversible injury of astrocytes from ischemia includes loss of their distal processes was made by Alzheimer in 1910 (and termed “clasmatodendrosis” by Cajal (Penfield, 1928)) and then largely lost in the modern literature. Accordingly, it is shown here to allow most immediate comparison to its redemonstration using current imaging strategies in hippocampal organ cultures (HOTCs). Alzheimer showed that astrocytes undergo clasmatodendrosis after brain ischemia in humans. Image above shows what resembles gold sublimate staining of astrocytes from human brain tissue. Two normal astrocytes are evident to left and right. The center astrocyte has lost its distal processes which have formed isolated bodies and were originally termed “filling bodies” (Penfield, 1928). (Reproduced from Penfield, 1928.)

Fig. 2.

Development of astrocytic clasmatodendrosis in hippocampal organ cultures (HOTCs) from exposure to acidic Ringer's solution and inhibition of mitochondrial function. Images show representative digitally restored volumes of GFAP-immunostained astrocytes from CA3 pyramidal cell body layer of HOTCs illuminated with DIC optics. A representative cell was selected from each image, copied and enlarged to facilitate observation of process length change. "Normal" astrocytes from control HOTCs (Normal) appear analogous to those from HOTCs exposed to Ringer's (Ringer) solution for 60 min (e.g., sham controls). Exposure to acidic (5.3 pH) Ringer's solution and mitochondrial inhibition with antimycin promptly caused a dramatic change in cell morphology that consisted of what appeared to be a comparable loss

of astrocytic processes at 15, 30, 45, and 60 min. However, isolated processes, e.g., consistent cell fragments originally termed “filling bodies” (Penfield, 1928), as seen in Figure 1 were less evident because of the filtering strategy that was employed. Cells are shown using a color palette that is complementary to the background color to present a visually attractive compromise between image contrast and the enhanced three-dimensional perspective afforded by color. Calibration bars are 100 μm .

Fig. 3.

Astrocytic clasmatodendrosis was most evident at the pyramidal cell body layer. Images show CA3 pyramidal cell area of hippocampal organ cultures (HOTCs). Paraffin-embedded HOTCs were sectioned at 4 μ m and stained with H&E (**A,B**) or GFAP immunostaining and toluidine blue counterstaining (**C,D**). Normal astrocytes (**A,C**) are shown (arrows) intermixed between CA3 pyramidal neurons. Exposure (60 min) to acidic Ringer's solution and mitochondrial inhibition (**B,D**) induced expected classic H&E changes (**B**) of neuronal death that include eosinophilic neurons with diminished nuclear definition (small arrow) and with heterogeneous changes in astrocytic size and staining intensity (large arrows). The preferential localization of astrocytic clasmatodendrosis to the pyramidal cell body area is evident from glial fibrillary acidic protein (GFAP) immunostaining (**D**). Astrocytes in the stratum radiatum still largely show detailed processes, while only fragments of GFAP⁺ cells or cells without comparable processes (arrow) are seen at the cell body area. Generally, exposure to acidic Ringer's solution and mitochondrial inhibition increased the number of GFAP⁺ cells, an effect that may be due to altered immunogenicity of the GFAP protein caused by acidity. Scale bar = 100 μ m.

Fig. 4.

Astrocytic clasmotodendrosis in primary cultures. To demonstrate further that astrocytes undergo clasmotodendrosis when irreversibly injured, cells in primary culture were transfected with green fluorescent protein (GFP). Then, 3–5 days later, astrocytes were transformed into process bearing cells by exposure to DcAMP so that they more closely reflected the morphology of their counterparts in HOTCs and in vivo. Cultures were placed in a standard recording chamber at 37°C and fluorescent images acquired every 3 min for 60 min. Changes from two typical astrocytes (left-hand column and right-hand column are shown under control conditions (top), 15 min (center), and 30 min (bottom) after exposure to acidic Ringer's and mitochondrial inhibition. Insets show enlargement of distal process changes. Note that, under

control conditions, fluorescence extends throughout the process. They then showed a progressive reduction in process diameter, followed by a complete break in the continuity of process fluorescence. This led to isolated fluorescent bodies that showed no fluorescent attachment to their original cell bodies. A change that supports the original description of such cell fragments found in vivo as “filling bodies” (Penfield, 1928). This is analogous to that seen by Alzheimer more than 90 years ago, providing dynamic confirmation that plasma membranes of parent cells and their isolated processes can remain intact after the onset of clasmotodendrosis. Scale bar = 20 μm .