Activity-dependent changes in MAPK activation in the Angelman Syndrome mouse model

Irina Filonova, Justin H. Trotter, Jessica L. Banko, and Edwin J. Weeber

Department of Molecular Pharmacology and Physiology, Morsani College of Medicine, University of South Florida, Byrd Alzheimer’s Institute, Tampa, Florida 33613, USA

Angelman Syndrome (AS) is a devastating neurological disorder caused by disruption of the maternal UBE3A gene. Ube3a protein is identified as an E3 ubiquitin ligase that shows neuron-specific imprinting. Despite extensive research evaluating the localization and basal expression profiles of Ube3a in mouse models, the molecular mechanisms whereby Ube3a deficiency results in AS are enigmatic. Using in vitro and in vivo systems we show dramatic changes in the expression of Ube3a following synaptic activation. In primary neuronal culture, neuronal depolarization was found to increase both nuclear and cytoplasmic Ube3a levels. Analogous up-regulation in maternal and paternal Ube3a expression was observed in Ube3a-YFP reporter mice following fear conditioning. Absence of Ube3a led to deficits in the activity-dependent increases in ERK1/2 phosphorylation, which may contribute to reported deficits in synaptic plasticity and cognitive function in AS mice. Taken together, our findings provide novel insight into the regulation of Ube3a by synaptic activity and its potential role in kinase regulation.

Results

Ube3a expression changes in primary neurons immediately after neuronal depolarization

Maternal Ube3a protein is present in the neuronal nucleus, soma, and dendrites, and is enriched in the postsynaptic compartment (Dindot et al. 2008; Gustin et al. 2010). Subcellular fractionation was used to isolate soluble and nonsoluble nuclear, membrane, and cytosolic compartments (Fig. 1A). To verify the specificity of the Ube3a antibody, hippocampi from Ube3a-deficient mice (Ube3a m−/p−) and littermate controls were fractionated. The antibody shows specificity to Ube3a-deficient mice (Ube3a m−/p−) and littermate controls were fractionated. The antibody shows specificity to Ube3a (~110 kDa) with a complete absence of reactivity in Ube3a m−/p− mice. We observed a single unidentified protein band of a slightly higher molecular weight detected in the nuclear and chromatin-bound fractions. We confirmed quality of fractions by detection of HSP90, ApoER2, NeuN, and histone H3 to the cytosolic, membrane, nuclear, and chromatin-associated fractions, respectively (Fig. 1A). Consistent with previous reports (Gustin et al. 2010), we find that Ube3a is enriched in the cytosol with lower detection in the nucleus and membrane compartments. Next, we determined if synaptic
depolarization could modulate Ube3a protein expression in neuronal culture. Mixed primary cultures (DIV 8-10) prepared from E17-18 wild-type brains were depolarized with 55 mM KCl for 5 and 30 min. Western blot analysis revealed that Ube3a expression following KCl treatment is significantly increased in the nucleus and cytosol as early as 5 min (nuclear soluble, 201 ± 21.42%, P < 0.05; cytosol, 153.5 ± 16.59%, P < 0.05) and remained elevated up to 30 min (nucleus, 182.4 ± 34.78%, P < 0.05; cytosol, 167.3 ± 17.51%, P < 0.01) (Fig. 1B–F). Ube3a was slightly down-regulated in the membrane fraction at 30 min; however, the decrease failed to reach statistical significance. These data demonstrate that KCl-induced depolarization affects Ube3a protein levels resulting in increased Ube3a expression in the cytosol and the nucleus.

Consequences of Ube3a absence on depolarization-induced kinase activation

The wide variety and severity of AS symptoms suggests that the loss of Ube3a causes a global disruption of synaptic function. This is supported in the AS mouse model with identified synaptic disruption in the visual cortex, cerebellum, and hippocampus (Jiang et al. 1998; Heck et al. 2008; Yashiro et al. 2009). A multitude of Ca2+-dependent signal transduction pathways are stimulated by synaptic depolarization. Previously, basal levels of several kinases important for LTP induction and maintenance in the hippocampus of AS mice were examined in the attempt to identify direct or indirect targets of Ube3a ubiquitination. In this study, we sought to revisit the p44/p42 extracellular signal-regulated kinase (ERK1/2) and evaluate its response to neuronal depolarization in wild-type (Ube3a m/+/p+) and Ube3a-deficient mice (Ube3a m−/−p+). The necessity of ERK p44/p42 (ERK1/2) is well established for synaptic plasticity and memory formation (English and Sweatt 1997; Atkins et al. 1998; Thomas and Huganir 2004).

Acute hippocampal slices from Ube3a m−/−p+ mice and wild-type littermates were treated with 55 mM KCl for 5 or 30 min. Following Western blotting, the levels of total and phosphorylated ERK1/2 (p-ERK, Thr202/Tyr204) and mitogen-activated kinase 1/2 (MEK1/2, p-MEK [Ser217/221]) were analyzed. Under basal conditions, Ube3a m+/p+ and Ube3a m−/p+ groups (n = 6-7 slices, three animals per genotype) had similar levels of total ERK1/2 and p-ERK (Fig. 2A,B). ERK1/2 activation was determined by same blot p-ERK to total ERK ratio and standardized to nontreated controls within genotype. All treated groups show elevated p-ERK after depolarization; however, p-ERK levels in Ube3a m−/−p+ slices (p-ERK1, 311 ± 20.83%; p-ERK2, 207 ± 16.98%; P < 0.01, two-way ANOVA) failed to reach wild-type levels (p-ERK1, 477.2 ± 57.65; p-ERK2, 354 ± 80%) at 5 min post depolarization (Fig. 2C,D). Interestingly, levels were elevated from basal levels in both groups 30 min post depolarization, but were not significantly different (n = 6 per genotype). Basal expression, phosphorylation, and activation of MEK were not affected by the absence of Ube3a (Fig. 2E,F). These data suggest that after neuronal depolarization only the immediate activation of ERK is impaired.

Spatial and temporal properties of Ube3a expression following fear conditioning training

Next, we wanted to determine if similarities in the observed changes exist for Ube3a and ERK1/2 in vivo. Recent reports suggest a dynamic regulation of Ube3a and a close integration of this regulation with synaptic plasticity in wild-type mouse brain (Greer et al. 2010). The Ube3a allele is believed to show biallelic expression in all cell types, with the exception of neurons. However, maternal deletion shows a near 100% absence of Ube3a expression in all cell types, with the exception of neurons. How-ever, maternal deletion shows a near 100% absence of Ube3a expression in all cell types, with the exception of neurons. Therefore, we sought to determine if both maternal and paternal copies respond to neuronal activation.
The heterozygous Ube3a-Yellow Fluorescent Protein (Ube3a-YFP) fusion protein reporter mouse allows the ability to distinguish paternal from maternal protein expression by increasing the molecular weight of a single allele with the addition of the YFP. Figure 4A shows that paternal Ube3a-YFP is resolved higher than maternal due to the presence of the 20 kD YFP tag. Using a three foot-shock paradigm. To verify that training paradigm leads to robust memory formation, a group of naive mice (handled only) was examined and showed no difference (data not shown). To determine if paternal Ube3a-YFP is affected by the inclusion of YFP, maternal Ube3a-YFP animals were generated and Ube3a-YFP was compared to naive Ube3a. We found reduced maternal (86.6 ± 3.38%, $P < 0.05$) or paternal Ube3a (56 ± 13.56%, $P < 0.01$) expression of the Ube3a-YFP fusion protein compared to naive wild-type protein (Fig. 4E,F).

Taken together our results suggest that both maternal and paternal alleles are activated by synaptic activity. It appears that paternal Ube3a expression has a specific temporal expression profile that is similar to the maternal activation in hippocampus, but not in cortex. Paternal Ube3a comprises ~3%–5% of total Ube3a in neurons; thus, the amount of paternal protein increase is negligible. Nevertheless, it is critical to consider and further characterize the paternal Ube3a allele, especially given the growing interest in gene-based therapy approaches.

Activity-dependent p-ERK disregulation in Ube3a deficient mouse model

Reduced ERK activation in response to neuronal stimulation may contribute to the cognitive disruption present in Ube3a maternal deficient mice and human AS. Numerous studies report that reduction in ERK activity disrupts associative fear conditioned memory formation (Blum et al. 1999; Selcher et al. 2003). Next we determined if hippocampal-dependent associative learning in adult Ube3a m−/p+ showed reduced p-ERK activation in area CA1 following a three foot-shock FC paradigm. This experimental approach has previously shown increased ERK activation in area CA1 of the hippocampus (Chwang et al. 2006). We subjected both Ube3a m−/p+ (n = 5 control, n = 6 experimental) and Ube3a m−/p− (n = 5 for control, n = 4 experimental) 3- to 4-mo-old mice to FC and measured p-ERK levels in CA1 60 min after to FC training. No significant change in total ERK1/2 or p-ERK1/2 was observed at baseline (Fig. 5B).

Significant differences in p-ERK activation became apparent when the samples were compared at 60 min post training. Consistent with published data (Chwang et al. 2006) area CA1 of Ube3a m−/p+ hippocampi showed a significant increase in p-ERK2 levels (143.4 ± 14.19% of Ube3a m−/p+, $P < 0.05$) compared to control group. By comparison, p-ERK2 activation was blunted in Ube3a m−/p− animals (82.10 ± 4.6% of Ube3a m−/p− controls). Analysis of CA3 and DG areas did not reveal changes in total or p-ERK in either wild-type or in transgenic littermates (data not shown).

Discussion

A preponderance of imprinted genes is associated with placental regulation and represents a genetically unique method of regulating protein expression. However, maternal Ube3a levels
can undergo further regulation beyond paternal allele silencing. For example, Ube3a is a target of itself, suggesting a negative feedback for regulating protein level. This form of biochemical regulation may be in response to the recent discovery of altered neuronal Ube3a expression following glutamatergic activation and neuronal depolarization. A wide variety of activators, including N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and brain-derived neurotrophic factor (BDNF), can initiate an increase of Ube3a mRNA within 5 h in primary cultures (Greer et al. 2010). In light of direct and indirect alterations of Arc and CaMKII, both major players in synaptic plasticity, changes in Ube3a expression suggest a role in synaptic function that supersedes the traditional roles of an E3 ubiquitin ligase. We sought to determine if Ube3a is altered in response to neuronal depolarization and in vivo learning and memory. Furthermore, we wanted to investigate if other signaling proteins modulated by calcium influx were likewise altered during synaptic activity in the Angelman Syndrome mouse model.

Although we did not specifically delineate isoform expression, we found increased total Ube3a in response to KCl treatment at 5 and 30 min post application in both cytosolic and nuclear fractions isolated from primary neurons, suggesting that this is not an isoform-dependent phenomenon. Although ubiquitous expression, Ube3a is altered in response to neuronal depolarization and in vivo learning and memory. Furthermore, we wanted to investigate if other signaling proteins modulated by calcium influx were likewise altered during synaptic activity in the Angelman Syndrome mouse model.

Figure 3. Ube3a expression in Ube3a m+/p+, Ube3a m−/p+, and Ube3a m−/p− mouse brains. (A) Paternal Ube3a is expressed at low levels throughout the brain. (HIP) Hippocampus, (PFC) prefrontal cortex, (COR) cortex, (STR) striatum, (CER) cerebellum. Immunostaining (B) and subsequent analysis (C) of coronal sections of mouse brain demonstrated near absence of paternal Ube3a expression in Ube3a m−/p+ mice (n = 3) and no Ube3a expression in Ube3a m−/p− (n = 2) compared to wild-type controls (n = 3) (one-way ANOVA, post hoc Tukey’s test, *P < 0.05).

Identifying activity-dependent regulation of Ube3a protein raises the possibility that Ube3a is involved in activities other than housekeeping protein degradation. It stands to reason that Ube3a may be involved in modulating pathways that are involved in synaptic plasticity, and this may explain why our earlier work investigating kinase alterations in the Ube3a deficient mouse only revealed an alteration in α-CaMKII phosphorylation (Weeber et al. 2003). Those previous experiments were performed during basal conditions. The use of KCl-induced neuronal depolarization should effectively activate multiple signaling pathways leading to ERK activation. Interestingly, we demonstrate reduced phosphorylation of ERK in response to KCl depolarization in the Ube3a m−/p+ mouse, suggesting that Ube3a is involved in a signaling cascade that targets ERK phosphorylation. Curiously, we did not find a concurrent difference in either total MEK or p-MEK levels, the kinase responsible for phosphorylation and activation of ERK. This result may reflect a direct or indirect action of Ube3a on MEK’s ability to phosphorylate ERK or an Ube3a-dependent regulation of phosphatases. The exact mechanism for this difference in ERK activation is currently under investigation.

There has been extensive investigation into the regulation of ERK in mice performing various behavioral tasks. Depending on the learning paradigm, the p-ERK levels are shown to increase as early as 1 min post training and stay elevated for up to 9 h (Blum et al. 1999; Chwang et al. 2006; Trifilieff et al. 2006, 2007). Both memory formation and hippocampal synaptic plasticity are sensitive to inhibition of ERK activation and reduced p-ERK levels correlate nicely with deficits in memory retention (Blum et al. 1999; Selcher et al. 1999). Our finding that p-ERK levels are reduced following contextual fear conditioning in Ube3a m−/p+ mice provides an additional rationale to explain
the well-established associative learning deficit exhibited by these mice (Jiang et al. 1998; van Woerden et al. 2007; Huang et al. 2013).

In conclusion, the present study suggests that Ube3a protein levels can change significantly in response to neuronal depolarization and, importantly, during the processes of associative learning and memory. Increased levels in both maternal and paternal Ube3a are intriguing and raise important questions about regulatory mechanisms of both maternal and paternal alleles. Furthermore, subtle changes in the paternal Ube3a allele, its distribution, isoform production, and function, should be addressed before considering it as a potential therapeutic target.

**Materials and Methods**

**Animals and reagents**

All animals utilized in the experiments were handled in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of South Florida. To generate paternally imprinted Ube3a-YFP, C57 female mice were bred to Ube3a-YFP males. Maternal Ube3a-YFP mutants were obtained by the opposite crosses. pUbe3a-YFP males and females that were 3–3.5 mo old were used in fear conditioning experiments to establish Ube3a expression profiles. 129SV/C57 hybrid Ube3a m/+/p and wild-type littermates 2–4 mo old were used to assess p-ERK activation. Antibodies: E6-AP (Bethyl lab); actin, ERK1/2, p-ERK1/2, MEK1/2, p-MEK1/2, Histone H3, and HSP90 (Cell signaling); NeuN (Millipore); ApoER2 (Abcam). Chemicals: KCl (50 mM, Sigma, P9594).

**Fear conditioning and tissue collection**

Paternal Ube3a-YFP mice were handled 5 min/day for 5 d in the room where fear conditioning training took place. On the day of experiment, each animal was placed in a sound attenuation chamber (SAC) for 4–5 min; after 3-min habituation three 0.5-mA shocks with 30-sec intervals were applied to a floor grid. Mice were euthanized with isoflurane at 5 min, 15 min, 1 h, 3 h, and 6 h following conditioning (three animals per group, males and females). Mouse brains were removed and cortex and hippocampus were dissected in ice-cold PBS containing Halt protease and phosphatase inhibitors cocktail (Thermo Scientific, 78440). Dissected tissue was frozen on dry ice and stored at −80°C until further processing. A control group of mice was subjected to fear conditioning training and was tested 24 h later. Freezing behavior was recorded and scored by AnyMaze software.

**Western blot**

Frozen tissue and primary neuronal cultures were homogenized by sonication in ice-cold RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% triton ×100) containing a Halt protease and phosphatase inhibitors cocktail. The lysates were clarified at 22,000g for 30 min. The protein concentrations were determined using the BCA Protein Analysis Kit (Pierce). Samples were resolved by 10% TGX gels (Bio-Rad) and transferred to PVDF membrane. Membranes were blocked with 5% milk in TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) for 1 h and incubated with primary antibodies at 4°C overnight. After three 10-min washes with TBST, secondary antibodies were applied for 1 h at room temperature. Blots were detected with film for chemiluminescence using Pierce ECL. Developed images were analyzed using ImageJ software. Statistical analysis (Student t-test, ANOVA) was performed using GraphPad Software.

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Subcellular fractionation

Mouse brains and murine mixed primary neuronal cultures were fractionated using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific, 78840) according to manufacturer manual.

Primary neuronal culture

Mixed (cortical and hippocampal) cultures were used for Western blot analysis and subcellular fractionation. Brains from E16–E18 wild-type 129Sv embryos were removed and dissected under microscope in ice-cold isotonic buffer (137 mM NaCl, 5.4 mM KCl, 170 μM NaH2PO4, 148 μM KHPO4, 5.5 mM glucose, 58.4 mM sucrose). Dissected tissue was pooled together in 15-mL tube and incubated with 1 mL 0.25% trypsin for 5 min at 37°C. Trypsin was removed and 5 mL dissociation media was added (DMEM, high glucose, 10% heat inactivate FBS, 1× antimycotic/antibacterial solution). Hippocampi were dissociated by slowly pipetting through glass pipettes. After brief centrifugation, dissociation media was replaced by neurobasal medium supplemented with 2% B27 and 0.5 mM glutamax. Cells were plated in six well (2,500,000/well) plates coated with poly-L-lysine. Cultures were treated with 55 mM KCl on DIV 7-10.

Nonfluorescent immunohistochemistry

Mice were transcardially perfused with saline solution (0.9% NaCl in water) followed by 4% PFA diluted in 0.1 M PBS. Removed brains were dehydrated via sucrose gradient (10%, 20%, 30%) and sectioned on a cryostat at 30 μm. Free-floating sections were blocked in 4% goat serum, 0.2% lysine in 0.1 M PBS and incubated with Ube3a primary antibody overnight (1:1000). Secondary antibody was added for 2 h and the avidin–biotin–HRP complex was formed with VECTASTAIN ABC kit (Vector labs). DAB was used to develop the stain. Sections were imaged with a Zeiss Mirax Scan 150 microscope. Identification of positive staining and percent area of positive stain was performed using Image Analysis software (created by Andrew Lesniak, Zeiss).

Acute hippocampal slice preparation and treatment

Two-month-old m+/p+ and m−/+p+ littermates were sacrificed by decapitation, the brains were rapidly removed and briefly submerged in ice-cold cutting solution (110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 28 mM NaHCO3, 0.5 mM CaCl2, 5 mM glucose, 0.6 M ascorbate) saturated with 95% O2 and 5% CO2. Hippocampal slices (400 μm) were cut on a vibratome and allowed to equilibrate in artificial cerebrospinal fluid solution (125 mM NaCl, 2.5 mM KCl, 1.24 mM NaH2PO4, 25 mM NaHCO3, 10 mM glucose, 2 mM CaCl2, 1 mM MgCl2) at 32°C for 2 h. Slices were treated with 55 mM KCl for 5 and 30 min, snap frozen, and stored at −80°C for further processing.

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