

# Chapter 5

## ***Ube3a* reinstatement identifies distinct developmental windows in a murine Angelman syndrome model**

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## Abstract

Angelman syndrome (AS) is a severe neurodevelopmental disorder that results from loss of function of the maternal ubiquitin protein ligase E3A () allele. Due to neuron-specific imprinting, the paternal copy is silenced. Previous studies in murine models have demonstrated that strategies to activate the paternal allele are feasible; however, a recent study showed that pharmacological gene reactivation in adulthood failed to rescue the majority of neurocognitive phenotypes in a murine AS model. Here, we performed a systematic study to investigate the possibility that neurocognitive rescue can be achieved by reinstating during earlier neurodevelopmental windows. We developed an AS model that allows for temporally controlled Cre-dependent induction of the maternal allele and determined that there are distinct neurodevelopmental windows during which restoration can rescue AS-relevant phenotypes. Motor deficits were rescued by reinstatement in adolescent mice, whereas anxiety, repetitive behavior, and epilepsy were only rescued when reinstated during early development. In contrast, hippocampal synaptic plasticity could be restored at any age. Together, these findings suggest that reinstatement early in development may be necessary to prevent or rescue most AS-associated phenotypes and should be considered in future clinical trial design.

## Introduction

Children with AS are typically diagnosed within the first year of life, due to developmental delay. The most prominent symptoms include motor impairments, epilepsy, intellectual disability, and absence of speech (1). AS is caused by loss of function of the maternally inherited *UBE3A* allele. In neurons, the maternally inherited *UBE3A* allele is the only active allele, since the paternally inherited *UBE3A* allele is silenced through cell type-specific imprinting. This imprinting results in the allele-specific and neuronally restricted expression of a large antisense RNA transcript (*UBE3A-ATS*), which selectively interferes with paternal *UBE3A* transcription through a *cis*-acting mechanism (2–6).

There is currently no effective treatment for AS, but the unique silencing mechanism of the paternal *UBE3A* allele holds great promise for developing novel therapeutic strategies. Two recent studies have shown that the paternal *UBE3A* allele can be pharmacologically reactivated (7, 8), which offers a unique molecular target with high clinical potential for the treatment of AS.

Notably, however, activation of the paternal *Ube3a* gene in adulthood appears insufficient to rescue the majority of neurocognitive phenotypes in the AS mouse model. The failure of phenotypic rescue in adult AS mice might have resulted from the incomplete reinstatement of *UBE3A* expression (35%–47% of WT levels) or, alternatively, because a neurocognitive rescue by *UBE3A* reinstatement requires early therapeutic intervention (8). Hence, for such a treatment strategy to be successful in the clinic, it is imperative to know whether there is a critical time window during which a disease-modifying therapy would be effective. This is particularly relevant for early-onset disorders, such as AS, whose causative gene is highly expressed in developing neural circuits.

Despite extensive knowledge of critical periods for the development of sensorimotor networks, much less is known about the critical periods for complex behaviors in neurodevelopmental disorders (9, 10). An inducible mouse model for Rett syndrome showed that adult activation of the *Mecp2* gene could rescue behavioral alterations and synaptic plasticity deficits, suggesting a broad window of therapeutic opportunity (11). In contrast, adult reactivation of the *Syngap1* gene in a mouse model of intellectual disability and autism did not reverse any of the core behavioral deficits related to anxiety and behavioral flexibility (12). However, to our knowledge, no previous study has been performed to systematically investigate the influence of critical developmental periods on

the ability to rescue disease-relevant behavioral phenotypes. Here, we explore the effect of gene reactivation across multiple developmental windows in a novel mouse model for AS. Our results demonstrate an essential role for *Ube3a* in neurodevelopment and define a critical period for therapeutic intervention during which *Ube3a* gene reactivation ameliorates the neurocognitive impairments of AS model mice

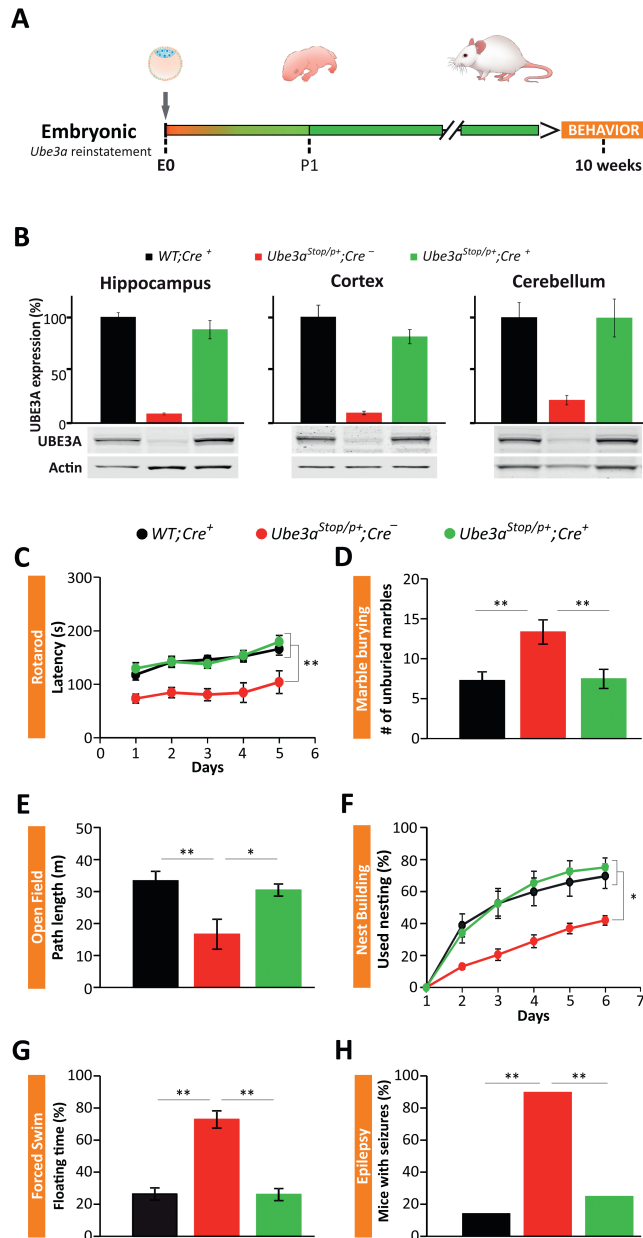
## Results

### *Generation and characterization of a conditional Ube3a mutant.*

To examine whether the therapeutic benefit of *Ube3a* gene reactivation is dependent upon the developmental stage at which gene expression is restored, we generated a conditional AS mouse model to allow temporally controlled reactivation of the *Ube3a* gene upon Cre-mediated deletion of a floxed transcriptional stop cassette inserted within intron 3 by homologous recombination (*Ube3a*<sup>Stop/p+</sup>) (Supplemental Methods and Supplemental Figure 1)

We first investigated the efficiency of the transcriptional stop cassette in blocking *Ube3a* expression. Female *Ube3a*<sup>Stop/p+</sup> mice were crossed to a constitutive Cre-expressing line with an early embryonic onset of recombination (ref. 13 and Figure 1A). Because the paternal *Ube3a* allele is epigenetically silenced, mice with a maternally inherited stop cassette without Cre expression (*Ube3a*<sup>Stop/p+</sup>/*Cre*<sup>-</sup> mice) showed a severe loss of UBE3A protein (also known as E6-associated protein [E6AP]), comparable to the reduction in UBE3A expression observed in the traditional AS mouse model with a maternally inherited deletion of *Ube3a* (*Ube3a*<sup>m-/p+</sup>) (Figure 1B; see also Figure 2D for a comparison to *Ube3a*<sup>m-/p+</sup> mice). Immunohistochemical staining of *Ube3a*<sup>Stop/p+</sup>/*Cre*<sup>-</sup> brain slices was indistinguishable from that of *Ube3a*<sup>m-/p+</sup> mouse samples (Supplemental Figure 1B). These results confirm that the floxed stop cassette is highly effective in blocking transcription of the maternal *Ube3a* allele, while preserving the normal epigenetic silencing of the paternal allele.



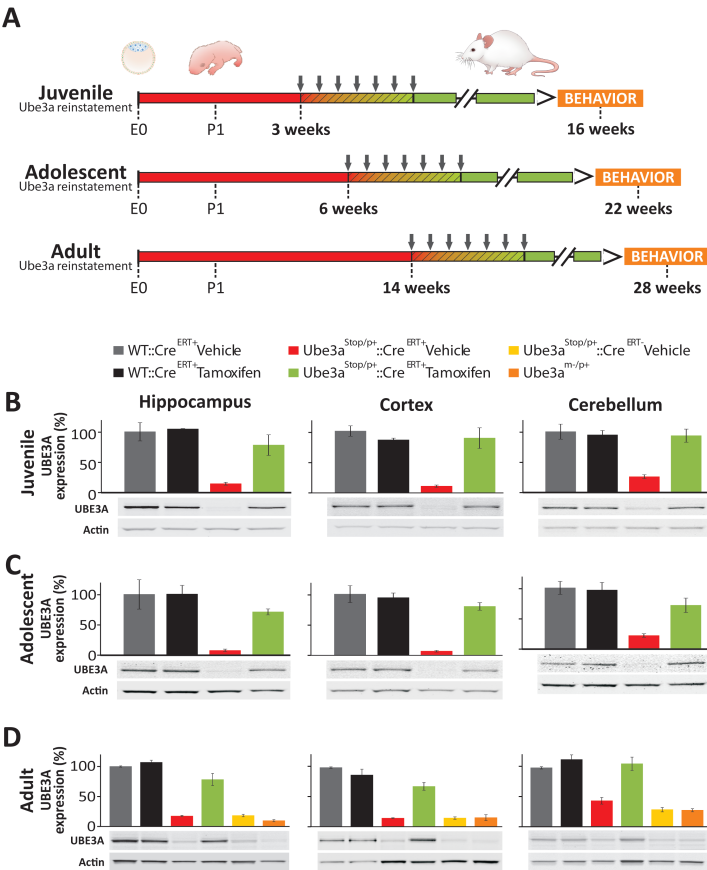


**Figure 1: Embryonic reactivation of *Ube3a* expression rescues AS-like behavioral phenotypes.** (A) Schematic representation of *Ube3a* reactivation (indicated by the gray arrow) during mouse embryonic development and time point of behavioral testing. (B) Western blot analysis of hippocampus (n = 4 per genotype), cortex (n = 5), and cerebellum (n = 5) from *Ube3a*<sup>Stop/p+</sup> and WT littermates crossed with an embryonically active Cre line. (C–H) *Ube3a*<sup>Stop/p+;Cre-</sup> mice show robust behavioral AS-relevant phenotypes, which can be fully rescued by embryonic reactivation of the *Ube3a* gene in the *Ube3a*<sup>Stop/p+;Cre+</sup> mice. Number of mice (WT;Cre<sup>+</sup>/*Ube3a*<sup>Stop/p+;Cre-</sup>/*Ube3a*<sup>Stop/p+;Cre+</sup>): accelerating rotarod, n = 14/8/17; marble burying test, n = 24/18/28; open field test, n = 14/8/17; nest building test, n = 7/7/7; forced swim test, n =

14/8/17; epilepsy test, n = 7/10/8. All data represent mean  $\pm$  SEM. ANOVA with genotype as independent variable was used for statistical comparisons. A significant effect of genotype was identified in all behavioral tests (see Supplemental Table 1). \*P < 0.05, \*\*P < 0.01; Bonferroni's post hoc analysis.

*Embryonic reactivation of Ube3a expression rescues AS-like behavioral phenotypes.*

Next, we investigated the efficiency of *Ube3a* reactivation upon Cre-mediated deletion of the floxed stop cassette. UBE3A protein levels were reinstated in *Ube3a*<sup>Stop/p+;Cre+</sup> mice to 89% of WT levels in the hippocampus, 82% in the cerebral cortex, and 99% in the cerebellum. Furthermore, the subcellular distribution of UBE3A was indistinguishable between *Ube3a*<sup>Stop/p+;Cre+</sup> and WT;Cre<sup>+</sup> mice, validating the functionality of the *Ube3a* reactivation method (Figure 1B and Supplemental Figure 1B).



**Figure 2: Molecular analysis of *Ube3a*<sup>Stop/p+;Cre<sup>ERT</sup></sup> mice reveals successful reactivation of the maternal *Ube3a* gene upon tamoxifen induction. (A) Schematics representing *Ube3a* reactivation achieved by tamoxifen treatment (gray arrows) in each experimental group. (B–D) *Ube3a*<sup>Stop/p+;Cre<sup>ERT</sup></sup> mice with**

postnatally induced gene reactivation express UBE3A at levels comparable to those achieved with early embryonic gene reactivation in hippocampus (juvenile,  $n = 3$  per genotype; adolescent,  $n = 4$  per genotype; adult,  $n = 4$  per genotype), cortex (juvenile,  $n = 3$  per genotype; adolescent,  $n = 4$  per genotype; adult,  $n = 3-4$  per genotype), and cerebellum (juvenile,  $n = 3$  per genotype; adolescent,  $n = 4$  per genotype; adult,  $n = 5$  per genotype). Data represent mean  $\pm$  SEM.

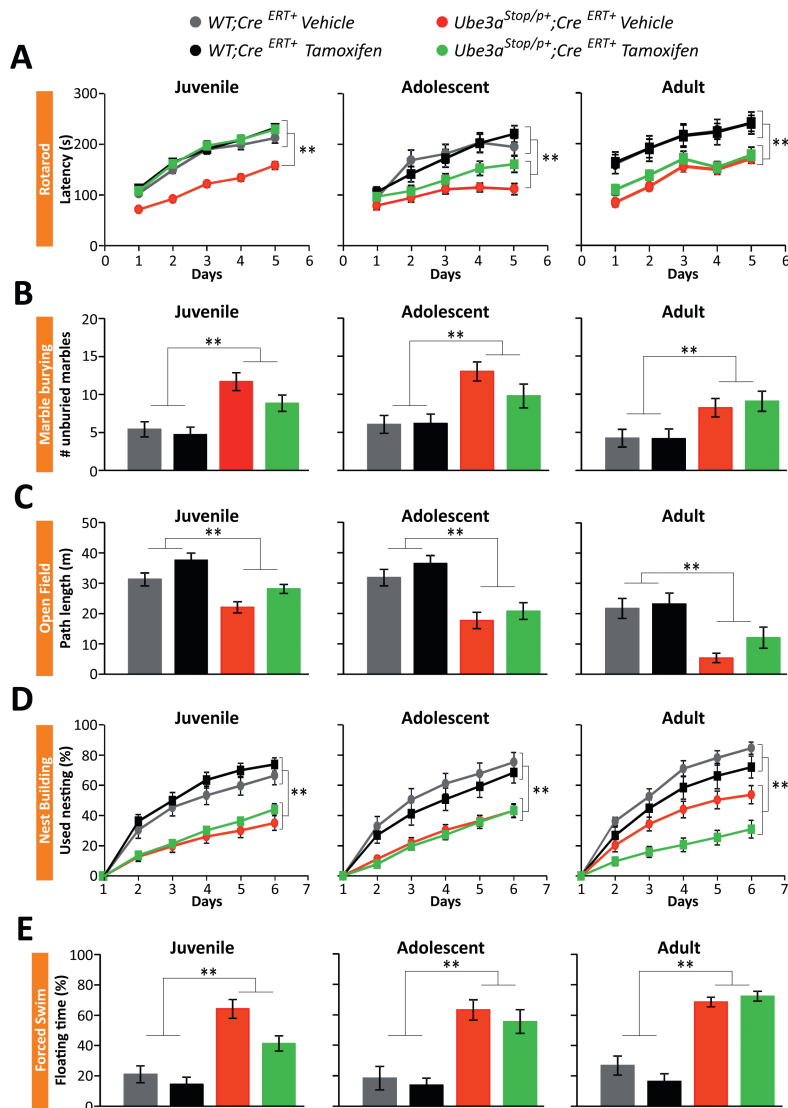
### *Early embryonic gene reactivation prevents the manifestation of AS phenotypes.*

Impaired motor coordination, autistic traits, anxiety, and epilepsy are hallmarks of AS patients, for which analogous phenotypes are well established in AS model mice (3, 14–16). As expected based on the loss of *Ube3a* expression, *Ube3a*<sup>Stop/p+;Cre-</sup> mice exhibited significant alterations in rotarod performance (Figure 1C), marble burying (Figure 1D), open field exploration (Figure 1E), nest building (Figure 1F), and audiogenic seizure threshold (Figure 1H), all of which are also present in the classical *Ube3a*<sup>m-/p+</sup> mouse model of AS (3,14). In addition, we identified a highly robust phenotype in the forced swim test present in both the classical *Ube3a*<sup>m-/p+</sup> mutant (data not shown) and the conditional *Ube3a*<sup>Stop/p+</sup> AS mouse model (Figure 1G).

Consistent with the therapeutic potential of *Ube3a* gene reactivation, *Ube3a*<sup>Stop/p+;Cre+</sup> mice exhibited a full rescue of all of these neurological and behavioral abnormalities, confirming that embryonic reactivation of UBE3A protein expression is sufficient to prevent the manifestation of AS phenotypes across multiple domains (Figure 1 and Supplemental Table 1).

### *Gene reactivation in juvenile, adolescent, and adult animals reveals the presence of distinct critical periods.*

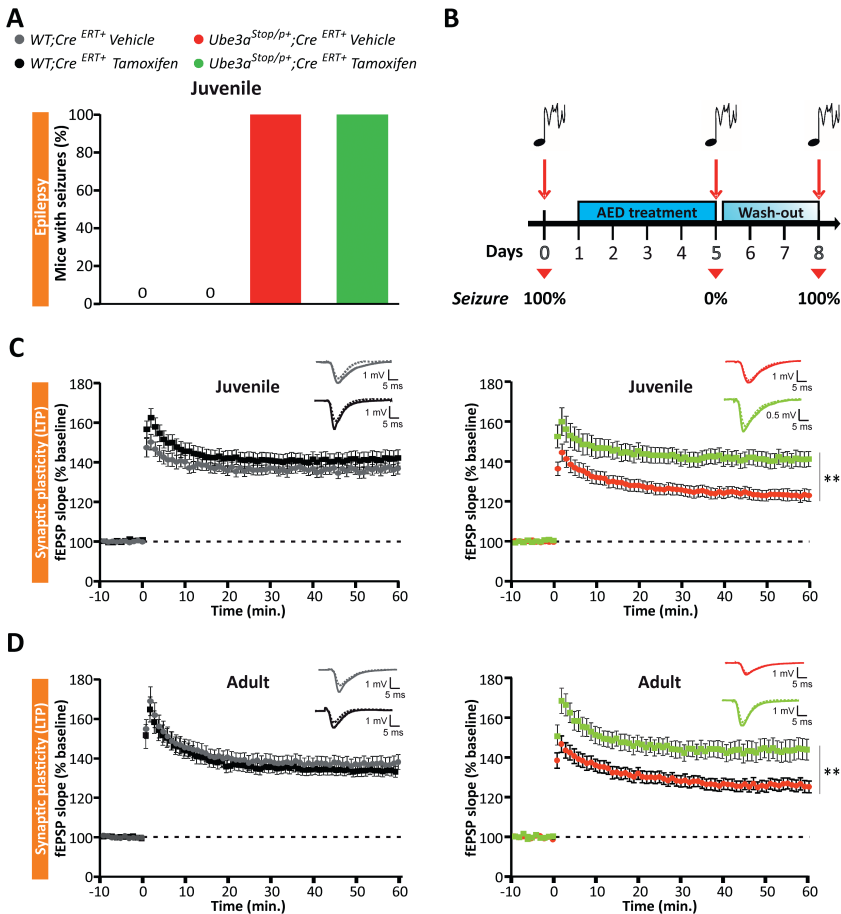
We next crossed the *Ube3a*<sup>Stop/p+</sup> mice with a tamoxifen-inducible *Cre*<sup>ERT+</sup> mouse line (17) to determine the efficacy of *Ube3a* reactivation at later stages of postnatal development. In particular, we induced *Ube3a* gene reactivation at 3 weeks (“juvenile mice”), 6 weeks (“adolescent mice”), and 14 weeks of age (“adult mice”), with behavioral testing performed at a mean age of 16 weeks, 22 weeks, and 28 weeks, respectively (Figure 2A). Across these developmental time points, UBE3A protein levels in tamoxifen-treated *Ube3a*<sup>Stop/p+;CreERT+</sup> mice were reinstated to 70%–100% of wild-type levels, which is comparable to those achieved by early embryonic reactivation (Figure 2, B–D, Supplemental Figure 2, and Figure 1B). Importantly, UBE3A expression in vehicle-treated *Ube3a*<sup>Stop/p+;CreERT+</sup> mice was also similar to that observed in *Ube3a*<sup>Stop/p+;Cre-</sup> and *Ube3a*<sup>m-/p+</sup> mice (Figure 2D and Supplemental Figure 3), demonstrating the tight control of gene reactivation.



**Figure 3: Postnatal reactivation of *Ube3a* expression reveals a critical period for behavioral rescue.** (A–E) Behavioral testing of *Ube3a*<sup>Stop/p+</sup> and WT littermates treated with either vehicle (Veh.) or tamoxifen (Tamox.) shows distinct critical periods for recovery of the behavioral deficits. Numbers of mice (WT;Cre<sup>ERT+</sup> Veh./ WT;Cre<sup>ERT+</sup> Tamox./*Ube3a*<sup>Stop/p+</sup>;Cre<sup>ERT+</sup>Veh./*Ube3a*<sup>Stop/p+</sup>;Cre<sup>ERT+</sup> Tamox.): (A) Accelerating rotarod after juvenile (n = 22/20/22/22), adolescent (n = 11/11/10/11), and adult (n = 11/9/12/13) gene reactivation. (B) Marble burying test after juvenile (n = 21/20/20/20), adolescent (n = 20/20/21/23), and adult (n = 13/11/14/15) gene reactivation. (C) Open field test after juvenile (n = 21/21/22/22), adolescent (n = 11/11/10/11), and adult (n = 10/8/9/10) gene reactivation. (D) Nest building test after juvenile (n = 12/13/14/13), adolescent (n = 14/13/16/17), and adult (n = 9/8/9/8) gene reactivation. (E) Forced swim test after juvenile (n = 17/19/18/19), adolescent (n = 11/11/10/11), and adult (n = 11/8/9/9) gene reactivation. All data represent mean ± SEM. Two-way ANOVA or repeated-measures 2-way ANOVA with genotype and treatment as independent variables was used for statistical comparisons. A significant effect of genotype was identified in all behavioral tests (see Supplemental Table 2). \*\*P < 0.01 for genotype significance.

Juvenile reactivation of *Ube3a* resulted in a full rescue of the motor coordination deficit. In contrast, adolescent reactivation only partially rescued motor coordination, while no improvement was observed with adult reactivation (Figure 3A). Together, these findings identify a critical period for *Ube3a*-dependent motor development, which closes between 3 and 6 weeks postnatally.

### Postnatal reactivation of *Ube3a* expression reveals a critical period for behavioral rescue.



**Figure 4: *Ube3a* reactivation in juvenile animals does not recover epilepsy susceptibility, but Schaffer collateral-CA1 LTP is fully recovered.** (A) Epilepsy susceptibility in *Ube3a*<sup>Stop/p+</sup>;Cre<sup>ERT+</sup> mice persists after *Ube3a* gene reactivation at a juvenile age (n = 8 mice/group). (B) The induction of tonic-clonic seizures induced by audiogenic stimulation (indicated by the red arrows) is efficiently treated by administration of AEDs (blue rectangle illustrates treatment administration and wash-out period) in adult *Ube3a*<sup>Stop/p+</sup>;Cre<sup>ERT+</sup> (n = 2) and *Ube3a*<sup>m-/p+</sup> (n = 12) mice. Seizures reappeared 3 days after cessation of treatment. Percentages indicate the amount of mutant mice that developed seizures upon audiogenic stimulation (see also

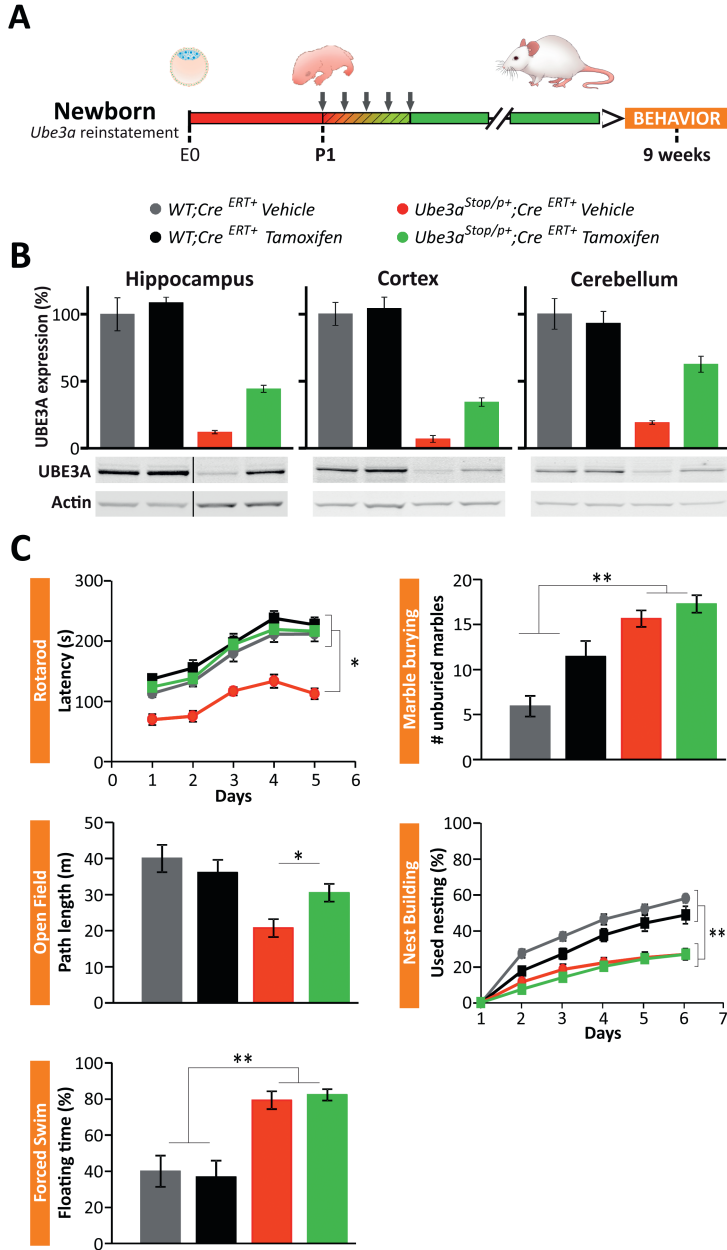
Supplemental Methods for more experimental details). Hippocampal plasticity deficit as measured by LTP in mutant mice is ameliorated upon gene reactivation at both (C) juvenile and (D) adult ages. Data represent mean  $\pm$  SEM. Two-way ANOVA with genotype and treatment as independent variables was used for statistical testing. All tests showed a significant effect of genotype (see also Supplemental Table 2 for statistical comparisons). Number of slices/mouse used: juvenile reactivation: WT;Cre<sup>ERT+</sup> Veh. (n = 16/4), WT;Cre<sup>ERT+</sup> Tamox. (n = 25/4), Ube3a<sup>Stop/p+;CreERT+</sup> Veh. (n = 22/4), Ube3a<sup>Stop/p+;CreERT+</sup> Tamox. (n = 22/5); adult reactivation: WT;Cre<sup>ERT+</sup> Veh. (n = 18/6), WT;Cre<sup>ERT+</sup> Tamox. (n = 37/8), Ube3a<sup>Stop/p+;CreERT+</sup> Veh. (n = 23/4), Ube3a<sup>Stop/p+;CreERT+</sup> Tamox. (n = 15/4). \*\*P < 0.01.

The critical window for rescuing motor coordination deficits was distinct from the window for rescuing autism- and anxiety-related phenotypes such as the marble burying task, open field test, nest building test, and forced swim test, which could be rescued by embryonic reactivation (Figure 1, D–G, and Supplemental Table 1) but not upon juvenile, adolescent, or adult reactivation (Figure 3, B–E, and Supplemental Table 2).

The epilepsy phenotype was also refractory to postnatal *Ube3a* reactivation, as seizures persisted despite gene reactivation at a juvenile age (Figure 4A). Next, we sought to confirm whether the epilepsy phenotype in AS mice is differentially responsive to treatment with antiepileptic drugs (AEDs) in mice with *Ube3a* reactivation. Adult (>8 weeks) *Ube3a*<sup>m-/p+</sup> and *Ube3a*<sup>Stop/p+;CreERT-</sup> mice were treated for 5 days with either valproate or clonazepam using a within-subjects design. Prior to treatment, every AS mouse examined showed audiogenic seizures (Figure 4B). However, after 5 days of treatment with either of these AEDs, seizures were completely prevented in all mice. Moreover, 3 days after the cessation of AED treatment (wash-out period), all AS mice again showed audiogenic seizures (Figure 4B). These data confirm that seizures can be successfully treated in adult AS mice using conventional AEDs, but that they are nevertheless insensitive to postnatal *Ube3a* reactivation.

### *Ube3a reactivation in juvenile animals does not recover epilepsy susceptibility, but Schaffer collateral–CA1 LTP is fully recovered.*

To investigate the extent to which postnatal *Ube3a* reactivation is able to rescue electrophysiological phenotypes, we measured hippocampal long-term potentiation (LTP), a form of synaptic plasticity required for experience-dependent neurodevelopment. Intriguingly, we observed full recovery of hippocampal LTP following *Ube3a* gene reactivation at all time points examined (Figure 4, C and D, and Supplemental Table 2), indicating the absence of a critical period window for rescue of this important cellular phenotype.



**Figure 5: Partial reactivation of *Ube3a* expression during the first postnatal week attenuates the motor coordination and open field deficits.** (A) Schematics representing *Ube3a* reactivation achieved by tamoxifen administration (gray arrows) to the lactating dams starting on the day of delivery. (B) Western blot analysis of UBE3A expression in hippocampal (n = 4 per genotype), cortical (n = 5), and cerebellar (n = 5) tissues of mutant mice and their WT littermates. The thin black lines on the hippocampus blot indicate noncontiguous samples run on the same gel. (C) Rescue of the accelerating rotarod and open field impairments. Number of mice used (WT;Cre<sup>ERT+</sup> Veh./WT;Cre<sup>ERT+</sup> Tamox./*Ube3a*<sup>Stop/p+</sup>;Cre<sup>ERT+</sup> Veh./*Ube3a*<sup>Stop/p+</sup>;Cre<sup>ERT+</sup> Tamox.) for accelerating rotarod, marble burying test, open field test, and forced swim test: n =

14/12/14/11; for nest building,  $n = 12/12/12/11$ . All data represent mean  $\pm$  SEM. Two-way ANOVA or repeated-measures 2-way ANOVA with genotype and treatment as independent variables was used for statistical comparisons. A significant effect of genotype was identified in all behavioral tests (see Supplemental Table 2 for statistical comparisons). \* $P < 0.05$ ; \*\* $P < 0.01$ .

### *Partial gene reactivation in newborn animals rescues the motor coordination and open field phenotype.*

Our data suggest that UBE3A is required during a critical period between early embryogenesis and the third postnatal week in order to prevent a wide spectrum of AS-like deficits, with the notable exception of defects in motor coordination and hippocampal LTP. To further refine the critical period window, we next induced gene reactivation immediately following birth by administering tamoxifen to lactating dams (Figure 5A). The efficacy of this method of tamoxifen administration was reduced compared with direct treatment of offspring, yielding 44%, 34%, and 63% of WT UBE3A levels, respectively, in the hippocampus, cortex, and cerebellum (Figure 5B). Notably, however, even with a reduced level of UBE3A reactivation, motor coordination was entirely rescued and performance in the open field test was significantly improved (Figure 5C). However, AS-like deficits persisted in the other behavioral paradigms, suggesting that the *Ube3a*-dependent neurodevelopmental critical period for autism-related phenotypes in AS might not extend significantly beyond birth. Alternatively, given that we achieved only partial reactivation during the neonatal period, it remains distinctly possible that functional plasticity may extend beyond 3 weeks of age but is only evident with a higher efficiency of *Ube3a* reactivation.

## Discussion

Our results demonstrate an essential role for *Ube3a* in neurodevelopment and define critical periods during which *Ube3a* gene reactivation can ameliorate AS-like phenotypes. In particular, the window for improving motor coordination extends furthest into postnatal development, whereas the autism- and anxiety-related phenotypes appear to be established much earlier. In contrast, at the cellular level, there appears to be no critical window for reversing plasticity deficits. The finding that LTP could be fully recovered at all ages is consistent with previous findings showing that the hippocampal LTP deficit in adult AS mice is reversible upon acute pharmacological treatment with an ErbB inhibitor



or with ampakine cognitive enhancers (18, 19). However, expression of UBE3A in adult *Ube3a<sup>m-/p+</sup>* mice through a viral-mediated approach only partially recovered synaptic plasticity (20). This apparent discrepancy likely reflects the limited efficiency of in vivo virally mediated neuronal transduction, compared with the more homogeneous biodistribution of systemically administered pharmacological compounds.

Importantly, and consistent with our findings, to our knowledge no studies in AS mice have demonstrated the successful rescue of the behavioral phenotypes related to anxiety and repetitive behavior upon adult treatment, despite multiple efforts using a variety of different interventions. In addition, it is notable that the behavioral deficits related to anxiety and behavioral flexibility in the *Syngap1* mutant mouse model for intellectual disability were also not rescued by adult reactivation of *Syngap1* gene expression (12). These findings could suggest that the window during which gene activation can ameliorate autism-related phenotypes closes early in neurodevelopment. Importantly, however, these observations do not exclude the possibility that directly targeting downstream signaling pathways could have a broader window for therapeutic intervention (21). In fact, this possibility is very well demonstrated by the highly effective intervention of AEDs in preventing audiogenic seizures, in contrast to the failure of postnatal gene reactivation to alter the susceptibility to audiogenic seizures. Therefore, at least for seizure susceptibility, the therapeutic benefit of restoring the etiological loss of UBE3A was inferior to that achieved by targeting downstream mechanisms, in this case with AEDs. This finding strongly supports investigation of the targets of UBE3A and their downstream signaling pathways in order to develop drugs that can be applied as part of a complementary therapeutic strategy.

We believe that our results will be important for informing future AS clinical trials regarding the critical period for therapeutic intervention. However, there are two important limitations of our study. First, although our behavioral experiments were performed in an isogenic F<sub>1</sub> hybrid background of 129/Sv and C57BL/6 mice, we cannot exclude an effect of the many heterozygous mutations that are contributed by each of these inbred strains, such as the *Disc1* mutation, which is common to all 129/Sv substrains (22, 23). Such mutations may interact with the *Ube3a* mutation and interfere with the ability to obtain a behavioral rescue. To minimize such confounding effects, we included matched littermate control groups for all experiments performed. Moreover, we selected only

those behaviors that consistently exhibited a robust and reliably reproducible phenotype across all experiments and for which we have demonstrated that a full rescue could be obtained upon early embryonic gene reactivation. A second translational limitation of our study is the obviously profound difference in brain development and systems-level functioning between mice and humans. Whereas a 3-week-old mouse can take care for itself and adult maturity is complete by 6–8 weeks of age, humans have a very extended childhood even compared with other primates. Therefore, it remains highly uncertain how and to what extent the precise critical period windows we have identified can be translated to humans. A recent comprehensive comparative study of early brain maturation across multiple mammalian species estimated that the extent of brain maturation observed in a 3-week-old mouse pup is comparable to that in a 2-year-old human infant (24). Regarding critical period windows, among the most well-studied examples is ocular dominance plasticity. In mice, the critical period for acquiring binocular vision closes by 4 weeks of age. However, in humans this extends until approximately 7 years of age (25). Therefore, the window of therapeutic opportunity in human AS patients is likely to be much longer than in mice, and this may offer some reason for optimism that gene reactivation could be more effective in humans than we have observed in mice. However, regardless of the precise conversion of the developmental time scales, our studies suggest that early intervention is very likely to determine the extent to which gene reactivation is therapeutically effective.

In addition to demonstrating an important developmental role for UBE3A, our study provides a notable contrast to a similar study of gene reactivation therapy in Rett syndrome (11), another neurodevelopmental imprinting disorder that is clinically reminiscent of AS. Whereas we demonstrate that adult reactivation of *Ube3a* is only minimally efficacious as a therapeutic intervention in AS, adult reactivation of *Mecp2* appears to be highly effective for the treatment of Rett syndrome (11). This distinction not only emphasizes the unique neurodevelopmental requirements for *Ube3a* and *Mecp2*, but also illustrates the importance of systematically investigating disease-specific preclinical models, no matter how phenotypically similar, when the goal is to accurately inform therapeutic discovery and human clinical trials.

## Methods

### Mice

For all behavioral experiments except the epilepsy test, we crossed female *Ube3a*<sup>Stop/p+</sup> mice (in the 129S2/SvPasCrl background; Charles River) with either *TgCAG-Cre* mice (13) (herein referred as *Cre*<sup>+</sup> mice) or with *Tg(CAG-cre/Esrl\*)5Amc/J* (The Jackson Laboratory) (17) (herein referred as *Cre*<sup>ERT+</sup>), both kept in the C57BL/6J background (Charles River), to generate heterozygous *Ube3a*<sup>Stop/p+;Cre+</sup> and *Ube3a*<sup>Stop/p+;CreERT+</sup> mutants and littermate controls in the F<sub>1</sub> hybrid 129S2-C57BL/6 background.

### Tamoxifen treatment

One-day to 8-month-old *Ube3a*<sup>Stop/p+</sup> mice and their WT littermates (both sexes) were used in this study. *Ube3a*<sup>Stop/p+;CreERT+</sup> mutants and WT mice were given tamoxifen to induce Cre-mediated deletion of the stop cassette. Tamoxifen (Sigma-Aldrich) was diluted in sunflower oil at a concentration of 20 mg/ml. Each mouse received 0.10 mg tamoxifen per gram body weight, by daily i.p. injection. The control groups were treated with daily i.p. injections of sunflower oil (vehicle). The newborn group received tamoxifen through the milk of the mother, who received daily i.p. injections of tamoxifen for 5 consecutive days starting at the day of delivery. Juvenile, adolescent, and adult groups received 7 daily i.p. injections of tamoxifen.

### Behavioral analysis

All behavioral experiments were performed during the light period of the cycle. The experimenter remained blind to the genotype and treatment until final statistical analysis. For the accelerating rotarod test, mice were given two trials per day with a 45- to 60-minute inter-trial interval for 5 consecutive days. The maximum duration of a trial was 5 minutes. For the marble burying test, clean Makrolon cages (50 × 26 × 18 cm) were filled with bedding material at 4 cm thickness and 20 glass marbles, which were arranged in an equidistant 5 × 4 grid. Animals were given access to the marbles for 30 minutes. Marbles covered for more than 50% by bedding were scored as buried. For the open field test, mice were placed in a brightly lit 120-cm-diameter circular open field for 10 minutes. For the nest building test, mice were singly housed for a period of 5–7 days before the start of the experiment. Subsequently, 12 g extra-

thick filter paper (Bio-Rad) was added to the cage, and the unused nesting material was weighed for 5 consecutive days. From this, the percentage of used nesting material was determined. For the forced swim test, mice were placed for 6 minutes in a cylindrical transparent tank (18 cm diameter) with water (at  $26^{\circ}\text{C} \pm 1^{\circ}\text{C}$ ). The duration of immobility was assessed during the last 4 minutes of the test. For the epilepsy test, we used mice in the 129/Sv background, since epilepsy susceptibility in AS mice is dependent on the genetic background (16). Audiogenic seizures were induced by vigorously scraping scissors across the metal grating of the cage lid. This was done for 20 seconds or less if a tonic-clonic seizure developed before that time.

### *Electrophysiology.*

After the behavioral tests, animals were sacrificed, and hippocampal sagittal slices (400  $\mu\text{m}$ ) were obtained using a vibratome. Extracellular field recordings were obtained in a submerged recording chamber and perfused continuously with artificial cerebrospinal fluid (ACSF). LTP was evoked using the 10 theta burst protocol (10 trains of 4 stimuli at 100 Hz, 200 ms apart), performed at two-thirds of the maximum field excitatory postsynaptic potential (fEPSP).

### *Western blot analysis.*

Blotted nitrocellulose membranes were probed with antibodies directed against E6AP (E8655 Sigma-Aldrich; 1:1,000) and actin (MAB1501R, Millipore; 1:20,000). A fluorophore-conjugated goat anti-mouse antibody (IRDye 800CW, Westburg; 1:15,000) was used as secondary antibody, and protein was quantified using a LI-COR Odyssey Scanner and Odyssey 3.0 software.

### *Immunohistochemistry.*

Forty-micrometer-thick frozen sections were subjected to hydrogen peroxidase ( $\text{H}_2\text{O}_2$ ) treatment, placed in blocking solution (10% normal horse serum [NHS], 0.5% Triton X-100) for 1 hour and incubated overnight with the primary antibody (mouse  $\alpha$ -E6AP [E8655 Sigma-Aldrich, 1:2,000] in 2% NHS, 0.5% Triton X-100). The next day the slices were incubated with the secondary antibody ( $\alpha$ -mouse HRP; Dako; 1:200), which was detected by DAB as the chromogen.

*Statistics.*

All data were statistically analyzed using IBM SPSS software, and *P* values less than 0.05 were considered significant. Statistical analysis was performed using 1-way ANOVA or 2-way ANOVA with Bonferroni's post hoc comparison.

*Study approval.*

All animal experiments were approved by the Dutch Animal Experiment Committee (Dierexperimenten commissie [DEC]) and in accordance with Dutch animal care and use laws.

*Acknowledgments*

This work was supported by grants from the Angelman Syndrome Foundation (ASF), the Simons Foundation (SFARI, award 275234) to Y. Elgersma, and the Netherlands Organization for Scientific Research (NWO-ZoN-MW) to Y. Elgersma and B. Distel. We are grateful to the Dutch (Prader-Willi/Angelman Vereniging [PWAV]), Italian (Organizzazione Sindrome di Angelman [OR.S.A.]), and French (Association Française du syndrome d'Angelman [AFSA]) Angelman parent organizations for financial support to generate the mice. S. Silva-Santos was supported by Fundação para a Ciência e Tecnologia and Fundação Amélia de Mello. C.F. Bruinsma was supported by the Nina Foundation. We thank Minetta Elgersma, Jolet van de Bree, Erica Goedknecht, and Sanne Savelberg for technical assistance.

## Supplementary Material

### *Supplemental Methods*

#### *Generation and breeding of the Ube3astop/+ line.*

The Ube3astop/p+ mouse was generated as follows: the Ube3a genomic sequence (ENSMUSG00000025326) was obtained from Ensembl and used to design the primers for the targeting constructs. PCR fragments encompassing exon 3 using 5' primer: 5'-CCGCGGGCTCCACTAGTCAATTC-3' and 3' primer: 5'- GCGGCCGCACCACAGTCCCTGGAGTTC-3' (4.9 kb; exon denotation according to ENSMUSG00000025326) and exon 4 using 5' primer: 5'- GGCCGGCCGGAACCTACCATATCCTGTTTTAC-3' and 3' primer: 5'- GCGGCCGCAGCCGATCTAGGTATTC' (4.6 kb) were amplified using High Fidelity Taq Polymerase (Roche) on ES cell genomic DNA and cloned on either side of a Neomycin-stop cassette flanked by loxP sites (1). Exon 3 and 4 were sequenced to verify that no other mutations were introduced. For counter selection, the diphtheria toxin chain A (DTA) gene was inserted at the 5' of the targeting construct. The targeting construct was linearized and electroporated into embryonic day 14 (E14) ES cells (derived from 129P2 mice). Cells were cultured in BRL cellconditioned medium in the presence of leukemia inhibitory factor. After selection with G418 (200 µg/ml), targeted clones were identified by PCR (long-range PCR from neomycin resistance gene to the region flanking the targeted sequence). A clone with verified karyotype was injected into blastocysts of C57BL/6 mice. Male chimeras were crossed with female 129S2/SvPasCrl mice. The resulting heterozygous offspring was used for subsequent breedings. The Ube3astop/p+ was maintained by breeding heterozygous males with wild-type 129S2/SvPasCrl mice (Charles River). For all behavioural experiments except epilepsy tests, we crossed female Ube3astop/p+ with either TgCAG-cre mice (2) (hereafter referred as Cre+ mice) or with Tg(CAGcre/Esrl\*)5Amc/J (Jackson) (3) (here after referred as CreERT+ ) kept in the in C57BL/6J background (Charles River) to generate F1 heterozygous Ube3astop/p+ ;Cre+ and Ube3astop/p+ :CreERT+ mutants and littermate controls in the F1 hybrid 129S2-C57BL/6 background. For the epilepsy test, both Cre lines were crossed 5 times into 129S2/SvPasCrl and subsequently crossed with Ube3astop/p+ mice to obtain Ube3astop/p+ ;Cre+ and Ube3astop/p+ ;CreERT+ mutants and littermate controls in the 129S2 background. Mice were genotyped when they were 7-10 days, and re-

genotyped at the moment the mice were sacrificed. Genotyping records were obtained and kept by a technician not involved in the 2 experimental design, performance and analysis. All animals were kept at  $22\pm 2^{\circ}\text{C}$  with 12 hours dark and light cycle and were provided with food and water ad libitum. Mice were preferably group-housed (2-4) cage, unless when fighting between males was observed.

#### *Tamoxifen treatment and randomization.*

Cages were semi-randomly (alternatingly) assigned to either treat all mice within the cage with vehicle or with tamoxifen. Both male and female mice were used. The alternating randomization was adjusted if there was an imbalance of genotype or sex. One day to eight-month-old Ube3aStop/p+ mice and their WT littermates (both males and females) were used in this study. The group of Ube3aStop/p+ and WT mice crossed with CreERT+ transgenic mice were divided into 4 different experimental groups (classified as Newborn, Juvenile, Adolescent and Adult) based on the age of Tamoxifen administration to induce Cre-mediated deletion of the Stop-cassette. Tamoxifen (Sigma-Aldrich) was diluted in sunflower oil at a concentration of 20mg/ml. Each mouse received 0.10 mg Tamoxifen per gram body weight, by daily intraperitoneal (i.p.) injection. The control groups were treated with daily i.p. injections of sunflower oil (vehicle). The Newborn group received Tamoxifen through the milk of the mother, who received daily intraperitoneal (i.p.) injections of Tamoxifen for 5 consecutive days starting at the day of delivery. Tamoxifen treatment was initiated between 21-23 days of age in the Juvenile group; at 6 weeks of age in the Adolescent group; and at 14 weeks in the Adult group. These last 3 groups received 7 daily i.p. injections of Tamoxifen. The group of Ube3aStop/p+ and WT mice crossed with the embryonic active Cre+ transgenic mice ('Embryonic') received 3 vehicle injections when they were 6-8 weeks old.

**Behavioral analysis.** All behavioral experiments were performed during the light period of the cycle. All animal experiments were approved by the Dutch Ethical Committee and in accordance with Dutch animal care and use laws. The experimenter remained blind to the genotype and treatment until final statistical analysis. Both male and female mice were used for the experiments.



### *Behavioural assays*

All behavioural assays and scoring were done by an experimenter blind to genotype and treatment. Behavioral tests were typically run in the order as presented below. However the 3 marble burying and nest building test were added later to the battery, and a new cohort of adolescent and adult mice were used for these tests. Since the mice for the epilepsy test required a different genetic background (see above) we used a separate cohort for this test.

### *Accelerating Rotarod.*

Motor function was tested using the accelerating rotarod (4-40 rpm, in 5 minutes; model 7650, Ugo Basile Biological Research Apparatus, Varese, Italy). Mice were given two trials per day with a 45-60 min inter-trial interval for 5 consecutive days. For each day we calculated the average of the time spent on the rotarod, or the time until the mouse made 3 consecutive rotations on the rotarod. Maximum duration of a trial was 5 min. Number of mice used in this task: Number of mice used: Embryonic: Wt;Cre+ (n=14), Ube3aStop/p+;Cre- (n=8), Ube3aStop/p+;Cre+ (n=17), Inducible: Adult – WT;CreERT+ Veh. (n=11), WT;CreERT+ Tamox. (n=9), Ube3aStop/p+ ;CreERT+ Veh. (n=12), Ube3aStop/p+ ;CreERT+ Tamox. (n=13); Adolescent – WT;CreERT+ Veh. (n=11), WT;CreERT+ Tamox. (n=11), Ube3aStop/p+ ;CreERT+ Veh. (n=10), Ube3aStop/p+ ;CreERT+ Tamox. (n=11); Juvenile – WT;CreERT+ Veh. (n=22), WT;CreERT+ Tamox. (n=20), Ube3aStop/p+ ;CreERT+ Veh. (n=22), Ube3aStop/p+ ;CreERT+ Tamox. (n=22); Newborn – WT;CreERT+ Veh. (n=14), WT;CreERT+ Tamox. (n=12), Ube3aStop/p+ ;CreERT+ Veh. (n=14), Ube3aStop/p+ ;CreERT+ Tamox. (n=11).

### *Marble burying test.*

Clean open makrolon (polycarbonate) cages (50x26x18 cm) were filled with 4 cm of bedding material. On top of the bedding material 20 blue glass marbles were arranged in an equidistant 5 x 4 grid and the animals were given access to the marbles for 30 minutes. After the test the mice were gently removed from the cage. Marbles which were covered for more than 50% by bedding were scored as buried. Occasionally, a mouse managed to escape out of the cage during the test, and was excluded. Number of mice used in this task: Embryonic: Wt;Cre+ (n=24), Ube3aStop/p+;Cre- (n=18), Ube3aStop/p+;Cre+ (n=28), Inducible: Adult – WT;CreERT+ Veh. (n=13), WT;CreERT+ Tamox. (n=11), Ube3aStop/p+ ;CreERT+ Veh. (n=14), Ube3aStop/p+ ;CreERT+ Tamox. (n=15); Adolescent – WT;CreERT+



Veh. (n=20), WT;CreERT+ Tamox. (n=20), Ube3aStop/p+ ;CreERT+ Veh. (n=21), Ube3aStop/p+ ;CreERT+ Tamox. (n=23); Juvenile – WT;CreERT+ Veh. (n=21), WT;CreERT+ Tamox. (n=20), Ube3aStop/p+ ;CreERT+ Veh. (n=20), Ube3aStop/p+ ;CreERT+ Tamox. (n=20); Newborn – WT;CreERT+ Veh. (n=14), WT;CreERT+ Tamox. (n=12), Ube3aStop/p+ ;CreERT+ Veh. (n=14), Ube3aStop/p+ ;CreERT+ Tamox. (n=11).

### *Open Field test.*

To test locomotor activity and anxiety, the mice were individually placed in a brightly lit 120 cm diameter circular open field. The total distance travelled was recorded for 10 minutes (SMART software, Panlab, Barcelona). Number of mice used in this task: Embryonic: Wt;Cre+ (n=14), Ube3aStop/p+;Cre- (n=8), Ube3aStop/p+;Cre+ (n=17), Inducible: Adult – WT;CreERT+ Veh. (n=10), WT;CreERT+ Tamox. (n=8), Ube3aStop/p+ ;CreERT+ Veh. (n=9), Ube3aStop/p+ ;CreERT+ Tamox. (n=10); Adolescent – WT;CreERT+ Veh. (n=11), WT;CreERT+ Tamox. (n=11), Ube3aStop/p+ ;CreERT+ Veh. (n=10), Ube3aStop/p+ ;CreERT+ Tamox. (n=11); Juvenile – WT;CreERT+ Veh. (n=21), WT;CreERT+ Tamox. (n=21), Ube3aStop/p+ ;CreERT+ Veh. (n=22), Ube3aStop/p+ ;CreERT+ Tamox. (n=22); Newborn – WT;CreERT+ Veh. (n=14), WT;CreERT+ Tamox. (n=12), Ube3aStop/p+ ;CreERT+ Veh. (n=14), Ube3aStop/p+ ;CreERT+ Tamox. (n=11).

### *Nest Building test.*

To measure nest building, mice were single housed for a period of 5 to 7 days before the starting the experiment. Subsequently 12 grams (12±1) of compressed extra-thick blot filter paper (Bio-rad©) was added to the cage and cages were put back in the rack and undisturbed for 24h. For 5 consecutive days and approximately at the same time of the day, the unused nesting material was carefully cleaned, dried and weighed to determine the amount used for nestbuilding. Number of mice used in this task: Embryonic: Wt;Cre+ (n=7), Ube3aStop/p+;Cre- (n=7), Ube3aStop/p+;Cre+ (n=7), Inducible: Adult – WT;CreERT+ Veh. (n=9), WT;CreERT+ Tamox. (n=8), Ube3aStop/p+ ;CreERT+ Veh. (n=9), Ube3aStop/p+ ;CreERT+ Tamox. (n=8); Adolescent – WT;CreERT+ Veh. (n=14), WT;CreERT+ Tamox. (n=13), Ube3aStop/p+ ;CreERT+ Veh. (n=16), Ube3aStop/p+ ;CreERT+ Tamox. (n=17); Juvenile – WT;CreERT+ Veh. (n=12), WT;CreERT+ Tamox. (n=13), Ube3aStop/p+ ;CreERT+ Veh. (n=14), Ube3aStop/p+ ;CreERT+ Tamox. (n=13); Newborn – WT;CreERT+ Veh. (n=12), WT;CreERT+

Tamox. (n=12), Ube3aStop/p+ ;CreERT+ Veh. (n=12), Ube3aStop/p+ ;CreERT+ Tamox. (n=11).

### *Forced swim test.*

Mice were placed for 6 min in a cylindrical transparent tank (27cm high and 18cm diameter), filled with water (kept at 26±1 degrees Celsius) 15 cm deep. Since little or no immobility is observed during the first 2 min after the mouse has been placed in the water, the duration of immobility was only assessed during the last 4 min of the test. The mouse was considered to be immobile when he ceased to move altogether, making only movements necessary to keep its head above water. Number of mice used in this task: Embryonic: Wt;Cre+ (n=14), Ube3aStop/p+;Cre- (n=8), Ube3aStop/p+;Cre+ (n=17), Inducible: Adult – WT;CreERT+ Veh. 5 (n=11), WT;CreERT+ Tamox. (n=8), Ube3aStop/p+ ;CreERT+ Veh. (n=9), Ube3aStop/p+ ;CreERT+ Tamox. (n=9); Adolescent – WT;CreERT+ Veh. (n=11), WT;CreERT+ Tamox. (n=11), Ube3aStop/p+ ;CreERT+ Veh. (n=10), Ube3aStop/p+ ;CreERT+ Tamox. (n=11); Juvenile – WT;CreERT+ Veh. (n=17), WT;CreERT+ Tamox. (n=19), Ube3aStop/p+ ;CreERT+ Veh. (n=18), Ube3aStop/p+ ;CreERT+ Tamox. (n=19); Newborn – WT;CreERT+ Veh. (n=14), WT;CreERT+ Tamox. (n=12), Ube3aStop/p+ ;CreERT+ Veh. (n=14), Ube3aStop/p+ ;CreERT+ Tamox. (n=11).

### *Epilepsy test.*

Since epilepsy susceptibility in AS mice is dependent on the genetic background, these experiments were performed in a 129/sv background. For this test, mice were taken from their home cage and placed in a clean cage. To assess seizure susceptibility, audiogenic seizures were induced by producing a loud and continuous noise, achieved by vigorously screeching scissors across the metal grating of the cage lid. This was done for 20s or shorter if a tonic-clonic seizure developed before that time. Number of mice used in this task: Embryonic: Wt;Cre+ (n=7), Ube3aStop/p+;Cre- (n=10), Ube3aStop/p+;Cre+ (n=8), Inducible: Juvenile – WT;CreERT+ Veh. (n=8), WT;CreERT+ Tamox. (n=8), Ube3aStop/p+ ;CreERT+ Veh. (n=8), Ube3aStop/p+ ;CreERT+ Tamox. (n=8). Mean age of the mice used in the epilepsy susceptibility test was the following: Embryonic, 10 weeks; Juvenile, 9 weeks.

***Antiepileptic drug (AED) treatment.***

Mutant Ube3aStop/p+;Cre- (n=2), Ube3am-/p+ (n=12) and WT littermates (n=3) in the 129sv background were used for these experiments. Tonic-clonic seizures were verified in all mutant mice upon audiogenic stimulation. No seizures were observed in WT mice. Mutant mice were randomized into two treatment groups (sodium valproate and clonazepam), and drug administration started 24hrs after the first epilepsy test. 1 Ube3aStop/p+;Cre- , 7 Ube3am-/p+ and 2 WT mice were treated with 5 daily i.p. injections of sodium valproate (Alliance Healthcare™) at a concentration of 200mg/kg. The valproate was dispensed in sterilized water through agitation and syringes were filled while stirring. Valproate was freshly prepared daily. 6 The second treatment group composed of 1 Ube3aStop/p+;Cre-, 5 Ube3am-/p+ and 1 WT mouse, which were treated with 5 daily i.p. injections of 0.05mg/kg Clonazepam (Roche) dispensed in PBS-methylcellulose. Clonazepam was prepared fresh every two days. Two re-tests experiments were performed in both treatment groups; the first re-test was performed 30 min. after the last injection, on the fifth day of treatment, and the second re-test was done 3 days after treatment cessation.

***Electrophysiology.***

After the behavioural tests, animals were been sacrificed, sagittal slices (400  $\mu$ m) were made and submerged in ice-cold artificial CSF (ACSF) using a vibratome, and hippocampi were dissected out. These sagittal hippocampal slices were maintained at room temperature for at least 1.5 h to recover before experiments were initiated. Then they were placed in a submerged recording chamber and perfused continuously at a rate of 2 ml/min with ACSF equilibrated with 95% O<sub>2</sub>, 5% CO<sub>2</sub> at 31°C. ACSF contained the following (in mM): 120 NaCl, 3.5 KCl, 2.5 CaCl<sub>2</sub>, 1.3 MgSO<sub>4</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 D-glucose. Extracellular recording of field EPSP (fEPSPs) were made in CA1 stratum radiatum with platinum/iridium electrodes (Frederick Haer). A bipolar Pt/Ir was used to stimulate Schaffer collateral/commissural afferents with a stimulus duration of 100  $\mu$ s. LTP was evoked using the 10 Theta burst protocol (10 trains of 4 stimuli at 100Hz, 200ms apart), performed at two-third of the maximum fEPSP. fEPSP measurements were done once per minute. Potentiation was measured as the normalized increase of the mean fEPSP slope for the duration of the baseline. Only stable recordings were included, and this judgment was made blind to genotype. Average LTP was defined as the mean last 10

min of each protocol. Recordings showing unstable baselines were excluded from the experiment. Number of slices/mice used: Juvenile- WT;CreERT+ Veh. (n=16/4), WT;CreERT+ Tamox. (n=25/4), Ube3aStop/p+ ;CreERT+ Veh. (n=22/4), Ube3aStop/p+ ;CreERT+ Tamox. (n=22/5); Adult - WT;CreERT+ Veh. (n=18/6), WT;CreERT+ Tamox. (n=37/8), Ube3aStop/p+ ;CreERT+ Veh. (n=23/4), Ube3aStop/p+ ;CreERT+ Tamox. (n=15/4).

### ***Western Blots.***

To collect tissue for Western blot analysis, hippocampus, cortex and cerebellum were dissected and immediately frozen in liquid nitrogen. The lysates were prepared by adding lysis buffer (10mM Tris-HCL pH 6.8, 2.5% SDS) supplemented with protease inhibitor cocktail (Sigma-Aldrich®) to the tissue and homogenization was achieved by sonication. After centrifugation (6000 rpm for 5min.) supernatants were collected. The protein concentration of the supernatants was determined using a BCA kit (Pierce, Thermo Scientific). A total of 20µg of each sample was loaded on the gel and a wet transfer was performed. The blotted nitrocellulose membrane was probed with antibodies directed against E6AP (E8655 Sigma-Aldrich®, 1:1,000) and Actin (MAB1501R Millipore®, 1:20,000). A fluorophore-conjugated secondary Goat antimouse antibody (Westburg®, IRDye 800CW 1:15,000) was used and the protein was detected using Li-cor® Odyssey Scanner system. Quantification was done using Odyssey 3.0 software (Li-cor® Biosciences). Number of samples used for immunoblot analysis range from 2 to 5 per genotype/brain area.

### ***Immunohistochemistry.***

Brains from adult mice were fixed by transcardial perfusion with 4% paraformaldehyde. Immunocytochemistry was performed on 40µm thick frozen sections. The sections were subjected to a hydrogen peroxidase (H<sub>2</sub>O<sub>2</sub>) treatment, placed in blocking solution (10% normal horse serum (NHS), 0.5% Triton X-100) for 1h and incubated overnight with the primary antibody (mouse α-E6AP (E8655 Sigma-Aldrich, 1:2000) in 2% of NHS, 0.5% Triton X-100). The next day the slices were incubated with the secondary antibody (α-mouse HRP (Dako®; 1:200), which was detected by 3,3'-diaminobenzidine (DAB) as the chromogen. DAB sections were analysed and photographed using a Leica® DM-RB microscope and a Leica DFC450 digital camera. For overview pictures of the slices a Zeiss Stemi SV6 was used.

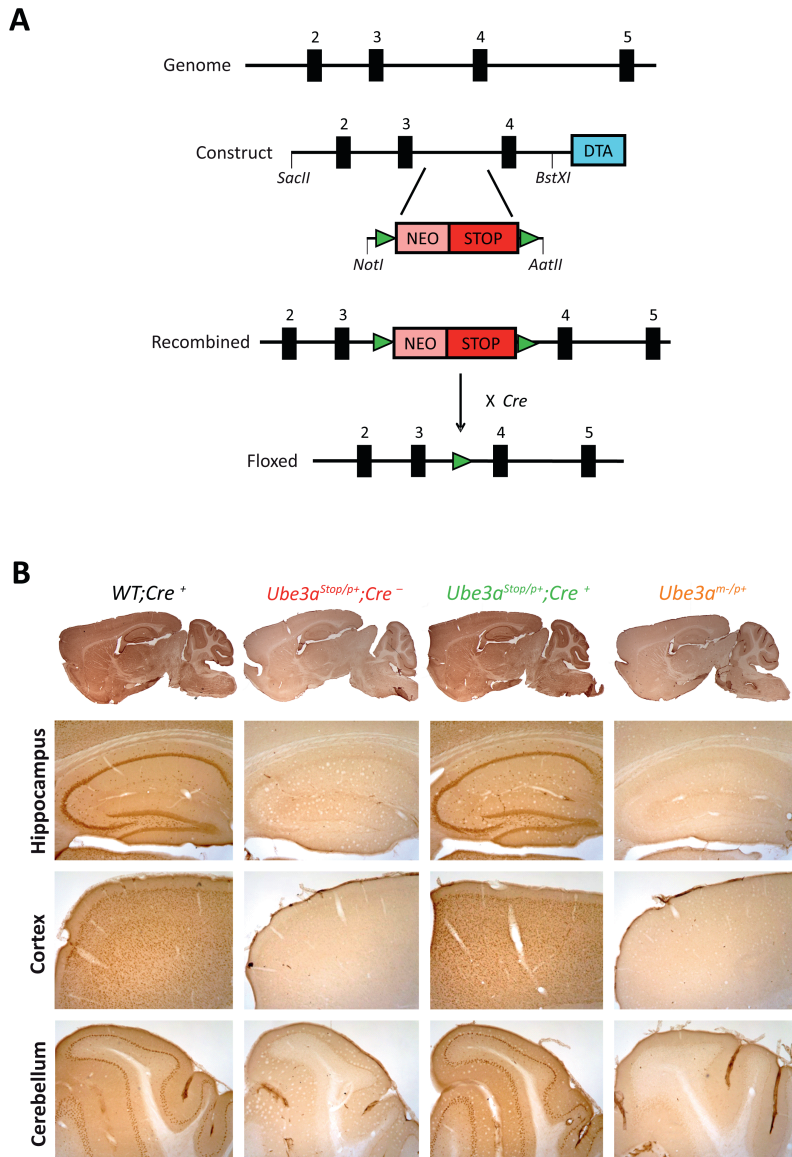
### *Data analysis and statistics.*

Values are represented as means  $\pm$  S.E.M. All the data was statistically analyzed using the IBM® SPSS software and P-values of

### *Supplemental References*

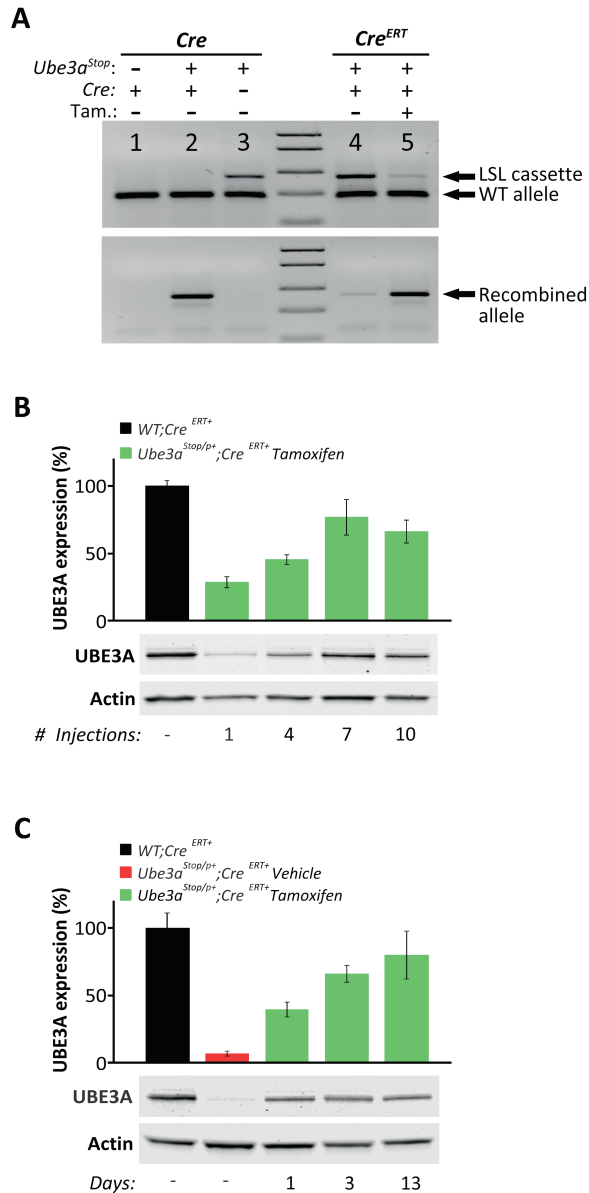
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Supplementary Figure 1:



**Supplemental Figure 1. Generation and histological validation of *Ube3a*<sup>Stop/p<sup>+</sup></sup> knock-in mice.** (A) Schematics depicting the generation of the inducible mouse model by the insertion of a floxed stop cassette into intron 3 of *Ube3a*, for which cre-mediated recombination leads to reinstatement of *Ube3a* gene expression. Black boxes correspond to *Ube3a* coding exons and green triangles represent the LoxP sites. (B) Immunohistochemical UBE3A stainings from *Ube3a*<sup>M-/p<sup>+</sup></sup> knock-out mice and *Ube3a*<sup>Stop/p<sup>+</sup></sup> and WT littermates crossed with a embryonically active cre-line. Brain overviews magnification = 1.6x (upper); zoomed-in pictures magnification = 5x (lower).

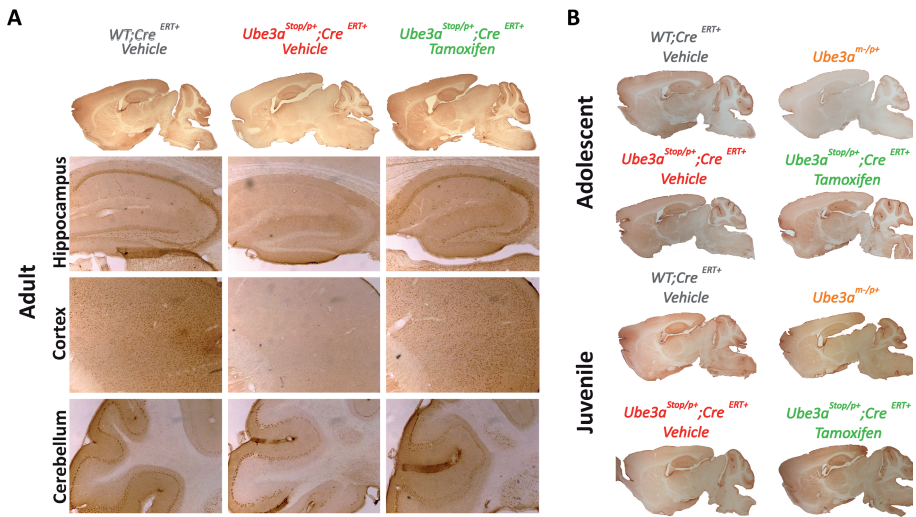
## Supplementary Figure 2:



**Supplemental Figure 2. High level of recombination is achieved by embryonically expressed cre ('Cre') and tamoxifen inducible cre expression ('Cre<sup>ERT</sup>'). (A) Polymerase chain reaction (PCR) of hippocampal tissue reveals successful recombination in mutant mice expressing Cag-Cre and in *Ube3a<sup>Stop/p+</sup>*; *Cre<sup>ERT</sup>* mutant mice injected 7 times with tamoxifen (Tam.). (B) Representative Western blot and corresponding quantification illustrates hippocampal UBE3A reinstatement achieved by successive tamoxifen injections in the *Ube3a<sup>Stop/p+</sup>*; *Cre<sup>ERT</sup>* mutant mice. (C) Time-course of UBE3A expression following gene reinstatement (days following the last seventh injection of tamoxifen). All data are represented as mean  $\pm$  S.E.M.**



Supplementary Figure 3:



**Supplemental Figure 3. Histological analysis of *Ube3a*<sup>Stop/p+</sup>;Cre<sup>ERT+</sup> mice reveals successful reactivation of the maternal *Ube3a* gene upon tamoxifen induction. (A-B)** Hippocampal, cortical, and cerebellar sections demonstrate brain-wide reactivation of *Ube3a* expression in tamoxifen-treated *Ube3a*<sup>Stop/p+</sup>;Cre<sup>ERT+</sup> mice. Brain overviews magnification = 1.6x; zoomed-in pictures magnification = 5x.



Supplementary Table 1:

Embryonic <i>Ube3a</i> gene reinstatement		Paradigm	Dependent variable	Statistical test	Independent variable	p	Post-hoc test	p
		RR	Latency to fall (s)	Repeated measures one-way ANOVA	Genotype	p<0.01	Ube3a <sup>Stop/p<sup>+</sup></sup> ;Cre <sup>+</sup>	p= 1.000
							Ube3a <sup>Stop/p<sup>+</sup></sup> ;Cre <sup>-</sup>	p<0.01
		Marble	Number unburied marbles	Univariate one-way ANOVA	Genotype	p<0.01	WT;Cre <sup>+</sup>	p= 1.000
							Ube3a <sup>Stop/p<sup>+</sup></sup> ;Cre <sup>-</sup>	p<0.01
		OF	Path length (m)	Univariate one-way ANOVA	Genotype	p<0.01	WT;Cre <sup>+</sup>	p= 1.000
							Ube3a <sup>Stop/p<sup>+</sup></sup> ;Cre <sup>-</sup>	p<0.05
		NB	Used nesting material (%)	Repeated measures one-way ANOVA	Genotype	p<0.01	WT;Cre <sup>+</sup>	p= 1.000
							Ube3a <sup>Stop/p<sup>+</sup></sup> ;Cre <sup>-</sup>	p<0.01
		FST	Floating time (%)	Univariate one-way ANOVA	Genotype	p<0.01	WT;Cre <sup>+</sup>	p= 1.000
							Ube3a <sup>Stop/p<sup>+</sup></sup> ;Cre <sup>-</sup>	p<0.01
		Epilepsy	Presence of seizures	Univariate one-way ANOVA	Genotype	p<0.01	WT;Cre <sup>+</sup>	p= 1.000
							Ube3a <sup>Stop/p<sup>+</sup></sup> ;Cre <sup>-</sup>	p<0.01

**Supplemental Table S1** Summary of the statistical tests used for each behavioral paradigm 2 performed on the early embryonic reactivation group and statistical outcomes obtained. 3 Statistical significance (P < 0.05) is indicated by green shading.

Supplementary Table 1:

		Paradigm	Dependent variable	Statistical test	Independent variable	p
<b>Adult</b> <i>Ube3a</i> gene reinstatement	Induction at 14 weeks	RR	Latency to fall (s)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.602
					Genotype*Treatment	p= 0.613
		Marble	Number unburied marbles	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.751
					Genotype*Treatment	p= 0.722
		OF	Path lenght (m)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.203
					Genotype*Treatment	p= 0.411
		NB	Used nesting material (%)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.05
					Genotype*Treatment	p= 0.377
		FST	Floating time (%)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.499
					Genotype*Treatment	p= 0.154
		LTP	fEPSP slope (% from baseline)	Univariate two-way ANOVA	Genotype	p= 0.849
					Treatment	p= 0.071
					Genotype*Treatment	p<0.01
<b>Adolescent</b> <i>Ube3a</i> gene reinstatement	Induction at 6 weeks	RR	Latency to fall (s)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.284
					Genotype*Treatment	p= 0.284
		Marble	Number unburied marbles	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.249
					Genotype*Treatment	p= 0.221
		OF	Path lenght (m)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.240
					Genotype*Treatment	p= 0.413
		NB	Used nesting material (%)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.308
					Genotype*Treatment	p= 0.530
		FST	Floating time (%)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.374
					Genotype*Treatment	p= 0.822
	Induction at P21-23	RR	Latency to fall (s)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.01
					Genotype*Treatment	p<0.01
		Marble	Number unburied marbles	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.080
					Genotype*Treatment	p= 0.537
		OF	Path lenght (m)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.01
					Genotype*Treatment	p= 0.948
		NB	Used nesting material (%)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.173
					Genotype*Treatment	p= 0.708
		FST	Floating time (%)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.01
					Genotype*Treatment	p= 0.135
		LTP	fEPSP slope (% from baseline)	Univariate two-way ANOVA	Genotype	p= 0.088
					Treatment	p<0.01
					Genotype*Treatment	p= 0.055
<b>Newborn</b> <i>Ube3a</i> gene reinstatement	Induction at P1	RR	Latency to fall (s)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.01
					Genotype*Treatment	p<0.05
		Marble	Number unburied marbles	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.05
					Genotype*Treatment	p= 0.124
		OF	Path length (m)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.365
					Genotype*Treatment	p<0.05
		NB	Used nesting material (%)	Repeated measures two-way ANOVA	Genotype	p<0.01
					Treatment	p<0.05
					Genotype*Treatment	p= 0.208
		FST	Floating time (%)	Univariate two-way ANOVA	Genotype	p<0.01
					Treatment	p= 0.987
					Genotype*Treatment	p= 0.663

**Supplemental Table S2** Summary of the statistical tests applied for each behavioral paradigm performed on the postnatal reactivation groups. Statistical significance ( $P < 0.05$ ) is indicated by green shading.

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