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Neonatal Estradiol Stimulation Prevents Epilepsy in Arx Model of X-Linked Infantile Spasms Syndrome

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Abstract

Infantile spasms are a catastrophic form of pediatric epilepsy with inadequate treatment. In patients, mutation of ARX, a transcription factor selectively expressed in neuronal precursors and adult inhibitory interneurons, impairs cell migration and causes a major inherited subtype of the disease X-linked infantile spasms syndrome. Using an animal model, the *Arx*^{(GCG)10+7} mouse, we determined that brief estradiol (E2) administration during early postnatal development prevented spasms in infancy and seizures in adult mutants. E2 was ineffective when delivered after puberty or 30 days after birth. Early E2 treatment altered mRNA levels of three downstream targets of *Arx* (*Shox2*, *Ebf3*, and *Lgi1*) and restored depleted interneuron populations without increasing

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Data and materials availability: All data and materials are available. Contact the author for information about the transgenic mice.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/6/220/220ra12/DC1

Materials and Methods

Fig. S1. Early E2 does not alter cortical VGAT immunoreactivity in adult *Arx* mutants.

Fig. S2. Early E2 does not alter NPY immunoreactivity of mossy fiber or hilar interneurons in *Arx* mutants.

Table S1. E2 treatment from P5 to P40 seizure and spike rate individual data.

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Movie S1. Digital video depicting a representative major spontaneous motor spasm typical of *Arx*(GCG)10+7 mutants between P7 and P11.

GABAergic synaptic density. Postnatal E2 treatment may induce lasting transcriptional changes that lead to enduring disease modification and could potentially serve as a therapy for inherited interneuronopathies.

INTRODUCTION

Infantile spasms syndrome (ISS) is a devastating form of childhood epilepsy characterized by involuntary, massive motor spasms during early infancy that herald a lifelong disorder of severe seizures and intellectual disability. Mutations in a growing list of genes critical for the establishment of proper neural networks during development have been associated with its many inherited forms (1). ISS responds poorly to typical anticonvulsant drugs, and despite the lack of a known mechanism, the synthetic glucocorticoid prednisone and adrenocorticotrophic hormone (ACTH) remain the primary initial treatments (1–3). Although short-term spasm reduction can be achieved, high relapse rates, cognitive impairment, and long-lasting side effects highlight the need for more effective therapy (2, 4, 5).

Mutations in *Aristaless*-related homeobox [*ARX*, Online Mendelian Inheritance in Man (OMIM): 300382] cause X-linked infantile spasms syndrome (ISSX; OMIM: 308350). A GCG triplet repeat expansion in *ARX*, resulting in seven additional alanine residues in the first polyalanine tract, is the most commonly inherited error (6–10). The transcription factor encoded by *ARX*, which is under transcriptional control of *Dlx1/2*, is selectively expressed in interneurons where it regulates their tangential migration and maturation by modulating the expression of cell-intrinsic downstream genes, including *Lmo1*, *Ebf3*, *Shox2*, *Lhx7*(8), *Cxcr4*, and *Lgi1* (11–15). Interneurons are a diverse set of local circuit cells that release γ -aminobutyric acid (GABA), various neuropeptides, or acetylcholine and have complex braking and modulatory control over activity within central neural networks. Inherited defects affecting this cell group are emerging as major etiologies of this and related human neurodevelopmental diseases (1, 16). A knock-in mouse engineered with the human 7-Ala expansion, designated *Arx*^{(GCG)¹⁰⁺⁷}, displays infantile motor spasms that resemble those of the human disorder, spontaneous seizures with frequent interictal spike discharges, cognitive and behavioral impairments, and a selective, lamina-specific depletion of cortical and striatal interneurons, presumably caused by a subset of precursors that fail to migrate and mature (17, 18). This loss is not associated with evidence of cell death, suggesting that early intervention might rescue this phenotype.

In mice, interneuron migration and cortical laminar positioning occur between embryonic day 9 (E9) and postnatal day 10 (P10). In developing male brains, this interval overlaps with a surge of intrinsic E2 accompanied by aromatase-mediated local conversion of testicular testosterone into 17 β -estradiol (E2) and estrogen receptor (ER) expression in specific brain regions (19–22). As a short-acting neurosteroid, E2 modulates neuronal excitability and neurotransmitter release within seconds after administration (23, 24). In addition, E2-induced changes in gene expression also exert enduring effects on developing neurons. Long-lasting, transcriptional, and epigenetic actions of E2 via ER- α and ER- β have been suggested to play a role in precursor cell proliferation, neuronal migration, dendritic spine formation, synaptogenesis, and survival in a temporally regulated and region-specific

manner (23). Although not fully understood, these neuroprotective effects suggest that E2 may have unexplored therapeutic potential (23, 25–30). Given the lack of effective treatment for ISSX, we hypothesized that the maturation-enhancing properties of E2 in the male brain during the perinatal period could be harnessed to improve the complex neurological phenotype caused by the $Arx^{(GCG)10+7}$ mutation.

RESULTS

Effect of early E2 treatment on epilepsy in Arx mutants

We treated male $Arx^{(GCG)10+7}$ mutants with daily subcutaneous injections of E2 for 35 days between P5 and P40 and then monitored the mice by video-electroencephalography (VEEG) for 3 weeks. Control $Arx^{(GCG)10+7}$ mutants displayed, on average, 283.3 ± 109.7 aberrant cortical spike discharges per hour (mean $\pm$ SEM, $n = 11$), whereas E2-treated mutants exhibited 21.3 ± 6.2 discharges per hour ($n = 9$), a 93% decrease in the treated group (two-tailed t test, $P = 0.0383$). Seizures were also decreased. The median seizure incidence in the control mutants was 1.0 seizure per mouse (range, 0 to 12; $n = 11$), whereas E2-treated mutants displayed a median of zero seizures per mouse (range, 0 to 1; $n = 9$) (two-tailed Mann-Whitney, $P = 0.0027$) (table S1). Fewer individuals treated with E2 exhibited seizures. We found that 9 of 11 control mutants had at least one seizure, whereas only 1 of 9 E2-treated mutants displayed one seizure (two-sided Fisher's exact, $P = 0.0055$). The reduction in spikes and seizures with E2 treatment indicated that early, prolonged activation of ERs exerts a robust and enduring anti-epileptogenic effect on $Arx^{(GCG)10+7}$ mutants.

To determine whether this disease-modifying effect was developmentally sensitive and persisted as the animal grew, we designed two complementary protocols. We injected E2 or vehicle into neonatal littermates for only 1 week, either early, between days P3 and P10, or later, between days P33 and P40, and then monitored the mice by VEEG in adulthood (Fig. 1A). Early-treated (P3 to P10) $Arx^{(GCG)10+7}$ mutants ($n = 8$) exhibited 64% fewer abnormal cortical discharges than vehicle-treated mutants ($n = 11$; one-tailed t test, $P = 0.0200$) (Fig. 1, B and D, and table S2). Seizure incidence in the treatment group was significantly decreased ($n = 8$ to 11; one-tailed Mann-Whitney, $P = 0.0221$; Fig. 1E and table S2). As we found with the prolonged E2 treatment protocol, fewer individuals treated with early E2 showed seizures. We found that only 1 of 8 of the treated mutants displayed one seizure, whereas 6 of 11 vehicle-treated controls had seizures, but this did not reach statistical significance (two-sided Fisher's exact, $P = 0.1473$). In contrast, there was no difference in the number of spike discharges (one-tailed t test, $P = 0.3380$) or seizure incidence (one-tailed Mann-Whitney, $P = 0.2170$) between late E2-treated (P33 to P40) $Arx^{(GCG)10+7}$ mutants ($n = 9$) and vehicle-treated mutants ($n = 9$) (Fig. 1, F and G, and table S3). Wild-type mice treated with E2 either early or late did not exhibit any seizures or cortical spike discharges ($n = 6$). Seizures and spikes are unprovoked spontaneous events recorded from freely moving mice; the variability in the data shown here reflects their unpredictable nature in epileptic brains. These results indicate that brief, early post-natal E2 stimulation is sufficient to limit cortical hyperexcitability in $Arx^{(GCG)10+7}$ mutants, whereas treatment of adult mutants has little effect, suggesting that E2's protective effects depend on exposure during a critical developmental period.

Effects of selective ER- α and ER- β agonists

The partially overlapping expression patterns of the ER isoforms, ER- α and ER- β , in developing brain indicate that either receptor isoform could be mediating the antiepileptogenic effect (19, 21). We explored this possibility by treating $Arx^{(GCG)10+7}$ littermates between P3 and P10 with an ER- α -selective agonist [propylpyrazoletriol (PPT)], an ER- β -selective agonist [s-diarylpropionitrile (s-DPN)], or both in combination. Treatment with either agonist alone failed to significantly reduce hyperexcitability (Fig. 2B and table S4). However, when combined, PPT and s-DPN significantly decreased spike discharges by 77% [$n = 9$; one-way analysis of variance (ANOVA), $F = 3.431$, $df = 3$, $P = 0.0278$; Fig. 2B and table S4], similar to the decrease produced by early E2 treatment. Seizure incidence in the combined treatment group was decreased compared to the vehicle control group but did not reach statistical significance (Kruskal-Wallis, $P = 0.1083$). The number of mutants per treatment group that displayed seizures was 8 of 12 (vehicle), 4 of 8 (s-DPN), 2 of 9 (PPT), and 1 of 9 (PPT and s-DPN combined) (Fig. 2C and table S5) (χ^2 contingency, $\chi^2 = 8.236$, $df = 3$, $P = 0.0414$). Because ER- α and ER- β have different affinities for their respective agonists (31), the fact that we used equimolar dosages make a direct comparison of the effectiveness between PPT and s-DPN difficult. Nevertheless, these results suggest that both ER- α and ER- β contribute to an overall antiepileptogenic effect and that neither agonist appears to exacerbate the epilepsy phenotype. Crossbreeding $Arx^{(GCG)10+7}$ mutants with ER knockout mice may be necessary to dissect their individual contributions.

Effect of early E2 treatment on infantile spasms in mutants

Between P7 and P11, $Arx^{(GCG)10+7}$ mutant pups transiently exhibit abrupt, massive truncal flexor contractions involving all four limbs, mimicking the infantile spasms provoked by the same *ARX* mutation in humans (10, 17). We examined whether early E2 treatment altered this phenotype in $Arx^{(GCG)10+7}$ mutant pups. Although 5 of 16 vehicle-treated mutants displayed spontaneous motor spasms during the 30-min video-monitoring period, none of the 16 E2-treated mutants exhibited this behavior (Fisher's exact, $P = 0.0434$; Fig. 3, movie S1, and table S6), indicating that early E2 treatment of $Arx^{(GCG)10+7}$ mutants decreased the occurrence of motor spasms. Wild-type littermates displayed no spasms.

Effect of E2 treatment on cortical interneurons

We observed an antiepileptogenic effect only in the early treatment group, in which E2 was administered before interneuron migration, synaptogenesis, and maturation are complete. This is consistent with the hypothesis that E2 intervention might modify regional circuit defects associated with the $Arx^{(GCG)10+7}$ mutation. To evaluate this possibility, we quantified interneurons immunolabeled for neuropeptide Y (NPY), calbindin_{28K} (Cb), and parvalbumin (Pv) in the somatosensory cortex of adult mice early-treated with E2 or vehicle and found that E2 restored the immunostaining profile in some of the affected interneuronal pools. Compared to wild-type mice, vehicle-treated mutants showed a 36% decrease in the cortical NPY-positive interneuron density, whereas E2-treated mutants displayed an increase of 30% in NPY-positive neuron density across all cortical layers ($n = 8$; one-way ANOVA, $F = 9.662$, $df = 2$, $P = 0.0011$; Fig. 4, A to D, and table S7). Cortical Cb-positive

interneurons are normally found in a nonuniform laminar distribution, with layers 1 to 4 displaying a higher density of cell bodies than layers 5 to 6 (Fig. 4, E to G). Our analysis revealed a significant 42% increase in the Cb-positive cell density in layers 5 to 6 compared to wild type ($n = 10$; one-way ANOVA, $F = 7.612$, $df = 2$, $P = 0.0024$; Fig. 4H and table S8). In layers 1 to 4, there was no difference in Cb-positive cell density between vehicle-treated mutants and wild type (Fig. 4H). Finally, Pv-positive interneurons, which are spared in $Arx^{(GCG)10+7}$ mutants (17), were not significantly altered by E2 (Fig. 4, I to L, and table S9). Analysis of wild-type mice treated with E2 from P3 to P10 showed a 37% increase in Cb interneurons of cortical layers 5 to 6 compared to wild-type control but not in other cell types. Treated wild-type mice had 232.3 ± 11.8 cells/mm² and wild-type controls displayed 169.9 ± 13.6 cells/mm² ($n = 4$; two-tailed t test, $P = 0.0130$). These results suggest that E2 promotes recovery of the wild-type immunostaining profile among some of the interneurons affected by the Arx mutation. The effects of E2 were also detected in wild-type mice, indicating a mutation-independent effect of E2 on the Cb-positive interneuron population.

We considered whether E2 treatment of $Arx^{(GCG)10+7}$ mutant cortex induced sprouting of inhibitory neuron terminals, thus providing a structural basis for its prolonged antiepileptogenic effect. To investigate this, we examined the anatomical distribution of punctate staining for vesicular glutamic acid transporter (VGAT), a specific marker of GABAergic terminals, in the somatosensory cortex of wild-type, early E2-treated, and vehicle-treated mutants (32). No significant macroscopic differences in overall terminal laminar density were found among these groups (fig. S1 and table S12). We also found no significant difference in VGAT mRNA levels among all groups (table S14). Terminal innervation patterns from a small subset of Arx -positive interneurons might still escape detection amidst the larger remaining interneuron pool, so it remains possible that E2 induced collateral sprouting from impaired neurons, as well as reactive axonal sprouting and synaptogenesis from neighboring unaffected interneurons (33–36).

In the hippocampus, hilar NPY interneurons as well as ectopic NPY expression in mossy fibers can be induced by E2 and inhibit epileptic network activity (25, 37). We examined this possibility in our animals, and despite the rescue of cortical NPY⁺ interneurons, we found no change in their number in the hippocampus ($n = 6$ to 9; one-way ANOVA, $F = 0.2380$, $df = 2$, $P = 0.7907$) or enhancement of mossy fiber expression in E2- or vehicle-treated Arx mutants (fig. S2 and table S11). These results suggest that the E2-induced increase in cortical NPY immunostaining likely reflects restorative cell-autonomous effects on Arx -deficient interneurons rather than a global induction of NPY expression.

Effect of E2 treatment on striatal cholinergic interneurons

Striatal cholinergic interneurons are reduced in Arx^{KO} and Arx expansion mouse models (17, 38, 39). Quantification of choline acetyl-transferase (ChAT)-positive striatal interneurons in $Arx^{(GCG)10+7}$ mutants (P3 to P10) revealed that vehicle-treated mutants displayed a 28% decrease in ChAT-positive cells relative to wild-type mice, whereas E2-treated mutants had essentially the same number as wild type ($n = 10$ to 14; one-way ANOVA, $F = 7.621$, $df = 2$, $P = 0.0020$; Fig. 5 and table S10). Thus, early E2 treatment restored normal cholinergic immunoreactive cell profiles in the $Arx^{(GCG)10+7}$ model. These

cells modulate the excitability of medium spiny neurons, the primary output of the striatum (40); cholinergic dysfunction is implicated in the pathophysiology of dyskinesias, including spasms (41). Thus, the recovery of ChAT immunoreactive profiles in response to E2 is consistent with a prominent role for cholinergic interneurons in spasm generation. Similar analysis of striatal NPY interneurons, previously found to be reduced in mutants (17), showed no treatment effect. Compared to wild-type mice, which showed 22.7 ± 3.4 NPY cells/mm², vehicle-treated mutants displayed 13.9 ± 1.6 cells/mm² and E2-treated mutants exhibited 16.9 ± 2.6 cells/mm² ($n = 7$ to 8 ; one-way ANOVA, $F = 2.701$, $df = 2$, $P = 0.0916$).

Effect of E2 treatment on mRNA levels of Arx targets

We next evaluated whether the E2-induced phenotypic improvement of *Arx*^{(GCG)10+7} mice could be attributed to ER-driven transcriptional pathways that converge on *Arx* downstream targets. *Arx* represses *Ebf3* (early B cell factor), *Lmo1* (LIM domain only 1), and *Shox2* (short stature homeo-box 2) and activates *Lhx7(8)* (LIM homeo-box 7), *Cxcr4* (chemokine receptor 4), *Cxcr7* (chemokine receptor 7), and *Lgi1* (leucinerich glycine inactivated 1) expression in interneurons (11, 13, 15). Quantitative transcriptional analysis of embryonic stem cells differentiated into neurons has demonstrated that a triplet repeat expansion similar to that of our mouse model in *Arx* protein leads to selective loss of its repressive activity (42). Whether *Arx*-mediated transcriptional regulation is disrupted as a result of abnormal *Arx* protein in postnatal brain is unknown. We used quantitative reverse transcription polymerase chain reaction (qRT-PCR) (43) to determine the mRNA levels of *Arx* and seven of its downstream transcriptional targets, plus brain-derived neurotrophic factor (*Bdnf*) and aromatase (*Cyp19*), in the forebrain at age P3 (the starting point for E2 intervention), thus providing a baseline for comparison. Only one, *Shox2*, was significantly up-regulated by 92% in *Arx*^{(GCG)10+7} mutants compared to wild type ($n = 5$ to 7 ; two-tailed t test, $P = 0.0047$; Fig. 6A and table S13). To determine the effects of early E2, we analyzed the brains of mice treated from P3 to P10 on day 11. At that age, there were no differences between wild-type mice and vehicle-treated mutants (Fig. 6B and table S14), suggesting that transcriptional regulation of these genes by *Arx* is restricted to embryonic and neonatal periods (15). E2 treatment decreased *Shox2* mRNA expression by 73% relative to that of wild-type mice ($n = 5$ to 7 ; one-way ANOVA, $F = 6.74$, $df = 2$, $P = 0.0076$). *Ebf3* mRNA expression was repressed in E2-treated mutants by 36% compared to wild type ($n = 7$; one-way ANOVA, $F = 3.807$, $df = 2$, $P = 0.0418$). Mutant treatment with E2 resulted in up-regulation of *Lgi1* mRNA expression by 63% compared to wild type ($n = 6$; one-way ANOVA, $F = 18.39$, $df = 2$, $P < 0.0001$). These results suggest that ER activation converges on *Arx* downstream targets and that modulation of *Shox2*, *Ebf3*, and *Lgi1* expression could participate in the antiepileptogenic effect of E2 (Fig. 6B).

We examined whether brain expression of aromatase (*Cyp19*), the enzyme that converts testicular testosterone into E2, was altered in vehicle- and E2-treated mutants compared to wild-type mice. Our analysis found no differences between any of the treatment groups at either age P3 or P11, suggesting that the baseline ability to convert testosterone into E2 is not likely to be altered by the *Arx* mutation or E2 treatment (Fig. 6, A and B). Direct quantification of E2 and aromatase activity will be needed to test this possibility.

We also investigated the mRNA expression of other downstream targets of *Arx* (Fig. 6, A and B). *Lhx7(8)* is necessary for cholinergic fate determination in striatal interneurons (44). We tested whether E2 up-regulated *Lhx7(8)* expression and therefore might have played a role in the normalization of ChAT immunoreactivity in the mutant *Arx*^{(GCG)10+7} striatum. Similarly, we examined whether the expression of *Bdnf*, a pro-NPY growth factor in adult rodents (45), was altered by E2 treatment of *Arx*^{(GCG)10+7} mutants. No differences in the expression levels of either *Lhx7(8)* or *Bdnf* in any of the groups tested were detected, suggesting that their regulation does not make a major contribution to the *Arx*^{(GCG)10+7} phenotype or the E2 effect. Likewise, we found no treatment effect on the expression of the chemokine receptor genes *Cxcr4* and *Cxcr7* in *Arx*^{(GCG)10+7} mutants.

DISCUSSION

ISSX is a prototypical epileptic encephalopathy, a disorder of assembly and maturation of neural networks, and many of its debilitating clinical manifestations emerge at a time when it is believed to be too late to change the course of the disease. We show here that the plasticity of developing neurons in the neonatal brain presents an opportunity for clinical management by stimulating ER signaling. Although our understanding of the roles of estrogenic hormones (E2, estrone, and estriol) in brain development remains incomplete, human studies show that poor developmental outcomes that are associated with sudden loss of maternal estrogens in prematurely born infants can be improved by postnatal estrogenic supplementation (46). [Although the doses used in that study (46) and ours are comparable to fetal physiological plasma levels of E2, estrogen is a developmentally active hormone, so the possibility of undesirable long-term effects should be investigated before any therapeutic application is considered.]

We found that early, brief postnatal E2 administration suppresses infantile motor spasms and adult epilepsy while restoring diminished interneuron populations in male *Arx*^{(GCG)10+7} mutants. The E2 stimulation (P3 to P10) began shortly before, but outlasted the peak appearance (P7 to P12) of, motor spasms in our model. Thus, once spasms are detected, immediate E2 delivery could potentially be an effective treatment regimen in patients with ISSX. Moreover, the presence of immunoreactive interneurons within somatosensory cortex and striatum, along with the rescue of the epileptic phenotype more than a month after the E2 treatment ceased, indicates that long-lasting functional changes mediated by genomic mechanisms are likely to contribute to E2's effect on these, and potentially other, genetic disturbances of interneuron maturation.

Early postnatal E2 treatment increased the immunoreactivity for NPY, Cb, and ChAT interneurons in *Arx* mutants. This finding could be a result of an increase in the number of migrating interneurons that attain their correct positions [as reported for songbird neostriatum (47)], an increase in the amount of protein synthesis in each cell, or enhanced cell survival; these alternatives are not mutually exclusive. Price *et al.* (17) did not report a decrease in NPY cortical interneurons in these mice at age 4 weeks; the age difference in the mice may at least partially account for this discrepancy. E2 also has effects on wild-type interneurons. Our analysis showed an increase in layers 5 to 6 cortical Cb-positive interneurons, suggesting a laminar specificity of E2's effects on these interneurons. Chronic

E2 administration can increase NPY immuno-reactivity in both hippocampal and cortical interneurons (25, 48), whereas ER- β has been implicated in cortical interneuron migration (26, 49), and ER- α participates in neuroprotection via crosstalk with insulin-like growth factor 1 receptors (50). Alternative biochemical markers such as GAD_{65/67} and GABA are not substantially better than the endogenous neuropeptides or calcium-binding proteins for quantifying absolute interneuron density because they are also dynamically regulated. Genetic tagging of *Arx*^{(GCG)10+7}-expressing neurons with stable cellular reporters may be required to distinguish between these hypotheses.

Inherited interneuronopathies such as ISSX would be predicted to result in a corresponding loss of inhibitory synapses and functional dys-inhibition. Surprisingly, we found no evidence of overall altered number or laminar distribution of VGAT-containing puncta in adult E2-treated cortical synaptic terminals, or expression of synaptic terminal markers. It is possible that the affected terminal population is too small to discern, or that compensatory axonal sprouting, synaptic repositioning, or dendritic remodeling by other cell types had a partially homeostatic effect that was sufficient to prevent the emergence of hypersynchronization and epilepsy (33–36). From a functional standpoint, GABAergic neuro-transmission may also be impaired and contribute to seizures; however, quantification of synaptic inhibition in a multilayered cortical micro-circuit is complex. This issue was investigated at the single-cell level in *Dlx1*-null mice, which also display interneuron loss and epilepsy (51). Although layer II/III and CA1 adult pyramidal neurons showed decreased input of spontaneous inhibitory postsynaptic potential frequency and amplitude, inhibitory evoked responses were unaltered. In addition, immature interneurons were more excitable in this model than in wild-type mice, suggesting that inhibition may actually increase during development (52). Interneurons also inhibit each other, leading to nonlinear contributions within a cortical circuit (53). In the absence of a change in cortical GABAergic terminal density, as described here, the precise circuit mechanisms by which *Arx* mutations produce, and E2 prevents, epilepsy in cortical networks are difficult to isolate by analyzing single cells. New optogenetic techniques may help to better understand the contribution of specific impaired interneuron ensembles to overall network inhibitory strength and abnormal oscillatory behavior in the epileptic brain (54).

The genes targeted by *Arx* may vary across development. In *Arx*-null embryos, the transcription factors *Ebf3*, *Lmo1*, and *Shox2* are activated, whereas the promigratory chemokine receptors *Cxcr4* and *Cxcr7* are repressed, compared to wild type (11, 13, 55). In our *Arx*^{(GCG)10+7} mice, only *Shox2* was up-regulated in 3-day-old (P3) mutants compared to age-matched wild-type mice. In mice aged 11 days (P11), E2 repressed the mRNA levels of *Shox2* and *Ebf3*, whereas *Lgi1* expression was strongly up-regulated compared to wild-type and vehicle-treated mutants, suggesting partial convergence of ER and *Arx* pathways. *Shox2* is necessary for the normal development of TrkB-positive dorsal root ganglia neurons in the spinal cord, whereas it is strongly repressed in the normally developing telencephalon, suggesting that its aberrant telencephalic expression, as seen in the *Arx*^{(GCG)10+7} mutant, may contribute to the epileptic phenotype (56). In differentiated embryonic stem cells, mutant expanded *Arx* protein (*Arx*^E) derepressed *Ebf3*, but not *Lmo1* and *Shox2*. In *Arx*^{(GCG)7} mice, ectopic *Ebf3* expression in developing interneurons disrupted their

migration, whereas *Lmo1* and *Shox2* remained repressed (42). Normally, *Ebf1/2/3* genes appear to control embryonic migration of Cajal-Retzius cells (57). *Lgi1* is a secreted protein expressed by central nervous system neurons that participates in synapse organization and neuronal growth (58, 59), and loss-of-function mutations are associated with temporal lobe epilepsy in human patients (60). Nuclear localization of *Lgi1* in caudal ganglionic eminence cells, a major source of interneuron precursors, suggests that it may play a role in interneuron development (61).

Although much has been learned about the role of *Arx* in inter-neuron migration and maturation at critical ages, the large number of transcriptional targets and endophenotype complexity are reminders that our understanding of the epileptogenic role of this pleiotropic transcription factor is only beginning (62, 63). The findings described here identify early disease-modifying effects of gene expression remodeling by transient stimulation with an endogenous steroid hormone and a promising avenue for exploring new treatment options for a devastating infantile epilepsy syndrome.

MATERIALS AND METHODS

Study design

Sample size was estimated with a power level set at 0.8 to detect an effect of at least 50% for each variable tested. Although this study was not powered to detect small changes in outcomes, the goal was to ensure that the detected effect of the intervention was robust. Endpoints for EEG monitoring experiments were selected endophenotypes associated with epilepsy in *Arx*^{(GCG)10+7} mutants, namely, electrographic seizures and interictal cortical spike discharges (sample size, 8 to 11). The endpoint for immunohistochemistry experiments was the number of immunolabeled cells per square millimeter (sample size, 6 to 14) or the number of immunolabeled VGAT-positive puncta per brightfield (sample size, 4). The gene expression assay endpoint (qRT-PCR) was the relative expression of selected gene products relative to control (*Gapdh*). All qRT-PCR samples were run as triplicates in a 96-well qRT-PCR plate and then averaged before analysis (sample size, 5 to 8). The pre-specified hypothesis in this study was that exogenous E2 stimulation to the *Arx*^{(GCG)10+7} mutant would have a protective effect against epilepsy. After the confirmation of this hypothesis, we tested whether (i) the protective effect of E2 against epilepsy was developmentally sensitive, (ii) E2 had a protective effect against spasms in infancy, (iii) E2 altered the inter-neuron populations affected by the mutation, and (iv) E2 altered expression of downstream targets of *Arx*.

Arx^{(GCG)10+7} mice were maintained on amixed C57BL/6/129S5/SvEvBrd background as described (17). Mice were housed under constant temperature and humidity, with a 7:00 a.m. to 7:00 p.m. light/dark cycle. Animal care and use conformed to the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* and was approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

All experiments in this study were designed as controlled laboratory experiments. E2 or vegetable oil (control) was injected subcutaneously into mutant and/or wild-type mice. E2-treated mice were used for EEG experiments, spasm monitoring, immunohistochemistry, or

qRT-PCR experiments. Mice treated with ER-selective agonists were used for EEG experiments only. Experimenters were blinded to mouse genotype during the treatment and data collection periods by assigning an alphanumeric code to each sample.

Drug injections

Male mice were studied. E2 (Sigma) was diluted in vegetable oil and injected daily subcutaneously on P5 to P40, P3 to P10, or P33 to P40. The E2 dose used was 40 ng/g. Therefore, mouse pups aged 3 days (P3) and weighing 1.5 g received 60 ng of E2. On day P40, mice weighing 25 g received 1000 ng of E2 subcutaneously. Littermate controls were injected with oil only. In other groups, the ER-selective agonists PPT (1 mg/kg) and s-DPN (1 mg/kg), which selectively target ER- α and ER- β , respectively, were diluted in β -cyclodextrin and injected subcutaneously between P3 and P10.

Video-electroencephalography

VEEG monitoring was conducted according to established methods (17). Briefly, 6-week-old mice were implanted with Teflon-coated silver wire electrodes 2 mm in diameter, which are soldered to a micro-miniature connector. Before surgery, mice were anesthetized by an intraperitoneal injection of Avertin (0.02 ml/g). Electrodes were inserted in the subdural space over frontal, parietal, and temporal cortices and secured with superglue and dental cement. Mice were allowed to recover for at least 48 hours before monitoring (Harmonie v. 6.1cEN-0, Stellate Systems). VEEG monitoring was performed while mice moved freely in the cage. Each mouse was recorded for three periods of 4 hours over a span of 3 weeks, for a total of 12 hours. The experimenter was blinded to genotype and treatment. Electrographic seizures were defined as high-amplitude ($1.5 \times$ baseline EEG trace), repetitive EEG activity involving at least two nonneighboring channels for at least 10 s. Interictal spikes are defined as transient, high-amplitude ($2 \times$ baseline) EEG discharges, lasting no more than 400 ms and present in at least two nonneighboring channels.

Immunohistochemistry

Pairs consisting of one wild-type male and one vehicle- or E2-treated $Arx^{(GCG)10+7}$ mutant male were used for each experiment. Cryosections (50 μ m thick) of brains fixed with 4% paraformaldehyde and cryoprotected with 30% sucrose were used for free-floating immunohistochemistry. The primary antibodies used were mouse anti-Cb at 1:3000 (catalog no. 300, Swant), mouse anti-Pv at 1:1000 (PARV-19, Sigma-Aldrich), rabbit anti-NPY at 1:3000 (N9528, Sigma-Aldrich), goat anti-ChAT at 1:200 (AB144P, Millipore), and mouse anti-VGAT at 1:200 (131011, Synaptic Systems). Avidin-biotin-peroxidase complex immunostaining was done with Elite ABC Kits (Vector Laboratories). 3,3'-Diaminobenzidine substrate reactions were started and stopped simultaneously for both treatment and control sections. Sections were mounted on glass slides and analyzed by light microscopy with an Olympus model IX71 inverted microscope. Images were acquired with a digital camera (Olympus model C-5050) and analyzed with ImageJ software (NIH). Immunolabeled cell bodies were counted by adjusting the contrast threshold with the Particle Analyzer tool available in ImageJ. For each pair of wild type and treated mutant, the threshold, size, and circularity values were applied identically. Cell counts were obtained

from three nonconsecutive sections of striatum between bregma 0.5 and 1.0 and hippocampus and neocortex between bregma 2.0 and 2.5 (64). NPY- and Pv-positive cortical cells counted within 1.0-mm² windows of somato-sensory cortex were expressed as cells per square millimeter. Cb-positive cortical cells from layers 1 to 4 and layers 5 to 6 were counted within 0.5-mm² windows and expressed as cells per square millimeter. Striatal cells counted within the entire 10× brightfield (2.2 mm²) were expressed as cells per square millimeter. Hippocampal hilar cell counts were performed within the area defined by the subgranular zones and a straight line connecting the superior and inferior blades of the dentate gyrus, and expressed as cells per 0.5 mm². All immunohistochemistry was analyzed while the experimenter was blinded to the genotype and treatment of brain sections.

Behavioral analysis

Spontaneous movements and spasms were assessed in infant mice. Male pups from five different litters per treatment group were tested on P10, an age when massive spasms have been noted previously (17). Each pup was marked with a felt pen and placed in a clear plastic tray subdivided into smaller compartments measuring 5.5 × 5.5 cm. The pups were allowed to acclimate for 10 min and then monitored during a 30-min, time-stamped, digital video recording. The recordings were made during the lights-on period in a humidified, 35°C temperature-controlled environment, between 2:00 p.m. and 4:00 p.m. Movements consisting of major truncal flexion, abdominal contractions, bowing or axial twisting of the body, or simultaneous strong movements of three or more appendages were later quantified by an observer blinded to genotype and treatment condition, as described previously (17).

Quantitative real-time PCR

Mouse forebrains were dissected and homogenized in TRIzol (Invitrogen) and chloroform for mRNA extraction. Experimenters were blinded to genotype and treatment conditions by labeling samples with a nonidentifying code. After centrifugation, the nucleic acid-containing clear phase was transferred and precipitated with isopropyl alcohol. After centrifugation, the nucleic acid pellet was washed with 75% ethanol and resuspended in ribonuclease-free water. The presence of RNA was confirmed by detecting ribosomal RNA in an agarose gel. Samples that failed this criterion were excluded. After treatment with DNA-free Kit (Ambion), reverse transcription was performed with Phusion Kit (Thermo Fisher Scientific) according to the manufacturer's specifications. As a control, the reactions were repeated without the presence of the reverse transcriptase enzyme ("NoRT"). The presence of complementary DNA (cDNA) was confirmed by PCR using primers that targeted exon regions only. NoRT controls yielded no visible bands in the agarose gel. Samples that failed to meet these criteria were excluded. The cDNA was stored in -20°C. qRT-PCR was performed in a Real-Time PCR System (model 7500, Applied Biosystems). All probes were obtained from Applied Biosystems. *Gapdh* was used as the endogenous control gene. For every sample, NoRT and "no template" controls were added. Relative gene expression was analyzed according to standard methods (43).

Statistical analysis

Unless otherwise noted, data are expressed as means $\pm$ SEM. Prism 5 for Mac OS X statistical software version 5.0d (GraphPad) was used. Normal distribution of the data was confirmed with D'Agostino-Pearson omnibus test available in Prism 5. Student's *t* test (one- and two-tailed, $\alpha = 0.05$) was used to evaluate spike discharges. Mann-Whitney test (one- and two-tailed, $\alpha = 0.05$) was used to evaluate seizure incidence between two groups. One-way ANOVA ($\alpha = 0.05$) followed by Dunnett's multiple comparisons test was used to compare spike discharges across multiple mouse groups treated with ER-selective agonists. Because these data had unequal variances, they were log-transformed before performing the one-way ANOVA. Kruskal-Wallis test was used for comparing seizure incidence across multiple groups ($\alpha = 0.05$). One-way ANOVA ($\alpha = 0.05$) was used to evaluate cell counts, followed by Dunnett's multiple comparisons test. Two-way ANOVA ($\alpha = 0.05$) was used to evaluate VGAT-positive puncta density across the depth of the cortex. Fisher's exact test ($\alpha = 0.05$) was used to evaluate spontaneous spasms by pups and the presence versus absence of seizures in E2-treated mutants. χ^2 contingency test ($\alpha = 0.05$) was used to evaluate the presence versus absence of seizures in mutants treated with ER agonists (s-DPN and PPT). Student's *t* test (two-tailed, $\alpha = 0.05$) or one-way ANOVA ($\alpha = 0.05$) followed by Tukey's post hoc test was used to evaluate qRT-PCR data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Neonatal E2 prevents epilepsy in *Arx*^{(GCG)10+7} mutants

(A) Experimental design showing E2 injection and VEEG recording protocols. (B) EEG traces depicting spike discharges from an early vehicle-treated mutant (top) and an early E2-treated mutant (bottom). (C) Representative electrographic seizure in an early vehicle-treated *Arx*^{(GCG)10+7} mutant. (D) Summary of the number of cortical interictal spikes (discharges) in early vehicle-treated mutants (Veh) and E2-treated mutants (one-tailed *t* test, $P = 0.0200$). (E) Total number of seizures per early-treated mouse (Veh and E2) sampled over a 3-week period (one-tailed Mann-Whitney, $P = 0.0221$). (F) Summary of the number of cortical interictal spikes in late vehicle-treated mutants (Veh) and E2-treated mutants sampled over a 3-week period (one-tailed *t* test, $P = 0.3380$). (G) Total number of seizures per mouse (late treatment) over a 3-week period (one-tailed Mann-Whitney, $P = 0.2170$). * $P < 0.05$. NS, not significant.

Fig. 2. ER- α - and ER- β -selective agonists have antiepileptogenic effect

(A) Experimental design showing timeline of selective ER agonist treatment and VEEG monitoring. (B) Summary of cortical interictal discharges per hour recorded over a 3-week period from *Arx*^{(GCG)10+7} mutants treated with vehicle (Veh), PPT (ER- α -selective agonist), s-DPN (ER- β -selective agonist), or a combination of PPT and s-DPN (one-way ANOVA, Dunnett's post hoc test, $F = 3.431$, $df = 3$, $P = 0.0278$). (C) Summary of total number of electrographic seizures captured per mouse treated with vehicle (Veh), PPT, s-DPN, or PPT and s-DPN sampled over a 3-week period (Kruskal-Wallis, Dunn's post hoc test, $P = 0.1083$). * $P < 0.05$.

Fig. 3. E2 reduces motor spasms in *Arrx*^{(GCG)10+7} mutant pups

(**A**) Still frames selected from a digital video (movie S1) depict an early vehicle-treated *Arrx*^{(GCG)10+7} mutant pup aged 10 days (bottom row) having a 5-s motor spasm involving sustained flexion of all limbs. The top row shows a wild-type (WT) mouse. Major spasms were never observed in any E2-treated mutants. (**B**) Number of mutant pups treated early with vehicle or E2 that displayed motor spasms as defined in Materials and Methods ($n = 16$; $*P = 0.0434$, Fisher's exact test).

Fig. 4. E2 increases immunoreactive cortical interneurons in *Arx*^{(GCG)10+7} mutants
 (A to C) Representative cortical sections displaying NPY-positive cortical interneurons in (A) a WT mouse, (B) a vehicle-treated mutant, and (C) a E2-treated mutant. (D) Summary of NPY cell counts (mean ± SEM; one-way ANOVA, $F = 9.662$, $df = 2$, $P = 0.0011$). (E to G) Cb-positive cortical interneurons. (H) Summary of Cb cell counts in layers 1 to 4 (one-way ANOVA, $F = 1.982$, $df = 2$, $P = 0.1581$) and layers 5 to 6 (one-way ANOVA, $F = 7.612$, $df = 2$, $P = 0.0024$). (I to K) Pv-positive cortical interneurons. (L) Summary of Pv-positive cell counts (one-way ANOVA, $F = 0.0529$, $df = 2$, $P = 0.9486$). Scale bar, 200 μ m. * $P < 0.05$ (Dunnett's post hoc test; WT control).

Fig. 5. E2 increases profile of ChAT-immunopositive striatal interneurons in *Arx*^{(GCG)10+7} mutants

(A to C) Representative striatal sections showing ChAT-positive interneurons from vehicle-treated mutant (A), E2-treated mutant (B), and WT mice (C). (D) Summary of ChAT-positive cell counts (one-way ANOVA, $F = 7.621$, $df = 2$, $P = 0.0020$). Scale bar, 200 μ m.

* $P < 0.05$ (Dunnett's post hoc test; WT control).

Fig. 6. E2 represses the expression of *Arx* downstream targets *Shox2* and *Ebf3* and increases *Lgi1* expression in *Arx*^{(GCG)10+7} mutants

(A) Whole-forebrain mRNA levels of *Arx* and seven of its downstream targets plus *Bdnf* in untreated *Arx*^{(GCG)10+7} mutants aged 3 days compared with those of WT mice [internal control; glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*)]. (B) Relative mRNA levels of *Arx* downstream targets plus *Bdnf* and *Cyp19* in *Arx*^{(GCG)10+7} mutants aged 11 days treated daily with vehicle or E2 from P3 to P10 compared with WT (internal control; *Gapdh*). * $P < 0.05$ (Tukey's post hoc test).