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Research Report

Age-related changes in Usp9x protein expression and DNA methylation in mouse brain

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Abstract

Usp9x, a ubiquitin-specific protease implicated in synaptic development, was found to be more abundant in adult as compared to newborn mouse brain tissue. The *Usp9x* gene was less methylated in adults than in newborns in both the promoter and the protein coding region. Compared with newborns, the adult mouse brain also had lower levels of Dnmt1, the enzyme responsible for maintaining DNA methylation state. These age-associated changes in DNA methylation and ubiquitin system protein concentrations potentially contribute to developmental changes in brain structure and function.

Theme: Development and regeneration
Topic: Developmental genetics

Keywords: Proteasome; Chromatin remodeling; Intracisternal A particle (IAP); β-catenin; Spinophilin; Ubiquitin-activating enzyme E1 (ube1)

1. Introduction

Ubiquitin is a small protein, 76 amino acids, used by cells either to label proteins for degradation or to modify the conformation and activity of protein substrates [7]. To degrade a protein, a polyubiquitin chain is formed and the polyubiquitinated protein is recognized by a multisubunit proteolytic complex proteasome which cleaves the protein into small peptides [15]. Conjugation of ubiquitin to substrates is mediated by an enzymatic cascade, requiring ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). The rate and specificity of ubiquitination in a particular tissue are further regulated by deubiquitinating enzymes (DUBs), which remove covalently linked ubiquitins from protein substrates resulting in one of two opposite consequences: deubiquitination can either rescue a ubiquitinated protein from degradation, or accelerate protein degradation by disassembling the polyubiquitin chain before the protein substrate enters the proteasome.

The role of the ubiquitin–proteasome system in brain development and function has gained appreciation lately [20]. In particular, deubiquitinating enzymes have been linked to synapse formation and plasticity [8,13,33]. *Fat facets* (*faf*) is a deubiquitinating enzyme in *Drosophila* flies [16]. When *faf* is overexpressed in neurons, drastic changes are observed in the nervous system, including a large increase in the number of synapses, elaboration of the synaptic branching pattern, and disruption of synaptic transmission [8]. In rodents, ubiquitin-specific protease 9 (*Usp9x*, also known as *Fam* for *fat facets* in mouse), the mammalian orthologue of *faf* [18,34], is found in the presynapse and interacts selectively with epsin1, an adaptor protein involved in clathrin-mediated neurotransmitter endocytosis [5]. Depolarization induces Usp9x-mediated deubiquitination of epsin1, which in turn assumes a higher affinity for clathrin and initiates the assembly of an endocytosis vesicle coat. The enhanced endocytosis might contribute to the stabilization of newly formed synapses, in

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agreement with the phenotype of synaptic overgrowth seen in the *faf* overexpressor flies. In addition to espin1, AF6 and β-catenin have been identified as substrates of Usp9x, which Usp9x rescues from degradation by removing the polyubiquitin conjugation [29,30].

The number of synapses in the brain is known to change in development. In rats, for instance, the number of synapses in the dentate gyrus increases dramatically between days 7 and 30 due to active synaptogenesis [6], then remains relatively constant during adulthood [22]. Considering the effect of *faf* in determining the number of synapses in flies, I sought to determine whether the abundance of Usp9x protein changes with age, which might be part of the mechanism underlying developmental changes in synaptic number.

A possible cause of a change in protein expression with age is a change in the DNA methylation pattern of the gene which encodes the protein of interest. In particular, hypermethylation of the 5-position of cytosine in CpG dinucleotides in the promoter region is associated with repression in gene expression, presumably due to reduced binding of transcriptional factors to their DNA binding sites [28] and/or enhanced binding of transcriptional repressors to the methylated CpG residues [14]. Hypomethylation, in contrast, generally leads to enhanced transcription of the downstream gene. The genomic DNA methylation pattern is laid down during embryonic development by DNA methyl-transferase 3a and 3b (Dnmt3a, 3b; [21]), and is maintained, to a certain degree, by DNA methyl-transferase 1 (Dnmt1; [26]) which methylates the hemimethylated DNA strand after each round of cell division. DNA methylation varies with age in a tissue-specific fashion [25]. Therefore, once I found an age effect in the expression of Usp9x protein, I sought to compare the DNA methylation pattern of the *Usp9x* gene in the brain between neonates and adults. The DNA methylation state was assessed by using a methylation-sensitive restriction enzyme, *Hpa*II, which cleaves at its recognition site, CCGG, only if the internal cytosine is unmethylated. The restriction digest product was then examined by either Southern blotting or PCR reaction including both regular PCR and SYBR Green-based real-time PCR. I also measured levels of Dnmt1 in newborn and adult mouse brains to assess whether overall DNA methylation activity changes in the brain with development.

The expression of Usp9x protein was found to be higher in adult mouse brains compared with neonates. In addition, the *Usp9x* gene was less methylated in adults, and brain Dnmt1 levels were lower.

2. Materials and methods

2.1. Animals

Procedures for use of mice were approved by the UCLA Chancellor's Animal Research Committee. C57BL/6J mice were bred in the Life Science Vivarium at UCLA from stocks obtained from Jackson Lab (Bar Harbor, ME). They were kept at a 12:12 light:dark cycle with food and water available ad libitum.

Tissues were harvested from newborn and 1-year-old mice. The adult mice were anesthetized under CO_2 prior to tissue dissection. The tissues were immediately frozen on dry ice and stored at −80 °C until processing for protein or DNA.

Each brain sample was pooled from four adults or eight neonates. Each lung sample contained tissues from two adults or neonates.

2.2. Western blots

The tissues were homogenized in RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing the protease inhibitor cocktail Complete (Roche, Indianapolis, IN). The homogenates were centrifuged at 5500 × *g* for 20 min at 4 °C to remove cellular debris. Protein concentration was determined by a standard Bradford assay, and equal amounts of protein were loaded for each sample.

25 μg protein from each sample was loaded on a 4–12% Tris–Glycine gradient gel (Invitrogen, Carlsbad, CA) and run at 140 V. After electrophoresis, the gel was rinsed in transfer buffer (48 mM Tris, 39 mM Glycine, 20% methanol) and then the protein was electrophoretically transferred in the same buffer at 4 °C overnight onto a Immonbilon-P transfer membrane (Millipore, Billerica, MA). To detect the protein of interest, the Western blots were blocked first in Blotto buffer containing 5% nonfat milk and 0.1% Tween in phosphate buffered saline (PBST), then incubated in PBST containing a specific antibody. The concentrations of primary and secondary antibody followed manufacturer recommendations. Bands were visualized on BioMax XAR film (Kodak, Rochester, NJ) using an ECL Plus Western Blotting Detection Kit (Amersham, Piscataway, NJ). Blots were stripped at 60 °C in the buffer containing 62.5 mM Tris pH 6.7, 2% SDS, and 100 mM β-mercaptoethanol before being incubated with other antibodies.

The polyclonal anti-Usp9x antibody was kindly provided by Dr. Kozo Kaibuchi of Nagoya University. The specificity of the antibody has been tested by Taya et al. [29]. Other antibodies tested on the Western blots included monoclonal anti-Ube1x (# 05-624) and polyclonal anti-spinophilin (# 06-852, both from Upstate Biotechnology, Lake Placid, NY); anti-actin (A4700) and anti-β-catenin antibody (C2206, both from Sigma, St. Louis, MI); anti-Dnmt1 (AM1032a), anti-Dnmt3a, (AM1034a), and anti-Dnmt3b (AM1035a, all three from Abgent, San Diego, CA). These antibodies recognized bands at 260 kDa (Usp9x), 120 kDa (Ube1x in neonates), 42 kDa (actin), 94 kDa (catenin), 120 kDa (spinophilin), 200 kDa (Dnmt1), 120 kDa (Dnmt3a), and 110 kDa (Dnmt3b) on the Western blots, consistent with previous reports [29] or manufacturer data. The size of