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Tissue-specific variation of Ube3a protein expression in rodents and in a mouse model of Angelman syndrome

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ABSTRACT

Angelman syndrome (AS) is a neurogenetic disorder caused by loss of maternal *UBE3A* expression or mutation-induced dysfunction of its protein product, the E3 ubiquitin–protein ligase, UBE3A. In humans and rodents, *UBE3A*/*Ube3a* transcript is maternally imprinted in several brain regions, but the distribution of native UBE3A/Ube3a[1] protein expression has not been comprehensively examined. To address this, we systematically evaluated Ube3a expression in the brain and peripheral tissues of wild-type (WT) and *Ube3a* maternal knockout mice (AS mice). Immunoblot and immunohistochemical analyses revealed a marked loss of Ube3a protein in hippocampus, hypothalamus, olfactory bulb, cerebral cortex, striatum, thalamus, midbrain, and cerebellum in AS mice relative to WT littermates. Also, Ube3a expression in heart and liver of AS mice showed greater than the predicted 50% reduction relative to WT mice. Co-localization studies showed Ube3a expression to be primarily neuronal in all brain regions and present in GABAergic interneurons as well as principal neurons. These findings suggest that neuronal function throughout the brain is compromised in AS.

Introduction

Angelman syndrome (AS) is a neurogenetic disorder associated with profound intellectual disability, severe language impairment, movement and balance disorder, epilepsy, and a unique behavioral profile with frequent laughter and smiling (Williams et al., 2006). AS results from a deficiency of functional UBE3A (also known as E6-associated protein or E6-AP), an E3 ubiquitin ligase encoded by the *UBE3A* gene. *UBE3A* is an imprinted gene, (Knoll et al., 1989; Kishino et al., 1997; Matsuura et al., 1997; Sutcliffe et al., 1997), and most commonly, AS results from maternal deletions of varying size that include the *UBE3A* gene. Less common causes of AS are *UBE3A* mutations, uniparental paternal disomy (UPD), and imprinting center mutations (Laan et al., 1999; Williams et al., 2006). Although several Ube3a ubiquitination substrates have been described, none have been definitively linked to the pathogenesis of Angelman syndrome (Huibregtse et al., 1991; Kuhne and Banks, 1998; Nuber et al., 1998; Kumar et al., 1999; Reiter et al., 2006; Greer et al., 2010).

The first AS mouse model developed had partial UPD spanning the region including *Ube3a* on mouse chromosome 7, the region homologous to human chromosome 15 (Cattanach et al., 1997). *In situ* hybridization studies in these mice showed that *Ube3a* expression was undetectable in the hippocampus and cerebellar Purkinje neurons, suggesting predominant maternal expression in these regions. In other regions including the cerebral cortex, *Ube3a* expression was moderately reduced, while in regions such as the anterior commissure and optic chiasm, expression was indistinguishable from wild-type (WT) controls, suggesting biallelic *Ube3a* expression (Albrecht et al., 1997). A

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[1] Throughout the manuscript, following the standard nomenclature, *UBE3A* and UBE3A will denote the human gene and protein name, respectively, and *Ube3a* and Ube3a the rodent gene and protein name, respectively. As indicated here, UBE3A and Ube3a are synonymous with E6-AP.

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subsequent AS mouse model was generated by an insertional mutation of exon II of the *Ube3a* gene passed through the maternal germline of *Ube3a*, a null mutation leading to loss of Ube3a expression. Based on *in situ* hybridization studies in these mice, monoallelic maternal *Ube3a* expression was proposed for only the hippocampus and cerebellar Purkinje neurons, while biallelic expression was demonstrated in the piriform cortex and suggested for other brain regions (Jiang et al., 1998). Another AS mouse model was generated by deletion of part of exon 15 and all of exon 16, a modification predicted to inactivate Ube3a ligase function (Miura et al., 2002). These mouse models were shown to recapitulate many of the phenotypic features of AS, including motor dysfunction, increased seizure susceptibility, and hippocampal-dependent learning and memory deficits (Jiang et al., 1998; Miura et al., 2002; Van Woerden et al., 2007).

Based on these models, AS research has focused mainly on the hippocampus and cerebellum, where *Ube3a* deficiency was thought to be the most pronounced. However, recent work using a YFP-Ube3a fusion protein reporter mouse demonstrated that maternal predominant expression of Ube3a may not be restricted to the hippocampus, cerebellum, and olfactory bulbs, as paternal YFP reporter expression was shown to be weakly detected in temporal cortex, and reported to have similar expression in other brain areas including thalamus (Dindot et al., 2008). Moreover, Yashiro et al. (2009) recently used immunohistochemistry and immunoblotting to show that there is minimal cortical expression of Ube3a in AS mice, indicating that Ube3a is nearly exclusively expressed from the maternal chromosome in WT mice throughout the cortex.

These results underscore a need for a more comprehensive understanding of Ube3a protein expression patterns to help direct future research into the pathogenesis and treatment of AS. Therefore, we compared Ube3a expression in the CNS and peripheral tissues of WT rodents and AS mice by immunoblotting and immunohistochemistry. We demonstrate that Ube3a expression is nearly exclusively maternal derived in all brain regions and is expressed in both principal neurons and GABAergic interneurons.

Materials and methods

Mice and tissue preparation

All mice were maintained on C57/SV-129 mixed background. WT males (*Ube3a* M+/P+) were bred with AS females (*Ube3a* M−/P+) to generate WT and AS littermates. PCR analysis (described previously by Jiang et al., 1998) was performed on ear punches to genotype the offspring. Male and female mice were used in this analysis.

Brain tissue was prepared by decapitation and rapid removal of the brain onto ice. The appropriate brain regions were rapidly dissected and snap frozen on dry ice. The time from decapitation to freezing of the brain regions was less than 3 min. Peripheral tissues (heart, liver, and kidney) were also removed and snap frozen on dry ice. Tissue was stored at −80 °C until use.

Western blot analysis

Tissues were homogenized in Kontes glass or Wheaton Teflon tissue grinders in a 2% sodium dodecyl sulfate (SDS) buffer containing 10 μg/ml leupeptin and 1 μg/ml pepstatin. A BCA protein assay was run on the homogenates which were then diluted to a final concentration of 0.7–1 mg/ml. Samples were heated at 60 °C for 10 min and fractionated on SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred in 1× CAPS buffer onto nitrocellulose membranes. Membranes were stained with Ponceau-S and digitally scanned in order to compare total protein levels in each lane by densitometric scanning of the 50–116 kDa molecular weight range using ImageJ. Membranes were then blocked in 5% milk in Tris-Buffered Solution with Tween-20 (TTBS) at 4 °C overnight. Membranes were then incubated with primary antibody, anti-mouse Ube3a (E6-AP) (Sigma-Aldrich clone 330 Cat#E8655) diluted 1:1000, anti-rabbit Ube3a (E6-AP) (Bethyl labs Cat# A300-352A) diluted 1:1000, anti-mouse β-actin (Santa Cruz Biotechnologies Cat#SC-47778) diluted at 1:2000, in 5% milk in TTBS overnight at 4 °C. Membranes were washed 4× 10 min in TTBS, incubated with secondary antibody (goat anti-mouse IgG1-HRP (Santa Cruz Biotechnologies Cat#SC-2969) diluted 1:5000 or 1:3000) in 5% milk in TTBS for 1.5 h, washed 4× 10 min in TTBS, and developed using Western Lighting Enhanced Luminol Reagent-Plus (Plus-ECL). X-ray films exposed in a linear range were quantified using ImageJ for densitometric analysis as described previously (Brown et al., 2005). Western blot signals were then normalized for variations in total protein loading in the corresponding lane, as quantified from Ponceau-S stained membranes (see above), thereby avoiding potential problems associated with quantifying a single protein for use as a loading control. For example, levels of β-actin varied approximately 2-fold in total tissue homogenates of micro-dissected brain regions (Supplementary Fig. 4).

Immunohistochemistry/immunocytochemistry

For immunohistochemistry, animals were deeply anesthetized and transcardially perfused with 0.1 M PBS (pH 7.4) followed by 4% paraformaldehyde in PBS. Brains were removed and postfixed overnight, cryoprotected, and 40 μm coronal sections cut through the neuraxis.

Free-floating sections were washed in 50 mM Tris-buffered saline (TBS, pH 7.4) and then incubated for 20 min in 10 mM sodium citrate (pH 6.0) containing 0.05% Triton X-100 at 75–80 °F. Sections were then processed for immunoperoxidase staining as previously described (Bubser and Deutch, 1999). Primary antibodies used include the mouse anti-Ube3a (E6-AP) (1:2000; Sigma-Aldrich clone 330), rabbit anti-parvalbumin (1:10,000; Swant), rabbit anti-calbindin (1:10,000; Swant), rabbit anti-calretinin (1:10,000; Swant), and rabbit anti-GFAP (1:2000; Chemicon).

For immunocytochemistry, primary hippocampal cultures were prepared from PND 0-1 Sprague–Dawley rat pups. Briefly, brains were removed and placed in ice-cold modified Hank's balanced salt solution (HBSS). Hippocampi were dissected, cut into 3 mm cubes and incubated with 0.05% trypsin–EDTA in HBSS for 30 min at 37 °C. After trypsin treatment, tissue blocks were triturated with HBSS with 10% horse serum, and dissociated cells were plated onto polyornithene coated plates or coverslips. The culture medium contained 500 ml Neurobasal, 10 ml B27, 0.5 mM L-glutamine, and 25 μM penicillin/streptomycin. 4 μM AraC was added two times in the first week to reduce glial cell proliferation and cultures were maintained in a 5% CO_2 incubator for 2–3 weeks. For immunostaining, cells were washed with cold PBS, and fixed in 3.7% formaldehyde, then blocked with 8% bovine serum albumin in PBS. Primary antibodies used were rabbit anti-PSD-95 diluted 1:500 (Invitrogen), mouse anti-Ube3a (E6-AP) diluted 1:1000 (Sigma-Aldrich clone 330), rabbit anti-Ube3a diluted 1:500 (Bethyl), and rabbit anti-synapsin diluted 1:1000 (Chemicon). Anti-rabbit or anti-mouse fluorescent secondary antibodies were used (Molecular Probes). Labeled cells were imaged using a Zeiss 510 confocal microscope. For overlay analysis, dendrites from 10 neurons in 5 separate cultures were examined using MetaMorph software (Molecular Devices).

Subcellular fractionation

Hippocampal tissue was homogenized in Homogenization buffer (150 mM KCl, 50 mM Tris–HCl pH 7.5, mM DTT, 0.2 mM PMSF, 1 mM benzamidine, 1 μM pepstatin, 10 μg/ml leupeptin, and 1 μM microcystin-LR) using Wheaton Teflon or Kontes glass tissue grinders at 4 °C. Total homogenate was rocked for 30 min at 4 °C and spun down at 100,000×*g* for 1 h yielding an S1 fraction (soluble cytosolic