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Increased 5-hydroxymethylation levels in the sub ventricular zone of the Alzheimer's brain

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ABSTRACT

The subventricular zone (SVZ) is a site of neurogenesis in the aging brain, and epigenetic mechanisms have been implicated in regulating the "normal" distribution of new nerve cells into the existing cellular milieu. In a case-control study of human primary SVZ cultures and fixed tissue from the same individuals, we have found significant increases in DNA hydroxymethylation levels in the SVZ of Alzheimer's disease patients compared with nondiseased control subjects. We show that this increase in hydroxymethylation directly correlates to an increase in cellular proliferation in Alzheimer's disease precursor cells, which implicates the hydroxymethylation tag to a higher degree of cellular proliferation.

1. Introduction

It is well established that the act of self-renewal, particularly during neuronal differentiation, requires many intrinsic and extrinsic factors. Neurogenesis and its incorporation into the existing neuronal circuitry are a central event in the process of efficient aging (Lazarov et al., 2010). It has been shown that the rate of neurogenesis decreases as a function of age (Riddle and Lichtenwalner, 2007; Conover and Shook, 2011), contrary of what has been observed in Alzheimer's disease (AD) (Jin et al., 2004a, 2004b). Unlike normal aging, the AD brain is subjected to many environmental stimulants known to induce neurogenesis, that is, amyloid (Lopez-Toledano and Shelanski, 2004), donepezil (Kotani et al., 2008), memantine (Jin et al., 2006), nonsteroidal anti-inflammatory drugs (Monje et al., 2002), brain trauma (Chen et al., 2003b), and statins (Chen et al., 2003a), to name a few. Because the environment inside and outside the cell directly affects epigenetic mechanisms that regulate the expression of multipotent genes (Feil and Fraga, 2011; Dao et al., 2014), the increased multipotency observed in AD (Fitzsimons et al., 2014) implicates epigenetic mechanisms like 5-hydroxymethylation (Dao et al., 2014).

Epigenetic mechanisms encompass a wide array of functional roles, which in due course lead to the repression or expression of genes. Although the existence and biological functions of active methylation (generally mediating gene repression) and demethylation (generally inducing gene expression) are still in its infancy, 5hmC has been implicated in active DNA demethylation (Zhang et al., 2012), particularly in multipotent genes (Szulwach et al., 2011). The 5-hydroxymethylcytosine (5hmeC) mark is an oxidized form of 5-methylcytosine (5meC) (Tahiliani et al., 2009). Oxidation of 5meC to 5hmeC is catalyzed by a family of proteins (ten-eleven translocation) that have also been implicated in the regulation and maintenance of multipotency (Tahiliani et al., 2009; Freudenberg et al., 2012). Because the subventricular zone (SVZ) is a site of neurogenesis and gliogenesis (Ming and Song, 2011), our data identify an epigenetic mechanism that could account for the increase in multipotency observed in AD. These data provide the foundation for future gene-specific studies and a possible therapeutic approach to neuronal differentiation in AD SVZ.

2. Materials and methods

2.1. Autopsy brain tissue

Brain tissue was obtained through the Sun Health Brain and Body Donation Program (Sun City, AZ). Specimens at autopsy were collected under institutional review board–approved protocols and informed

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Table 1
Characteristics of cases used for quantitative assessment

Sex	Age expired	PMI	Control	AD	Braak score
F	90	3	Yes	No	II
M	86	2.5	Yes	No	II
M	86	2.66	Yes	No	II
F	90	2.5	Yes	No	II
M	83	2.16	Yes	No	III
F	96	3	Yes	No	III
F	86	2.5	No	Yes	IV
F	91	4.5	No	Yes	IV
M	85	5.75	No	Yes	IV
F	85	3.16	No	Yes	VI
M	79	1.83	No	Yes	VI
M	86	2.83	No	Yes	VI

consents that permitted use of the samples for research by the investigators. At expiration, subject ages ranged from 79 to 96 years old ($N = 12$), with a mean of 86.9 ± 1.1 (SEM) years. Postmortem intervals for the subjects averaged 3 hours ± 3 minutes. Diagnoses of patient condition included AD ($n = 6$) and neurologically/pathologically normal for age ($n = 6$) (Table 1). Subjects included in this study received antemortem evaluation by board-certified neurologists and postmortem evaluation by a board-certified neuropathologist. Evaluations and diagnostic criteria followed consensus guidelines for National Institute on Aging Alzheimer's Disease Centers.

After brain removal, gross surface neuropathological abnormalities were documented, 1-cm–thick frontal slabs were cut and photographed, and the slabs were bisected into hemispheres. Small blocks that included superior-lateral periventricular white matter were immediately dissected from right hemisphere slabs and processed for primary cell culture or for fixation and detailed immunohistochemical study (see below).

2.2. SVZ dissection, tissue dissociation, and primary cell culture

As previously published (Leonard et al., 2009), SVZ cultures were dissected from the superior lateral wall of the lateral ventricle, from the most anterior aspect of the lateral ventricle to approximately 3 cm posterior to that point. These dissections typically included approximately 1 cm of white matter but specifically excluded any striatal gray matter. For parallel, control cultures, neocortical tissue blocks from the same cases were dissected from frontal and/or parietal regions. These latter slabs always included all adjoining subcortical white matter, except for a 2-cm margin around the lateral ventricle that contained the SVZ. Eliminating the SVZ from these samples provided a control to evaluate whether neurosphere development in our SVZ cultures derived from the SVZ or from surrounding periventricular tissue necessarily included in the SVZ dissections.

Tissue was quickly transported in ice-cold Hibernate A medium (BrainBits, LLC, Springfield, IL) to a sterile laminar flow hood, mechanically dissociated into 1–2 mm pieces, and digested with 0.25% trypsin (Irvine Scientific, Santa Ana, CA) and 0.1% DNAse (Sigma, St. Louis, MO) in a shaking water bath at 30°C. Digestion was stopped with fetal bovine serum. After passing the cell and tissue suspension through progressively finer metal screens, it was diluted with complete Dulbecco modified Eagle medium (DMEM) (minus phenol red). Complete DMEM consisted of 500 mL DMEM (high glucose, plus or minus phenol red, as noted; Invitrogen-Gibco, Carlsbad, CA), 50 mL fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 10 mL HEPES (Irvine Scientific), 5 mL sodium pyruvate (Mediatech Cellgro, Herndon, VA), 5 mL penicillin/streptomycin (Invitrogen-Gibco), and 0.5 mL gentamycin (Irvine Scientific). Cells and debris were separated using 50% Percoll gradient (Amersham/GE Healthcare, Piscataway, NJ) centrifugation (13,000 rpm; refrigerated). The first layer of myelin and debris was discarded. The second layer of the gradient, which is rich in microglia and astrocytes (Lue et al., 1996) but also proved to be the most optimal source for neurospheres, was aspirated, washed, pelleted, gently triturated, washed a second time, resuspended in complete DMEM (plus phenol red), and transferred to a 75-mL tissue culture flask (Nunc, Rochester, NY).

Flasks with suspended cells were left undisturbed for 2–24 hours in a tissue culture incubator maintained at 37°C/7% CO_2. As previously reported (Lue et al., 1996), some 98% of microglia became adherent under these conditions, such that culture supernatants that were relatively free of microglia could be transferred to a second set of 75-mL flasks for plating. To estimate viability and density of the cells remaining in suspension, 50-μL aliquots of cell suspension were subjected to trypan blue exclusion counting using a hemocytometer.

The secondary flasks were left undisturbed, except for weekly medium replacement with complete DMEM, for 1–3 weeks in tissue culture incubators maintained at 37°C with 7% CO_2, after which a portion of the supernatant was seeded into various receptacles depending on experimental requirements. When flasks became confluent, neurospheres and lightly adherent cell clusters were mechanically dislodged by brief gentle shaking, gently pelleted and triturated, and plated into a new flask; neurospheres were not intentionally dissociated during passage. Characterization studies typically used 6- or 12-well uncoated tissue culture plates (Corning, Lowell, MA) or plates and culture dishes coated with 10 μg/mL poly-L-lysine (Sigma) or a combination of poly-L-lysine /10 μg/mL mouse laminin I (ATCC, Manassas, VA).

2.3. Immunohistochemistry and immunocytochemistry

For immunohistochemical analysis of tissue sections, periventricular white matter/SVZ blocks were collected at autopsy in the same manner as that for cell culture. Tissue blocks were immersion-fixed at 4°C for 24–36 hours in freshly made 4% paraformaldehyde/0.1 mol/L PO_4 (phosphate buffer, PB). The blocks were then washed extensively in PB, cryoprotected in 30% sucrose, sectioned serially at 20 μm or 40 μm on a cryostat, and stored at −20°C in ethylene glycol/glycerol/PB solution until needed.

2.3.1. 3,3'-diaminobenzidine immunohistochemistry

Forty-micrometer free floating sections were washed in phosphate-buffered saline Triton X-100, blocked in 1% hydrogen peroxide followed by 1-hour incubation in 3% bovine serum albumin (BSA), and then incubated at 4°C overnight in primary antibody (5hmeC; Active Motif, Carlsbad, CA; 1:5000 dilution) solutions containing 0.25% BSA. After incubation, the sections were washed; incubated in biotinylated, species-specific secondary antibodies (Vector) for 2 hours at room temperature (RT); washed 3× in phosphate-buffered saline Triton X-100; and incubated in avidin-biotin complex (Pierce) for 30 min. Following incubation with avidin-biotin complex, sections were washed 2× in 50 mmol/L Tris buffer and immersed in 3,3'-diaminobenzidine solution (500 μL 5 mg/mL 3,3'-diaminobenzidine, 2 mL saturated nickel, 10 μL 1% H202, fill to 50 mL with 50 mmol/L Tris buffer) for no longer than 10 minutes, followed 2 quick rinses in 50 mmol/L Tris to stop the reaction. AD and nondiseased (ND) sections were immunoreacted simultaneously using Netwells in well-less plates. Sections were mounted with Permount (Pierce).

2.3.2. Fluorescence immunohistochemistry

Briefly, extensively washed 40-μmol/L sections were blocked with 3% normal goat serum (NGS)/0.1% Triton X-100 and then incubated with rabbit anti-human 5hmeC, diluted 1:5000 (Active Motif, Carlsbad, CA) for 24 hours at 4°C. The diluent for all solutions and washes was 0.05 mol/L Tris-buffered saline, pH 7.4. After 3 washes, the sections were incubated with goat anti-rabbit Alexa-Fluor 488–conjugated secondary antibody (1:1500; Invitogen/Molecular Probes) for 2 hours at room temperature, washed, mounted on microscope slides, and coverslipped with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). All washes included 1% NGS and 0.1%