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Research Article

Genetics

Angelman syndrome (AS) is a severe neurodevelopmental disorder that results from loss of function of the maternal ubiquitin protein ligase E3A (*UBE3A*) allele. Due to neuron-specific imprinting, the paternal *UBE3A* copy is silenced. Previous studies in murine models have demonstrated that strategies to activate the paternal *Ube3a* allele are feasible; however, a recent study showed that pharmacological *Ube3a* 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 *Ube3a* during earlier neurodevelopmental windows. We developed an AS model that allows for temporally controlled Cre-dependent induction of the maternal *Ube3a* allele and determined that there are distinct neurodevelopmental windows during which *Ube3a* restoration can rescue AS-relevant phenotypes. Motor deficits were rescued by *Ube3a* reinstatement in adolescent mice, whereas anxiety, repetitive behavior, and epilepsy were only rescued when *Ube3a* was reinstated during early development. In contrast, hippocampal synaptic plasticity could be restored at any age. Together, these findings suggest that *Ube3a* reinstatement early in development may be necessary to prevent or rescue most AS-associated phenotypes and should be considered in future clinical trial design.

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

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Angelman syndrome (AS) is a severe neurodevelopmental disorder that results from loss of function of the maternal ubiquitin protein ligase E3A (*UBE3A*) allele. Due to neuron-specific imprinting, the paternal *UBE3A* copy is silenced. Previous studies in murine models have demonstrated that strategies to activate the paternal *Ube3a* allele are feasible; however, a recent study showed that pharmacological *Ube3a* 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 *Ube3a* during earlier neurodevelopmental windows. We developed an AS model that allows for temporally controlled Cre-dependent induction of the maternal *Ube3a* allele and determined that there are distinct neurodevelopmental windows during which *Ube3a* restoration can rescue AS-relevant phenotypes. Motor deficits were rescued by *Ube3a* reinstatement in adolescent mice, whereas anxiety, repetitive behavior, and epilepsy were only rescued when *Ube3a* was reinstated during early development. In contrast, hippocampal synaptic plasticity could be restored at any age. Together, these findings suggest that *Ube3a* 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 incom-

plete 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.

Authorship note: Geeske M. van Woerden, Caroline F. Bruinsma, and Edwin Mientjes contributed equally to this work.

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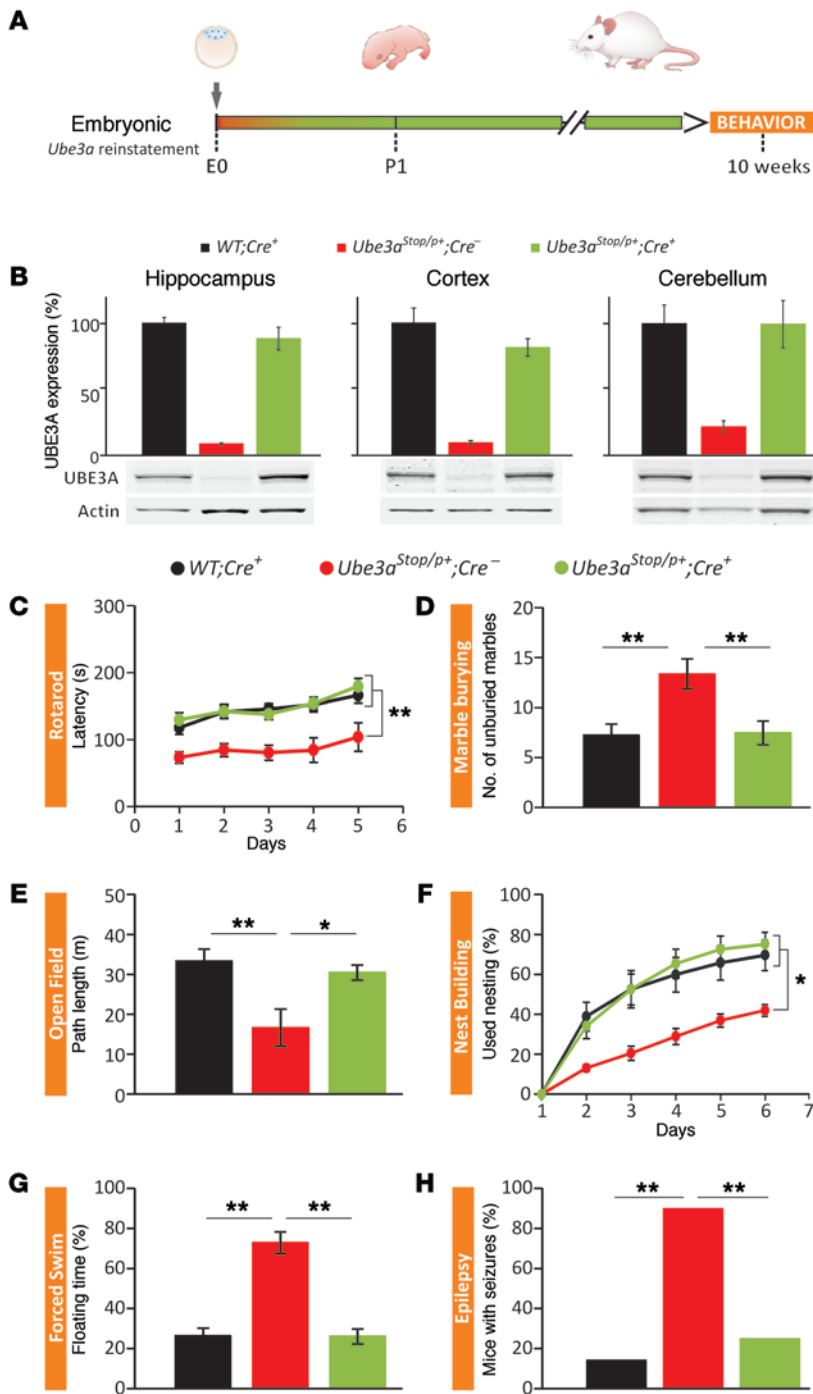


Figure 1. Embryonic reactivation of *Ube3a* expression rescues AS-like behavioral phenotypes. (A) Schematic representation of *Ube3a* reactivation (indicated by the gray arrow) during mouse embryonic development and time point of behavioral testing. (B) Western blot analysis of hippocampus ($n = 4$ per genotype), cortex ($n = 5$), and cerebellum ($n = 5$) from *Ube3a*^{Stop/p+} and WT littermates crossed with an embryonically active Cre line. (C–H) *Ube3a*^{Stop/p+};Cre⁻ mice show robust behavioral AS-relevant phenotypes, which can be fully rescued by embryonic reactivation of the *Ube3a* gene in the *Ube3a*^{Stop/p+};Cre⁺ mice. Number of mice (WT;Cre⁺/*Ube3a*^{Stop/p+};Cre⁻/*Ube3a*^{Stop/p+};Cre⁺): 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 ± 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.

We first investigated the efficiency of the transcriptional stop cassette in blocking *Ube3a* expression. Female *Ube3a*^{Stop/p+} 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*^{Stop/p+};Cre⁻ 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*^{m-/p+}) (Figure 1B; see also Figure 2D for a comparison to *Ube3a*^{m-/p+} mice). Immunohistochemical staining of *Ube3a*^{Stop/p+};Cre⁻ brain slices was indistinguishable from that of *Ube3a*^{m-/p+} 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.

Next, we investigated the efficiency of *Ube3a* reactivation upon Cre-mediated deletion of the floxed stop cassette. UBE3A protein levels were reinstated in *Ube3a*^{Stop/p+};Cre⁺ 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*^{Stop/p+};Cre⁺ and WT;Cre⁺ mice, validating the functionality of the *Ube3a* reactivation method (Figure 1B and Supplemental Figure 1B).

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*^{Stop/p+};Cre⁻ mice exhibited significant alterations in rotarod performance (Fig-

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*^{Stop/p+}) (Supplemental Methods and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI80554DS1).

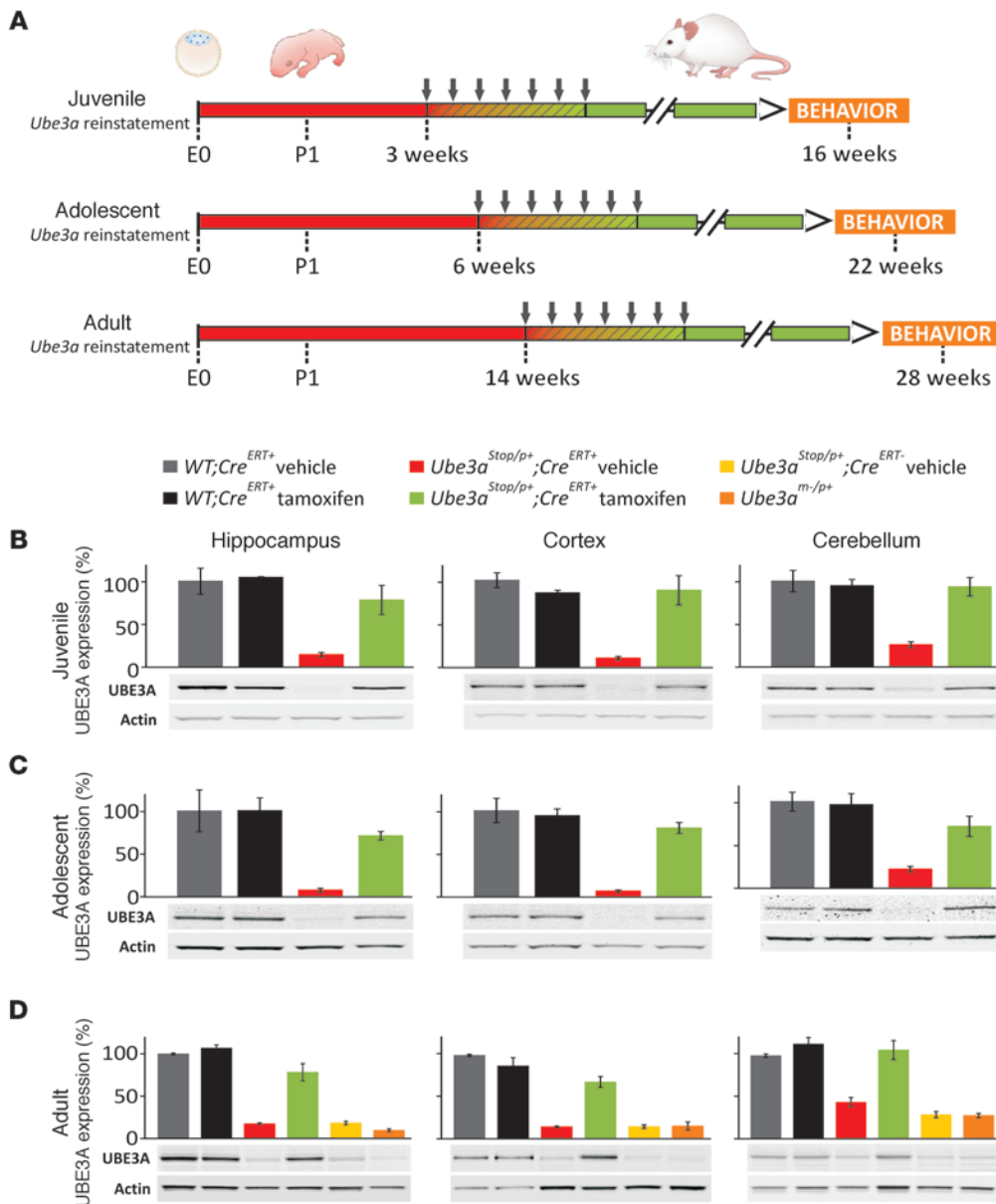


Figure 2. Molecular analysis of *Ube3a*^{Stop/p+};Cre^{ERT+} mice reveals successful reactivation of the maternal *Ube3a* gene upon tamoxifen induction. (A) Schematics representing *Ube3a* reactivation achieved by tamoxifen treatment (gray arrows) in each experimental group. (B–D) *Ube3a*^{Stop/p+};Cre^{ERT+}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

ure 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*^{m-/p+} 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*^{m-/p+} mutant (data not shown) and the conditional *Ube3a*^{Stop/p+} AS mouse model (Figure 1G).

Consistent with the therapeutic potential of *Ube3a* gene reactivation, *Ube3a*^{Stop/p+};Cre^{ERT+} 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*^{Stop/p+} mice with a tamoxifen-inducible Cre^{ERT+} 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*^{Stop/p+};Cre^{ERT+} 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*^{Stop/p+};Cre^{ERT+} mice was also similar to that observed in *Ube3a*^{Stop/p+};Cre^{ERT-} and *Ube3a*^{m-/p+} mice (Figure 2D and Supplemental Figure 3), demonstrating the tight control of gene reactivation.

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

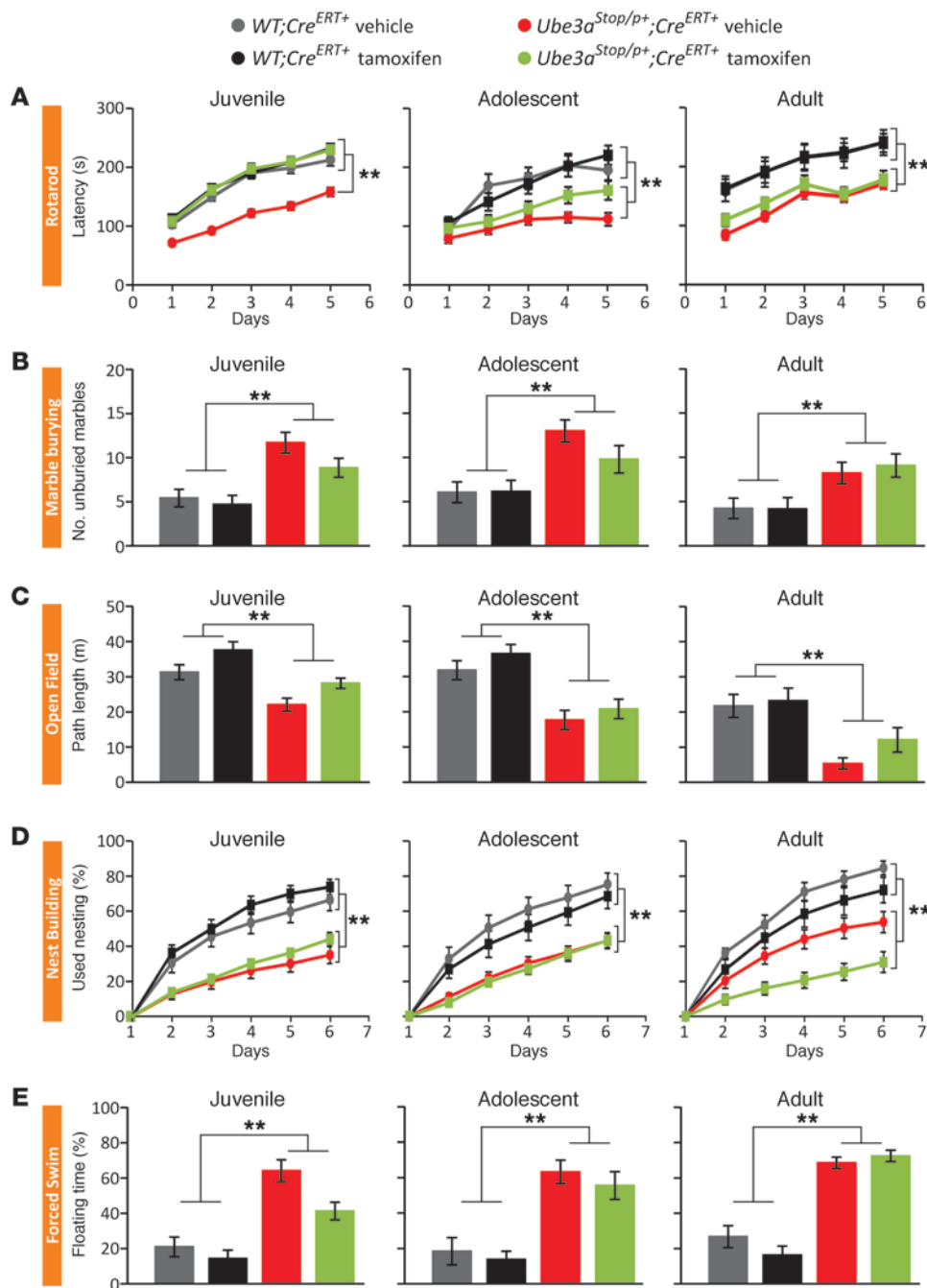


Figure 3. Postnatal reactivation of *Ube3a* expression reveals a critical period for behavioral rescue. (A–E) Behavioral testing of *Ube3a*^{Stop/p+} and WT littermates treated with either vehicle (Veh.) or tamoxifen (Tamox.) shows distinct critical periods for recovery of the behavioral deficits. Numbers of mice (WT;Cre^{ERT+} Veh./WT;Cre^{ERT+} Tamox./*Ube3a*^{Stop/p+};Cre^{ERT+} Veh./*Ube3a*^{Stop/p+};Cre^{ERT+} 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 \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). ** $P < 0.01$ for genotype significance.

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.

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*^{m-/p+} and *Ube3a*^{Stop/p+}; Cre^{ERT-} 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.

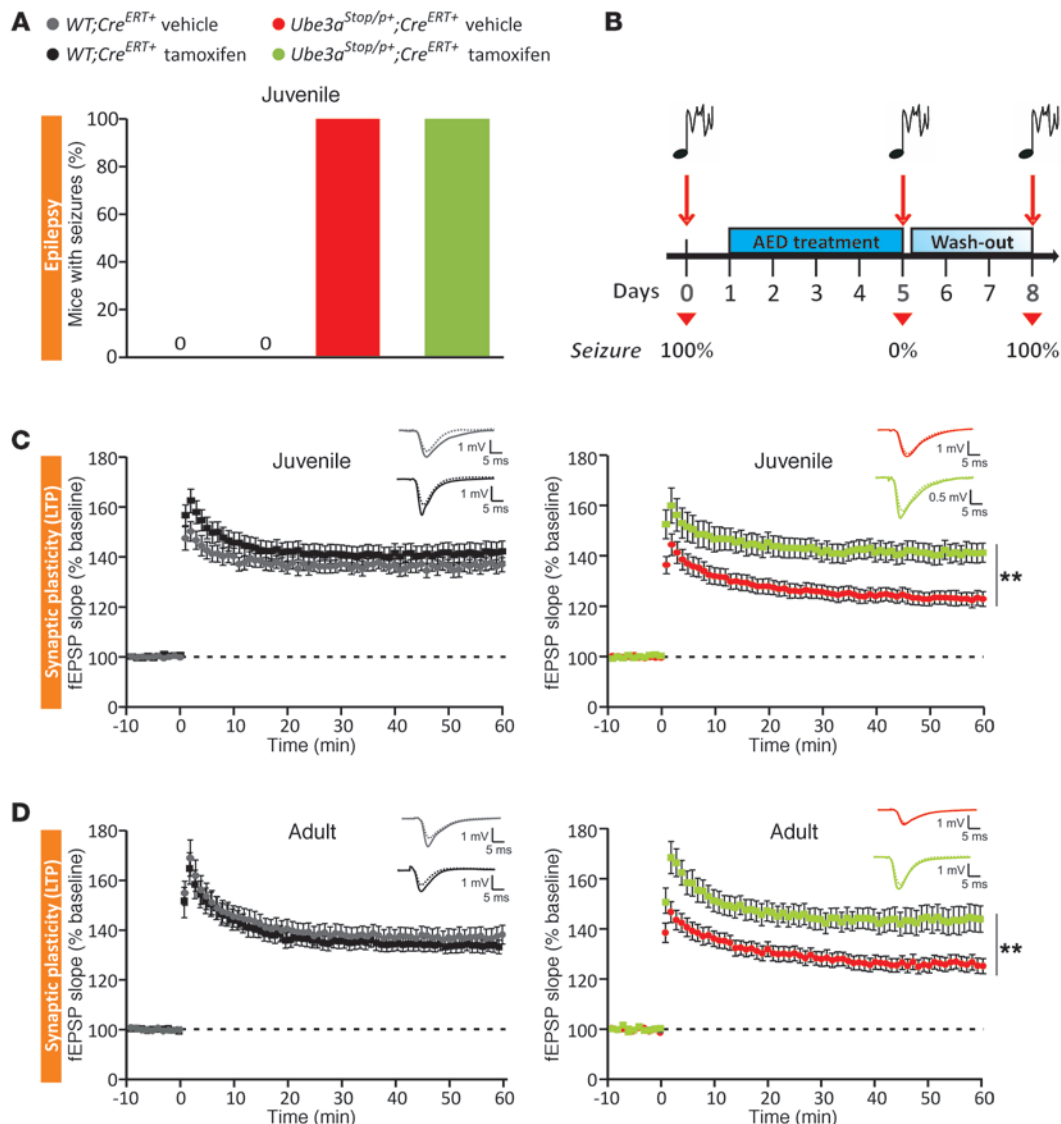


Figure 4. *Ube3a* reactivation in juvenile animals does not recover epilepsy susceptibility, but Schaffer collateral-CA1 LTP is fully recovered. (A) Epilepsy susceptibility in $Ube3a^{Stop/p+};Cre^{ERT+}$ mice persists after *Ube3a* gene reactivation at a juvenile age ($n = 8$ mice/group). (B) The induction 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 $Ube3a^{Stop/p+};Cre^{-}$ ($n = 2$) and $Ube3a^{m-/p+}$ ($n = 12$) 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: $WT;Cre^{ERT+}$ Veh. ($n = 16/4$), $WT;Cre^{ERT+}$ Tamox. ($n = 25/4$), $Ube3a^{Stop/p+};Cre^{ERT+}$ Veh. ($n = 22/4$), $Ube3a^{Stop/p+};Cre^{ERT+}$ Tamox. ($n = 22/5$); adult reactivation: $WT;Cre^{ERT+}$ Veh. ($n = 18/6$), $WT;Cre^{ERT+}$ Tamox. ($n = 37/8$), $Ube3a^{Stop/p+};Cre^{ERT+}$ Veh. ($n = 23/4$), $Ube3a^{Stop/p+};Cre^{ERT+}$ Tamox. ($n = 15/4$). ** $P < 0.01$.

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.

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

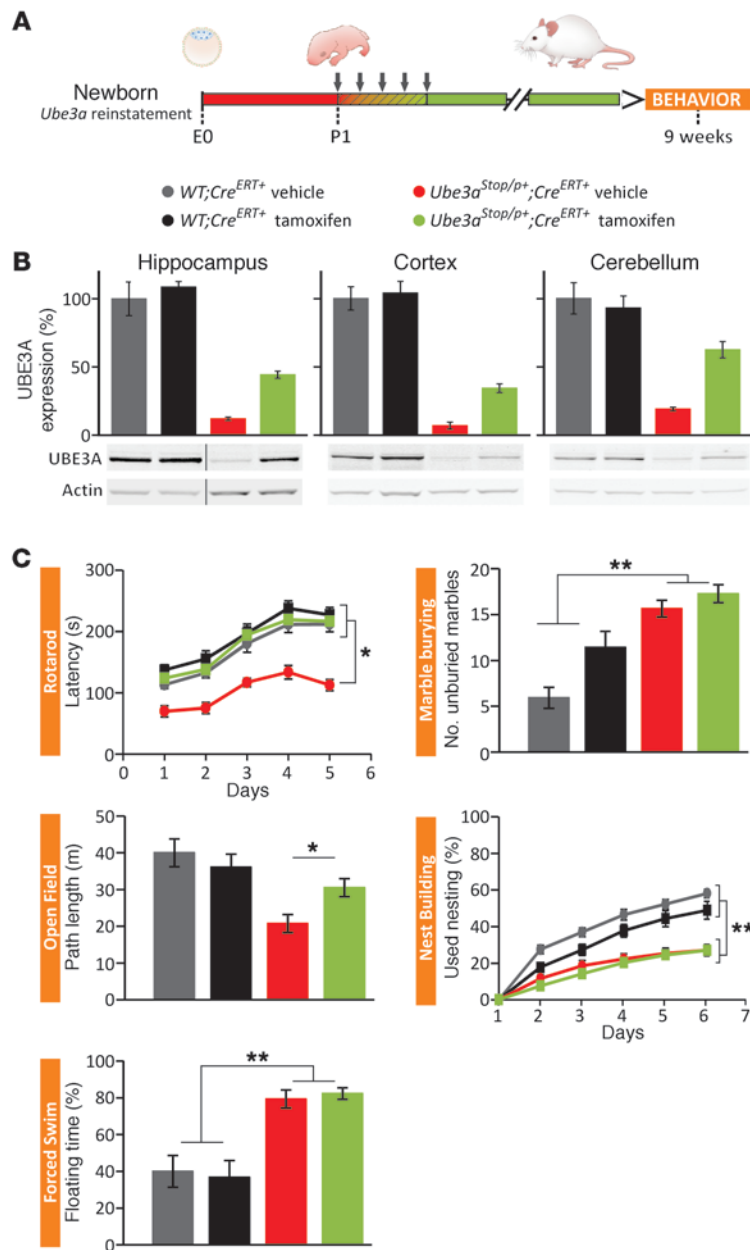


Figure 5. Partial reactivation of *Ube3a* expression during the first postnatal week attenuates the motor coordination and open field deficits. (A) Schematics representing *Ube3a* reactivation achieved by tamoxifen administration (gray arrows) to the lactating dams starting on the day of delivery. (B) 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^{ERT+}* Veh./WT;*Cre^{ERT+}* Tamox./*Ube3a^{Stop/p+}*;*Cre^{ERT+}* Veh./*Ube3a^{Stop/p+}*;*Cre^{ERT+}* 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). * $P < 0.05$; ** $P < 0.01$.

trast, 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^{m-/p+}* 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.

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 con-

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_1 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

Further details are provided in Supplemental Methods.

Mice. For all behavioral experiments except the epilepsy test, we crossed female *Ube3a*^{Stop/p⁺} mice (in the 129S2/SvPasCrl background; Charles River) with either *TgCAG-Cre* mice (13) (herein referred as

Cre⁺ mice) or with *Tg(CAG-cre/Esr1⁺)5Amc/J* (The Jackson Laboratory) (17) (herein referred as *Cre*^{ERT+}), both kept in the C57BL/6J background (Charles River), to generate heterozygous *Ube3a*^{Stop/p⁺}; *Cre*⁺ and *Ube3a*^{Stop/p⁺}; *Cre*^{ERT+} mutants and littermate controls in the F_1 hybrid 129S2-C57BL/6 background.

Tamoxifen treatment. One-day to 8-month-old *Ube3a*^{Stop/p⁺} mice and their WT littermates (both sexes) were used in this study. *Ube3a*^{Stop/p⁺}; *Cre*^{ERT+} 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°C ± 1°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 μm) 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 (H₂O₂) 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 α -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 (α -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.

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