Analysis of Cerebellar Function in Ube3a Deficient Mice Reveals Novel Genotype Specific Behaviors

Detlef Heck², Yu Zhao¹, Snigdha Roy², Mark S. LeDoux¹, and Lawrence T. Reiter¹,*

¹Department of Neurology, ²Department of Anatomy and Neurobiology, UTHSC, Memphis, TN

*Correspondence should be addressed to Lawrence T. Reiter
Department of Neurology
855 Monroe Ave., Link 415
Memphis, TN  38163
(901) 448-2635 (Phone)
(901) 448-7440 (FAX)
lreiter@utmem.edu (e-mail)
Abstract:

Angelman syndrome (AS) is a childhood-onset neurogenetic disorder characterized by functionally severe developmental delay with mental retardation, deficits in expressive language, ataxia, appendicular action tremors and unique behaviors such as inappropriate laughter and stimulus-sensitive hyperexcitibility. Most cases of AS are caused by mutations which disrupt expression of maternal UBE3A. Although some progress has been made in understanding hippocampal-related memory and learning aspects of the disorder using Ube3a deficient mice, the numerous motoric abnormalities associated with AS (ataxia, action tremor, dysarthria, dysphagia, sialorrhea, and excessive chewing/mouthing behaviors) have not been fully explored with mouse models. Here we use a novel quantifiable analysis of fluid consumption and licking behavior along with a battery of motor tests to examine cerebellar and other motor system defects in Ube3a deficient mice. Mice with a maternally-inherited Ube3a deficiency (Ube3a<sup>m-/p+</sup>) show defects in fluid consumption behavior which are different from Ube3a<sup>m-/p-</sup> mice as well. The rhythm of fluid licking and number of licks per visit were significantly different among the three groups and indicate that not only was fluid consumption dependent on Ube3a expression in the cerebellum, but may also depend on low levels of Ube3a expression in other brain regions. Additional neurological testing revealed defects in both Ube3a<sup>m-/p+</sup> and Ube3a<sup>m-/p-</sup> mice in rope climbing, grip strength, gait, and a raised-beam task. Long term observation of fluid consumption behavior is the first phenotype reported that differentiates between mice with a maternal loss of function vs. complete loss of Ube3a in the brain. The neuronal and molecular mechanisms underlying mouse fluid consumption defects specifically associated with maternally inherited Ube3a deficiency may reveal important new insights into the pathobiology of AS in humans.
Introduction

Angelman syndrome (AS; MIM 105830) is a severe neurodevelopment disorder with an incidence of ~1/20,000 and characterized by profound mental, motor and behavioral abnormalities. The molecular lesion in most AS patients is a defect in expression of the maternal copy of the E3 ubiquitin ligase gene *UBE3A*. Maternal deficiency or loss-of-function mutations in *UBE3A* are sufficient to cause AS (1, 2). The *UBE3A* gene exhibits maternal allele specific expression in the Purkinje cell layer of the cerebellum and the cell body of CA1-CA2 neurons of the hippocampus in mice and humans (3-5). Essentially, this means that the loss of function allele (be it a point mutation, imprinting center mutation or the more common large 15q11.2-q13 deletion) must be maternally derived to result in an AS phenotype or, in some cases, paternal uniparental disomy can prevent the maternal copy of *UBE3A* from being expressed (reviewed in (6)). Recent data also suggests that maternal imprinting is not restricted to neurons in the hippocampus and cerebellum but rather extends to all mature neurons in the brain. Furthermore, these data suggest that the paternal *Ube3a* allele may not be completely “off” in all neurons as previously reported (7). A great deal of research has been focused on understanding this complex imprinted regulation of *UBE3A* in the context of the molecular defects that cause AS (8).

Patients with AS consistently exhibit a movement disorder that can be ascribed, in large part, to cerebellar dysfunction (8). In affected individuals, truncal ataxia contributes to postural instability whereas appendicular ataxia is often associated with an action tremor. In AS, postural maintenance is associated with abnormal rhythmic bursts of muscle activity (9). Cerebellar defects in AS individuals clearly contribute to the motoric aspects of the disease. Furthermore, based on modern views of cerebellar function, cerebellar dysfunction could play a role in the various behavioral and cognitive deficits seen in AS (10-12). However, little progress has been made in the analysis of cerebellar function in mouse models of AS and how cerebellar defects in the mouse model relate to cerebellar motor and possibly cognitive defects present in AS individuals.

To date, two *Ube3a* loss of function mouse models of AS have been generated (13, 14). The *Ube3a* deficient mice generated by Jiang *et al.* via knock out of exon 2 of the *Ube3a* gene were analyzed for gross cerebellar defects via accelerating rotarod tests (13). Mice generated by Muria *et al.* were constructed by a knock-in strategy resulting in the replacement of exons 15 and
16 of the Ube3a gene with a β-geo reporter gene (14). These mice show similar rotarod defects and have been used to map regions of the brain that are subject to imprinted expression through the detection of the Ube3a-β-geo reporter gene. Although Ube3a shows clear maternal-specific expression in the cytoplasm of Purkinje neurons, Muria et al. also reported that Ube3aβ-geo expression occurred in neurons of the granular cell layer. The subsequent generation of a Ube3aYFP knock-in mouse has verified that maternal-specific expression of Ube3a is not restricted to Purkinje cell bodies, but can be detected in cerebellar neurons of both the molecular and granular cell layers (4). In fact, maternal expression and even low levels of paternal expression of Ube3a have been detected in other regions of the brain including the cortex, thalamus and olfactory bulb (7). The only region where biallelic expression could be detected was the GFAP-positive cells that line the ventricles.

Previous results from rotarod and balance beam testing suggest cerebellar related movement defects and electrophysiological studies have shown that cerebellar neuronal activity is abnormal in Ube3a deficient mice (15). Recently it has been shown that the rotarod defects in Ube3a deficient mice can be rescued by a mutation in the auto-phosphorylation domain of the α-CaMKII protein (16). However, accelerating rotarod testing may not be the most informative and sensitive behavioral assessment to identify primarily cerebellar defects since it does not reliably differentiate among defects in muscle, nerve, upper motor neuron, and cerebellar motor control. A quantifiable test with output metrics that are closely tied to cerebellar activity and outside of the context of locomotion is needed which, ideally, can be combined with electrophysiological measurements to understand the neuronal aspects of cerebellar defects in these animals.

Here we propose that fluid licking behavior in mice is a sensitive behavioral test for both motor and autonomic aspects of cerebellar function. Fluid licking is a naturally occurring behavior that is easy to analyze in the home-cage environment and we have previously shown that it can be combined with electrophysiological recordings (17). Licking behavior in mice is a highly stereotypic, rhythmic movement that is believed to be controlled by a central pattern generator circuit located in the brain stem (18). The mean inter-lick-interval is strain specific and changes little with changing environmental conditions (19, 20). Licking movements are widely represented in the neuronal activity of the cerebellum in rats (21) and mice (17).
The rhythmic fluid licking movements have been shown to be precisely coordinated with swallowing (22, 23) and respiratory movements (24). Dysphagia, i.e. difficulty in swallowing, is a common symptom in patients with acquired and hereditary cerebellar disease (25) and is present in as many as 74% of AS deletion patients (26). Functional magnetic resonance imaging (fMRI) studies indicate strong activation of the cerebellum during swallowing in humans (27). Respiratory patterns are abnormal in young cerebellar patients (28) and fMRI studies in adults show a strong cerebellar activation during respiratory challenges (29). Based on these observations, we predicted that mice with cerebellar defects due to Ube3a deficiency should demonstrate abnormal licking behavior due to impaired coordination of licking, breathing and swallowing movements. Furthermore, recent neuroimaging studies in humans have linked the cerebellum to the awareness of thirst (11, 30) suggesting that Ube3a deficient mice might have abnormal drinking patterns.

In this study, we used tests of motor coordination, balance, and motor power to characterize the effects of Ube3a deficiencies on cerebellar-dependent motor control. Long-term home-cage measurements of licking behavior were used to investigate licking and fluid consumption behavior. Here we show that Ube3a deficient mice have an ataxic gait as well as rope climbing defects. Furthermore, they exhibit significant differences in their licking rhythms and number of licks per visit to the water spout, a new quantitative phenotype that differentiates between the three genotypes. The quantitative differences in the number of licks per visit is the first reported behavioral measure of cerebellar function that can distinguish mice that are deficient for maternal specific expression of Ube3a from mice with complete absence of Ube3a expression.

Results

Ube3a<sup>m-/p-</sup> and Ube3a<sup>m-/p+</sup> mice exhibit an extended inter-lick interval and increased frequency of licks per visit.

Fluid licking behavior was measured over a three day period in 2-5 month old mice that were either homozygous for the Ube3a null allele (Ube3a<sup>m-/p-</sup>) or inherited the null allele through the maternal germline (Ube3a<sup>m-/p+</sup>). Wild type littermates of these animals were used as controls since they better represent the genetic background than Ube3a<sup>m+/p-</sup> mice, which would have to be generated from a separate crossing scheme. The average inter-lick interval is a measure of the
tempo of the lick rhythm. Both $Ube3a^{m-/p+}$ and $Ube3a^{m-/p-}$ mice exhibit significantly longer mean inter-lick intervals than their wild type littermates (Figure 1A). $Ube3a^{m-/p+}$ mice have a mean lick interval of 137ms which is approximately equal to the interval in $Ube3a^{m-/p+}$ but significantly different from the 118ms interval measured in wild type animals ($N_{wt} = 9, N_{Mat-Het} = 10, p_{lick interval} = 0.001, N_{KO} = 10, N_{Mat-Het} = 10, p_{lick interval} = 0.001$). This difference was stable over the 3-day uninterrupted measurement period. $Ube3a$ deficient mice differed from wild type mice also in the number of licks they would generate when they visited the water spout. When compared to controls, the total number of licks per visit to the waterspout was 42% greater in $Ube3a^{m-/p+}$ and 77% greater in $Ube3a^{m-/p-}$ mice (Figure 1B). The number of licks per visit was not only significantly different between $Ube3a^{m-/p+}$ and wild type animals, but also between $Ube3a^{m-/p-}$ and $Ube3a^{m-/p+}$ animals ($WT = 54.5 +/- 1.1, Mat-Het = 76.7 +/- 2.5$ and $KO = 96.2 +/- 2.3; N_{wt} = 9, N_{Mat-Het} = 10, p_{licks/visit} = 0.001; N_{KO} = 10, N_{Mat-Het} = 10, p_{licks/visit} = 0.001; N_{wt} = 9, N_{KO} = 10, p_{licks/visit} = 0.001$). We lacked the technical means to measure the small fluid intake volumes in mice, therefore, we could not determine if the increase in the number of licks per visit resulted in an increase in the amount of water consumed. However, we did notice a significant increase in the weight of $Ube3a^{m-/p+}$ mice that was not as pronounced in the $Ube3a^{m-/p-}$ mice (supplemental Figure 1). The affect of this weight difference on our testing paradigm will be addressed in detail in the discussion, however as wild type and $Ube3a^{m-/p-}$ mice had similar body weights it may be assumed that fluid consumption was within the normal physiological range for at least these two genotypes.

**Additional behavioral testing for motor coordination reveals ataxic gait, coordination and balance problems in both $Ube3a^{m-/p-}$ and $Ube3a^{m-/p+}$ mice.**

Behavioral tasks involving complex motor coordination like walking, climbing or balancing on a beam involve the orchestration of several sensory and motor regions of the brain such as motor and pre-motor cortices, brain stem and cerebellum, as well as the function of peripheral muscles guided by the signals from the spinal cord and peripheral nervous system. In order to evaluate abnormalities in motor coordination of $Ube3a$ deficient mice which may be the result of cerebellar defects we performed a battery of established motoric tests: accelerating rotarod assays, footprint assays for gait ataxia and beam assays for balance. In addition, a rope climbing assay was used to assess the coordination of hind- and forelimb movements in a task that has never been performed by the animals and, therefore, has not been subject to
improvement through practice. Finally, grip strength was measured to isolate neuromuscular from coordination defects.

In two previous reports, latency periods before mice fell off the accelerating rotarod were variable, but the end result indicated in both publications that \textit{Ube3a}^{m/-p-} and \textit{Ube3a}^{m/-p+} mice performed in a similar manner and fell off the rod before their wild type littermates (13, 14). Our analysis was in agreement with these results (Figure 2). During all of five trials using 8 mice of each genotype the rotarod performance of \textit{Ube3a}^{m/-p-} and \textit{Ube3a}^{m/-p+} mice was statistically identical (\(N = 8\) each genotype; \(p_{\text{session1}} = 0.121, p_{\text{session2}} = 0.770, p_{\text{session3}} = 0.837, p_{\text{session4}} = 0.399, p_{\text{session5}} = 0.756\)), but both strains fell off significantly earlier than wild type controls (for \textit{Ube3a}^{m/-p+} vs. wild type: \(p_{\text{session1}} = 0.011, p_{\text{session2}} = 0.002, p_{\text{session3}} = 0.002, p_{\text{session4}} = 0.0002, p_{\text{session5}} = 0.001\); for \textit{Ube3a}^{m/-p-} vs. wild type: \(p_{\text{session1}} = 0.0002, p_{\text{session2}} = 0.003, p_{\text{session3}} = 0.001, p_{\text{session4}} = 0.002, p_{\text{session5}} = 0.002\)). Rotarod assays, however, are not a direct assay of cerebellar function but could also result from peripheral nervous system defects.

Angelman syndrome patients exhibit tremor, ataxia and lack of coordination. Step width and stride length measurements are a common test for ataxic gait in rodents (31). We measured base width and hind stride length in wild type, \textit{Ube3a}^{m/-p+} and \textit{Ube3a}^{m/-p-} mice. Our findings indicate that both the hind stride length and hind base width are significantly increased in \textit{Ube3a} deficient mice (\(N = 8\) each genotype; \(p_{\text{stride length}} = 0.001, p_{\text{hind-base width}} = 0.017\) for \textit{Ube3a}^{m/-p+}; \(p_{\text{stride length}} = 0.003, p_{\text{hind-base width}} = 0.006\) for \textit{Ube3a}^{m/-p-}; Figure 3). There was no significant difference in fore base width between the three genotypes. This gait ataxia can be clearly seen on the footprint sheets (Figure 3A) and is similar for \textit{Ube3a}^{m/-p+} and \textit{Ube3a}^{m/-p-} genotypes (Figure 3B). These results are the opposite of those reported by Jiang \textit{et al.} for the same line of \textit{Ube3a} deficient mice. Those authors found that mice with maternal deficiency for \textit{Ube3a} had a shorter stride length as compared to wild type littermates (stride length 5.0+/-0.3 cm vs. 5.9+/-0.5 cm) (13). Possible reasons for the different outcomes are discussed below.

Mice were also analyzed in a raised-beam balance testing paradigm. These tests included both round and flat beams of varying width from 20-mm down to 8-mm. Mice with maternally inherited \textit{Ube3a} deficiency performed as well as their wild type littermates on square beams of decreasing size. However, the homozygous \textit{Ube3a} null mice took significantly longer to perform this task on all square beams from 20-mm down to 8-mm (\(N = 8\) each genotype; \(p_{20\text{-mm square}} = 0.036, p_{12\text{-mm square}} = 0.005, p_{8\text{-mm square}} = 0.0001\); Figure 4A). When these motor-
coordination tests were performed using round beams, the maternal heterozygotes again performed as well as their wild type littermates, but the homozygous Ube3a null mice took significantly longer to perform this task on 12-mm round beam (\(N = 8\) each genotype; \(p_{12\text{-}mm\text{ round}} = 0.042\); Figure 4B). In all beam sizes the Ube3a null mice took longer to reach the end of the beam than Ube3a\(^{m-/p^+}\) or wild type mice. No significant differences were detected in the number of slips during raised beam testing for all genotypes.

In an effort to analyze ataxia outside the context of balance we subjected the mice to a rope climbing assay which depends on both motor coordination deficits and grip strength. Mice with a maternally inherited Ube3a null allele actually performed statistically the same as homozygous Ube3a null mice. Both Ube3a deficient strains needed significantly more time to climb the rope than their wild type littermates (\(N_{\text{WT}} = 18, N_{\text{Het}} = 16\), \(p_{\text{rope climbing}} = 0.0001\) for Ube3a\(^{m-/p^+}\); \(N_{\text{WT}} = 18, N_{\text{KO}} = 15\), \(p_{\text{rope climbing}} < 0.0001\) for Ube3a\(^{m-/p^-}\); Figure 4C). In addition, both types of Ube3a deficient mice were resistant to training and had to be coaxed to climb the rope. Vertical rope climbing not only involves the coordination of fore and hind limb movements, but also requires grip strength to pull the animal up the rope. Therefore, we tested grip strength in the same mice to determine if differences in grip strength could account for the differences in the vertical rope climbing results. This test revealed a significant decrease of grip strength in both maternally inherited and homozygous Ube3a null mice compared to their wild type littermates (\(N_{\text{WT}} = 23, N_{\text{Het}} = 18\), \(p_{\text{grip}} = 0.0007\); \(N_{\text{WT}} = 23, N_{\text{KO}} = 13\), \(p_{\text{grip}} = 0.0038\); Figure 4D). It should be noted, however, that a retrospective analysis of the weight of Ube3a\(^{m-/p^+}\) mice used in this test revealed that they are on average 2-3 grams heavier than either wild type littermates or Ube3a null animals of the same age (range 3-5 months; Supplemental Figure 1). This weight difference is significant as compared to wild type animals (\(N_{\text{WT}} = 32, N_{\text{Het}} = 21\), \(p_{\text{weight}} = 0.007\)), but more careful analysis at multiple time points for age matched sib pairs will be required before the impact of this observation can be warranted.

**Discussion**

The main finding of this study is that long term observation of fluid licking behavior revealed a quantitative phenotypic difference in water consumption behavior which can differentiate between mice with maternally inherited Ube3a deficiency, Ube3a null mice and wild type littermates. There was a significant difference among all three strains in the number of
licks they generated each time they visited the waterspout to drink. Furthermore, both $Ube3a$ deficient strains licked significantly slower than their wild type litter mates. These quantifiable difference in what are presumably cerebellar regulated functions may open the door to more finite analysis of the phenotypic consequences of loss of $Ube3a$ protein in cerebellar neurons, where it is maternally expressed, vs loss in other regions of the brain where it is not only preferentially maternally expressed, but also expressed at lower levels from the paternal allele. To our knowledge this is the first behavioral assay that distinguishes between mice with maternally inherited $Ube3a$ deficiency and $Ube3a$ null mice. Thus, fluid consumption behavioral assays provide new experimental opportunities to study CNS deficiencies and their consequences for natural behaviors specific to maternally inherited $Ube3a$ deficiency.

Previous behavioral analysis of $Ube3a$ deficient mice have focused mostly on memory and learning phenotypes observed as hippocampal long term potentiation defects in these animals. Miura et al. reported deficits in spatial learning tasks typically associated with hippocampal dysfunction as well as abnormal electroencephalographic activity in the hippocampus (14). Possible cerebellar defects in these mice have been evaluated using the rotarod tests and lead to the conclusion that $Ube3a$ deficient mice suffer from defects in balance and coordination (13, 14). In addition, electrophysiological recordings from cerebellar Purkinje cells in mice with maternally inherited $Ube3a$ deficiency revealed abnormal oscillatory simple spike activity as well as oscillatory local field potential signals (15). In this study, however, we have been able to quantify cerebellar defects using a newly developed method to study home-cage licking behavior and interrogated $Ube3a$ deficient mice with an expanded repertoire of motor-behavioral testing designed to expose additional cerebellar-related motor defects (rope climbing, raised beam and quantitative gait analysis).

Although maternally imprinted expression of $Ube3a$ is found in mature neurons in all brain areas except possible neural stem cells lining the ventricles (4), maternal specific expression of $Ube3a$ is significantly reduced in the Purkinje cells, hippocampal neurons and mitral cells of the olfactory bulb in mice with UPD for the paternal allele (32). Based on current knowledge, it is highly unlikely that the hippocampus is involved in the control of water consumption. We therefore suggest that the differences in water consumption behavior that differentiate between the three genotypes are due to defects in cerebellar function.
Several lines of evidence have linked the cerebellum to the control of feeding and drinking behavior. Alterations in feeding behavior have been observed in rats after cerebellar lesions (33, 34). Electrophysiological studies in rats have demonstrated that the cerebellum modulates activity in feeding related hypothalamic neurons (35). Furthermore, a number of neuroanatomical studies in rodents and primates have demonstrated connections between the cerebellum and most hypothalamic nuclei (10, 35). In addition, the most direct evidence for cerebellar involvement in controlling lick rhythm comes from electrophysiological studies showing that licking movements are widely represented in the neuronal activity of the cerebellum in rats (21) and mice (17). We hypothesize that the role of the cerebellum is to coordinate licking with swallowing and respiratory movements. This hypothesis is derived from the combined results of animal and human studies. Animal studies have shown that licking movements are precisely coordinated with swallowing (22, 23) and respiratory movements (24) in rats. Clinically, dysphagia is common in cerebellar patients (25). Furthermore, the cerebellum is critically involved in swallowing and orobuccolingual and pharyngeal motor control (27). Thus, the slow lick rhythm in Ube3a deficient mice is possibly due to impaired cerebellar coordination of licking, breathing and swallowing movements. Consistent with our findings are the results of a previous study which found that as many as 74% of patients with AS as a result of maternally inherited deletions of 15q had swallowing difficulties (26).

It most interesting, however, that the number of licks per visit is a phenotype which is highly sensitive to the level of Ube3a expression and at least partially rescued by the residual paternal expression of Ube3a in Ube3a\(^{-/-}\)p\(^{-+}\) animals. This suggests that although the number of licks per visit may primarily is regulated by the cerebellum, other regions of the brain where Ube3a is expressed - albeit at lower levels - may also play a role in controlling this behavior (Figure 5) or that the lower than normal levels of Ube3a in the cerebellum of Ube3a\(^{-/-}\)p\(^{-+}\) mice do not accurately represent the complete loss of Ube3a phenotype.

Expression from the paternal allele of Ube3a can clearly still be detected in these neurons as well, but at much lower levels (7). It has been demonstrated that although Ube3a maternal specific expression in the cerebellum is primarily localized to the Purkinje cell layer, there are also neurons in both the molecular layer and granular cell layer where Ube3a is detected (7, 36). In addition, the cerebellar output, which originates in Purkinje cells, subsequently connects to the deep cerebellar nuclei and then to other regions of the brain where Ube3a expression may
also be imprinted. Altered function of these target areas due to complete loss of Ube3a may contribute to the increased defects in licking behavior in Ube3a knock out mice as compared to Ube3a\textsuperscript{m-\textbar p+}. The genotype specific phenotype we observed may, therefore, provide a unique opportunity to investigate the neuronal networks and molecular mechanisms specific to maternally inherited Ube3a deficiency as opposed to complete loss of Ube3a expression in all tissues, a genotype which has not been observed in humans.

We also found significant differences among genotypes in rope climbing and raised-beam tests but none of these tests differentiated between all three genotypes. For the raised beam balance tests the Ube3am-\textbar p+ mice actually performed more similar to wild type than to complete Ube3a null animals (Figure 4A & 4B). Perhaps these differences reflect the orchestration of balance through a network of neurons outside of the cerebellum which would show greater changes in Ube3a expression in the null animals. On the rope climbing task, both Ube3a null and maternally deficient Ube3a mice generally performed worse than wild type mice. These defects may be, in part, due to peripheral muscle weakness since the two Ube3a deficient strains performed similarly on grip strength analysis and in their rope climbing abilities (Figure 4C & D). However, it should be noted that we observed a significant weight gain in the Ube3a\textsuperscript{m-\textbar p+} animals which may have contributed to the large error bars in the rope climbing study (Supplemental Figure 1). Most mammals can compensate for cerebellar defects when performing an essential and repeated behavior such as walking, but when presented with a new task that requires cerebellar coordination like rope climbing the true extent of the coordination defects can be directly compared between these closely related groups. However, the combination of the weight gain and the general resistance of Ube3a deficient mice to perform in the rope climbing task complicates the interpretation of this test as an assay for cerebellar regulated coordination. In fact, additional testing for autistic behavior similar to the tests used in Rett syndrome and Fragile X mouse models may be needed in these animals to reveal that training for any learned behavior task in Ube3a deficient mice is not feasible (37).

In an earlier study Jiang et. al reported that Ube3a deficient mice had a narrow ataxic gait, but they did not provide footprint analysis diagrams to demonstrate this rather subtle difference reported (13). Our data show that Ube3a deficient mice have a wide-spaced ataxic gait, similar to the gait observed in AS patients (38). We are not sure why there is a discrepancy between our findings and the findings of Jiang and colleagues. One possibility is that the
animals in this study were older, and therefore, better able to compensate for their ataxia. Another possibility is that some of the gait ataxia was suppressed in the mixed genetic background of the previous study.

One of the most urgent questions in Angelman syndrome research, particularly in regard to possible interventions, is how the loss of a single protein like UBE3A predominantly in cerebellar Purkinje cells can result in the extremely debilitating ataxias of limb movements, balance and gait observed in AS patients. It has recently been reported that mutations in the α-CaMKII protein, which block auto-phosphorylation of the kinase, can rescue all previously reported seizure, motor and long term potentiation defects observed in Ube3a maternal deficient mice (16). The molecular mechanism of this suppression is still unclear and there is no evidence to date that Ube3a is actually phosphorlyated by α-CaMKII. Our lab has been actively identifying potential Ube3a substrates through proteomic analysis in Drosophila head extracts (36). One of these target proteins, Ect2, is clearly regulated by Ube3a in Purkinje cells and could therefore be an intermediate protein regulated by both α-CaMKII and Ube3a. An obvious question is whether the quantifiable defects in water consumption behavior reported here are also rescued by α-CaMKII mutations or if subsequent mutations in Ube3a target proteins like Ect2 are responsible for the phenotypic differences.

In summary, using novel, behavioral tests of fluid licking behavior, we have shown that Ube3a null mice and with maternally inherited Ube3a deficiency display significant quantitative differences in the number of licks generated per visit to the waterspout. Both groups also expressed similar defects with regards to motor coordination. We propose that long term observation of fluid consumption behavior provides a new experimental approach sensitive enough to detect the phenotypic consequences of complete loss of Ube3a vs the very low levels of paternal expression of Ube3a in the Ube3a<sup>m-/p+</sup> mice. In the future we hope to use these methods to investigate the behavioral, molecular and neuronal mechanisms associated with the debilitating cerebellar defects present in individuals with Angelman syndrome.

**Materials and Methods**

**Selection of Genotypes for Analysis**

The mice used for analysis were obtained from Dr. Arthur L. Beaudet (Baylor College of Medicine, Houston, TX). These mice have previously been published by Jiang et al. and show a
clear rotarod deficits indicative of cerebellar defects (13). Genotyping is performed on this colony using newly designed PCR primers (P13=TGC ATC GCA TTG TCT GAG TAG GTG TC; 06U= CAA GTA TGG GCT CAA GGT TGT ATG C; 06L= TCA CTA CTA CCT CCC AAA GTG TTA G) to detect a 380bp product from the knock out chromosome and 420bp product from the wild type chromosome (Scott Dindot, personal communication). Although testing is often done on these mice using $Ube3a^{m-/p+}$ mice to represent the AS disease condition and $Ube3a^{m/+p-}$ mice to represent the normal control, these two genotypes must be generated by two separate crossing schemes, and as such, are not littermates. In order to directly compare behaviors in littermates that differ only in the presence of absence of a functional $Ube3a$ gene on one or both alleles, we compared both the $Ube3a^{m-/p-}$ and $Ube3a^{m/+}$ mice to their $Ube3a^{m+/p+}$ littermates that are also generated in these crosses. The mice on our colony have been crossed to c57BL/6 wild type mice two times for initial colony management and so, are less like the mixed 129/SvEv mice reported earlier. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Tennessee Health Science Center.

**Home Cage Licking Behavior**

Two-three month old $Ube3a^{m-/p-}$ ($N = 10$), $Ube3a^{m/+}$ ($N = 10$) and wild-type littermates ($N = 9$) of these mice were analyzed for natural licking behavior over a 72hr period in home cages. Cages have been modified using a device developed in Dr. Heck’s laboratory, which detects contact between the mouse’s tongue and the water spout (17). Briefly, a metal wire mesh (~10 x 15 cm) was placed under the waterspout to serve as an electrical reference. The tongue-to-water spout contact (while the mouse stands on the grid) results in a voltage signal of 0.1 – 1 Volt that reflects the junction potential between the water and the metal spout. The spout and the wire mesh are connected to the two poles of an analogue to digital converter (CED 1401, Cambridge Electronic Design, Cambridge, UK) and the voltage signals were digitized (1200 – 2000 Hz) and stored on hard disk. Lick events were analyzed off line using Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Statistical analysis of lick data was performed using SigmaStat software (Systat, San Jose, CA). Mice were kept singly and had full access to food and water during the entire test period.
Additional Behavioral Assessments
For Rotarod, raised beam and footprint analysis, three month-old *Ube3a* maternal-deficient (m-/p+) (female $N = 4$; male $N = 4$) and knock-out (m-/p-) (female $N = 4$; male $N = 4$) mice, along with littermate controls (m+/p+) (female $N = 6$; male $N = 3$), were used for quantitative analyses of motor function. Additional mice from each genotype were tested in the rope climbing and grip strength assays to achieve statistical significance of the results (m+/p+ $N = 10$, m-/p+ $N = 8$ and m-/p- $N = 6$).

**Rotarod performance**
Mice were acclimated to a Rotamex-5 rotarod (Columbus Instruments, Columbus, OH, USA) rotating at 5 rpm for 5 min one day prior data acquisition. The assessment began at 1 rpm and accelerated at a speed of 1 rpm / 5 s to a maximum speed of 50 rpm. Mice were given five trials at the same time each day for 5 consecutive days. Median values were used for statistical comparison.

**Raised-beam task**
Mice were acclimated to an 80-cm long, 20-mm wide beam elevated 50 cm above a padded base. A 60W lamp at the start served as an aversive stimulus, whereas the opposite end of the beam entered a darkened escape box. Transversal time and number of slips were measured as mice traversed the beam. After initial testing with a 20-mm diameter square beam, mice were given follow-up tests using supplementary round (8-mm, 10-mm and 12-mm diameter) and square (8-mm and 12-mm diameter) beams. All testing was performed in triplicate and median values were used for subsequent statistical analyses.

**Footprint analysis**
Mouse paws were dipped in nontoxic water-based paints (forepaws in green and hind-paws in red paint). Mice were then allowed to walk down an enclosed runway lined with white paper. Three trials were performed on three separate days within one week. Two to four steps from the middle portion of each run were measured for (1) hind-stride length, (2) fore-base width (the distance between the right and left forelimb strides) and (3) hind-base width (the distance
between the right and left hind-limb strides). At least nine steps were measured for each mouse. Mean values were used for statistical analysis.

Vertical rope climb

Mice were acclimated to a vertical, 40-cm long, 10-mm thick nylon rope prior to testing. The bottom of the rope was suspended 15-cm above a padded base and the top entered into a darkened escape box. Five trials with a 5-min inter-trial interval were completed for each mouse. Median times were used for statistical analysis.

Grip strength analysis

To measure grip strength, the mice were held by the scruff of the neck with one hand and the base of the tail with the other hand. The mice were then free to grasp a metal grid attached to a Columbus instruments force meter (Columbus Instruments, Columbus, OH) as they were moved along the axis of the grid. Maximal strength (grams) with which mice pulled the grid was measured in triplicate trials with a minimal inter-trial interval of 5 min.

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References


Figure Legends

Figure 1. Quantitative analysis of fluid consumption and licking behavior in wild type and \textit{Ube3a} deficient mice. (A) The mean inter-lick intervals were significantly longer in both groups of \textit{Ube3a} deficient mice (dark grey bar, m-/p-, light grey bar, m-/p+) compared to wild type (black bar, m+/p+). (B) All three groups also differed significantly in the number of licks generated per visit to the water spout. Both groups of \textit{Ube3a} deficient mice generated significantly more licks than wild type mice and knock out mice licked significantly more often than maternal heterozygotes. Error bars are standard error of the mean (SEM). Statistical significance was determined with the Mann-Whitney U test. Number of animals tested: m+/p+: \textit{N}=9, m-/p+: \textit{N}=10, m-/p-: \textit{N}=10.

Figure 2. Rotarod analysis of motor functioning in wild type and \textit{Ube3a} deficient mice.
Eight mice from all three genotypes were tested on an accelerating rotarod with the same destination speed for five consecutive days. The latencies from rotation onset until the mice fell off the rod were measured. Wild type mice (black circles) managed to stay significantly longer on the accelerating rotarod than \textit{Ube3a} $m-/p+$ (light grey inverted triangles) and \textit{Ube3a} $m-/p-$ (grey squares) mice. Differences between \textit{Ube3a} $m-/p+$ vs wild type littermates ($p < 0.05$) and between \textit{Ube3a} $m-/p-$ vs wild type littermates ($p < 0.05$) were significant for all data points in a 1-way ANOVA test. Each data point represents the average across 8 mice. Error bars are SEM.

Figure 3. Footprint assay of gait abnormalities in wild type and \textit{Ube3a} deficient mice. A) Examples of footprint assays from each of the three genotypes. Forepaws were marked with green ink, hind paws with red ink. The stride length was measured using the left hind paw, while the stride width was measured using both forepaw and hind paw widths. B) The stride length and the hind-base width of \textit{Ube3a} $m-/p+$ (light grey bars) and \textit{Ube3a} $m-/p-$ (dark grey bars) mice were significantly longer compared to \textit{Ube3a} $m+/p+$ (black bars). Significant differences and $p$-values (1-way ANOVA test) are indicated by brackets. Error bars are SEM.
Figure 4. Quantitative analysis of grip strength, vertical rope climbing, and raised beam task in wild type and Ube3a deficient mice. A) Ube3a$^{m/p_-}$ mice (dark gray bars) needed significantly more time to cross the 80-mm raised square beams of all three different widths (20, 12, and 8-mm) than did wild type (black bars) or Ube3a$^{m/p_+}$ mice (light gray bars). B) Ube3a$^{m/p_-}$ mice (dark gray bars) needed significantly more time than wild type or Ube3a$^{m/p_+}$ mice to cross the 80-mm raised round beam of 12-mm diameter. There were no significant differences between the three genotypes for round beam diameters 10 and 8 mm. C) The vertical rope climbing assay revealed significant differences between both Ube3a deficient genotypes and wild type animals. Ube3a deficient mice climbed significantly slower than wild type animals. Ube3a deficient mice were also resistant to climbing and some could not be trained, resulting in abnormally large error bars for the measurements in Maternal Heterozygotes ($N_{M-het} = 16$; light gray bar) and Ube3a null mice ($N_{KO} = 15$; dark gray bars). D) Grip strength analysis revealed significantly reduced grip strengths in Ube3a$^{m/p_+}$ mice and Ube3a$^{m/p_-}$ mice compared to wild type animals. There was no significant difference in grip strengths between Ube3a$^{m/p_+}$ and Ube3a$^{m/p_-}$. Each data point represents the average across all mice within each group ($N_{WT} = 23$; $N_{M-Het} = 18$; $N_{KO} = 13$). Error bars are SEM. Significant differences and p-values (Mann-Whitney U statistical test) are indicated by brackets.

Figure 5. The magnitude of the quantitative differences in licking behavior correspond to the degree of loss of Ube3a. A) In the wild type mouse the maternal Ube3a allele is expressed everywhere in the brain with enhanced expression in the hippocampus and cerebellum. B) Loss of the maternal allele will result in complete loss of maternal Ube3a expression but low-level expression of the paternal allele remains throughout the brain. C) In the knock out mice Ube3a expression is completely eliminated from all brain areas, even those where Ube3a expression is normally low. The differences in Ube3a expression in wild type mice, maternal heterozygotes and null mice are directly correlated with the quantitative differences in the licks-per-visit phenotype between these three genotypes. The cerebellum is the brain structure most likely to be responsible for the phenotype in Ube3a$^{m/p_+}$ mice. Complete loss of Ube3a likely amplifies the phenotype because the complete loss of expression eliminates residual function in the cerebellum and in cerebellar projection areas in the rest of the brain.
A

Mean lick interval (msec)

- m+/p+
- m-/p+
- m-/p-

B

Licks / visit

- p < 0.001
- p < 0.001
- p < 0.001

Legend:
- m+/p+: Black
- m-/p+: Light gray
- m-/p-: Dark gray
Abbreviations

Angelman syndrome = AS
Functional magnetic resonance imaging = fMRI