In inherited neurodevelopmental diseases, pathogenic processes unique to critical periods during early brain development may preclude effectiveness of gene modification therapies applied later in life. We explored this question in a mouse model of DYT1 dystonia, a neurodevelopmental disease caused by a loss-of-function mutation in the \textit{TOR1A} gene encoding torsinA. To define the temporal requirements for torsinA in normal motor function and gene replacement therapy, we developed a mouse line enabling spatiotemporal control of the endogenous torsinA allele. Suppressing torsinA during embryogenesis caused dystonia-mimicking behavioral and neuropathological phenotypes. Suppressing torsinA during adulthood, however, elicited no discernible abnormalities, establishing an essential requirement for torsinA during a developmental critical period. The developing CNS exhibited a parallel “therapeutic critical period” for torsinA repletion. While restoring torsinA in juvenile DYT1 mice rescued motor phenotypes, there was no benefit from adult torsinA repletion. These data establish a unique requirement for torsinA in the developing nervous system and demonstrate that the critical period genetic insult provokes permanent pathophysiology mechanistically delinked from torsinA function. These findings imply that to be effective, torsinA-based therapeutic strategies must be employed early in the course of DYT1 dystonia.
TorsinA restoration in a mouse model identifies a critical therapeutic window for DYT1 dystonia

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ABSTRACT

In inherited neurodevelopmental diseases, pathogenic processes unique to critical periods during early brain development may preclude effectiveness of gene modification therapies applied later in life. We explored this question in a mouse model of DYT1 dystonia, a neurodevelopmental disease caused by a loss-of-function mutation in the \textit{TORIA} gene encoding torsinA. To define the temporal requirements for torsinA in normal motor function and gene replacement therapy, we developed a mouse line enabling spatiotemporal control of the endogenous torsinA allele. Suppressing torsinA during embryogenesis caused dystonia-mimicking behavioral and neuropathological phenotypes. Suppressing torsinA during adulthood, however, elicited no discernible abnormalities, establishing an essential requirement for torsinA during a developmental critical period. The developing CNS exhibited a parallel “therapeutic critical period” for torsinA repletion. While restoring torsinA in juvenile DYT1 mice rescued motor phenotypes, there was no benefit from adult torsinA repletion. These data establish a unique requirement for torsinA in the developing nervous system and demonstrate that the critical period genetic insult provokes permanent pathophysiology mechanistically delinked from torsinA function. These findings imply that to be effective, torsinA-based therapeutic strategies must be employed early in the course of DYT1 dystonia.
INTRODUCTION

During critical periods, the normally developing nervous system is uniquely sensitive to sensory stimuli that drive circuit plasticity. A unique landscape of molecular and circuit activities is responsible for critical periods. This special landscape also renders the maturing nervous system uniquely vulnerable to specific insults and receptive to particular therapeutic interventions.

A limited number of examples of critical period vulnerability to a pathogenic insult have been defined in mouse models of disease. Embryonic deletion of the Angelman syndrome protein Ube3a causes abnormal mouse behavioral phenotypes, while removal from juvenile (3-week-old) or adult (12-week-old) mice does not significantly influence behavior (1). A selective developmental window of vulnerability to neurodegeneration exists for ethanol exposure, corresponding to the period of rapid synaptogenesis (2, 3). Sensitivity to Smn1 loss in spinal muscular atrophy models is exclusive to an early developmental period prior to the maturation of neuromuscular synapses (4). Beyond simply delineating critical periods of vulnerability, these studies illustrate how such efforts can improve understanding of disease pathogenesis by linking pathogenic insults to specific neurodevelopmental processes. Some neurodevelopmental diseases impair the CNS without temporal selectivity, however. Loss of MeCP2 in mice produces Rett syndrome phenotypes whether initiated during early development or in adulthood (5).

Early pathogenic events that selectively disrupt developmental processes can produce a cascade of events that cause permanent circuit dysfunction mechanistically distinct from the initial insult. Paralleling the period of vulnerability, juvenile re-expression of Ube3a expression rescues a broad range of behavioral phenotypes in Angelman syndrome models – but adult re-expression is much less efficacious (6). Analogous to MeCP2 inducing abnormal phenotypes when deleted from juvenile or adult mice, genetic restoration at any age is efficacious (7).

Dystonia is a CNS disease that manifests as abnormal involuntary twisting. Considerable evidence implicates striatal dysfunction in dystonia pathogenesis (8-12). The natural history of DYT1 dystonia, an inherited form of the disease, strongly suggests a critical period of vulnerability, but this question has not been tested experimentally. The disease is incompletely penetrant, with only ~1/3 of mutation carriers developing symptoms (13, 14). Symptom onset typically occurs between 6-12 years of age. Critically, mutation carriers that do not develop symptoms as juveniles typically remain symptom-free for life (known as non-manifesting carriers; (15)). These clinical data indicate that the DYT1 mutation selectively disrupts events essential for the maturation of motor circuits.

DYT1 dystonia is caused by a mutation in the TOR1A gene deleting a single glutamic acid residue (ΔE) from the torsinA protein (16). The ΔE mutation disrupts torsinA function through multiple mechanisms (17-19). Several in vivo studies suggest the existence of a critical period of CNS vulnerability to torsinA loss-of-function (LOF), but this question has never been explicitly addressed. These data have been collected in models in which torsinA is conditionally deleted from different parts of the CNS because construct valid DYT1 dystonia model mice (i.e., Tor1aΔE/+ ) do not exhibit motor abnormalities (20). The time course of cellular and behavioral phenotypes characteristic of these models is consistent with a unique role for torsinA in the developing CNS. These phenotypes emerge during in late embryonic and early postnatal life, but
resolve in the first 3-4 postnatal weeks (21, 22). For example, nuclear membrane abnormalities develop in post-migratory maturing neurons (17) but resolve in the first 3 postnatal weeks (22). Similarly, several of these models exhibit behavioral and neuropathological phenotypes that emerge during the first ~1-3 postnatal weeks but do not subsequently worsen and may even improve at later ages (17, 23, 24).

Here, we explicitly test whether torsinA function is uniquely necessary during a neurodevelopmental critical period, and whether genetic rescue is similarly confined to an analogous therapeutic critical period. To rigorously address these questions, we developed a novel mouse reagent that enables spatiotemporal control of the endogenous torsinA allele. We find that in this novel system, embryonic suppression of torsinA causes overt dystonia-mimicking motor and neuropathologic phenotypes. In contrast, torsinA suppression in adult mice (sustained for up to 6 months) causes no apparent behavioral or neuropathologic abnormalities. TorsinA rescue of motor and neuropathological phenotypes similarly exhibited striking developmental dependence. Restoring torsinA expression in symptomatic juvenile mice reversed abnormal motor phenotypes and halted the progression of neuropathologic change. In contrast, torsinA repletion during adulthood had no discernible effect. Our findings establish a requirement for torsinA function unique to an early critical period and suggest that torsinA-based therapeutics may need to be targeted early in the course of DYT1 dystonia.
RESULTS

Cre- and tetracycline-based spatiotemporal control of the endogenous Tor1a locus. To test for a 
torsinA critical period and explore whether torsinA repletion can reverse or suppress DYT1 
phenotypes, we generated a novel mouse line that allows control of the endogenous Tor1a locus. 
We created this line by targeting the endogenous Tor1a allele with a cassette that confers Cre and 
tetracycline responsivity (“Tet(TorA)” (25). This cassette, containing a “floxed stop” element 
followed by a tetracycline operator (TetO), was targeted just 5’ to the Tor1a start site (Figure 1A).

We first tested the ability of a tetracycline-controlled transcriptional silencer (tTS) to suppress 
torsinA expression from the Tet(TorA) allele, and the ability of doxycycline (DOX) to displace 
tTS from the TetO sequence and enable gene expression (26). Our approach was based on Dlx-
CKO (Dlx5/6-Cre; Tor1a<sup>flx<sup>c</sup>) mice (27). The Dlx5/6-Cre field includes cortical inhibitory neurons, 
striatal cholinergic interneurons, striatal GABAergic interneurons, and medium spiny project 
neurons, populations implicated in the corticostriatal circuit dysfunction underlying dystonic 
movements (28-30). We used the ubiquitously expressed β-actin-tTS allele (31) to generate 
Dlx5/6-Cre; Tor1a<sup>Tet<sup>flx</sup>β-actin-tTS</sup> mice, a model analogous to Dlx-CKO mice but using the new 
Tet(TorA) allele. In these animals, Cre selectively deletes the floxed Tor1a allele from all striatal 
neurons, as well as the floxed stop cassette within the Tet(TorA) allele. In this configuration the 
Tet(TorA) allele should be suppressed by tTS, creating a Dlx5/6-Cre conditional null, but the allele 
should also be DOX regulatable selectively within the Dlx5/6-Cre field. We administered DOX to 
these animals from conception (in the mother’s chow) until P70 to test its ability to maintain 
normal torsinA expression. We then withdrew DOX at P70 to test the ability of tTS to suppress 
the Tet(TorA) allele (Figure 1B). P250 Dlx-Tet(TorA) mice continuously administered 
doxycycline displayed normal striatal torsinA expression. In contrast, torsinA expression was 
undetectable in P250 Dlx-Tet(TorA) mice switched to regular chow at P70. These results confirm 
that the Tet(TorA) allele is efficiently suppressed by tTS, and that this suppression is relieved by 
DOX. Striatal levels of torsinA were essentially undetectable in Dlx-Tet(TorA) mice never 
administered DOX, further demonstrating the ability of tTS to effectively suppress the Tet(TorA) 
gene (Supplemental Figure 1A-B). Comparing the expression of a single WT or Tet(TorA) allele 
in the absence of tTS demonstrated that they express at comparable levels (compare Dlx5/6-Cre; 
Tor1a<sup>Tet<sup>flx</sup></sup> and Dlx5/6-Cre; Tor1a<sup>flx<sup>c</sup></sup> β-actin-tTS mice; Supplemental Figure 1A-B). These 
findings demonstrate that the Tet(TorA) allele expresses at levels indistinguishable from the wild-
type Tor1a allele and is efficiently suppressed by tTS.

We next tested the ability of DOX to derepress torsinA expression in the entire CNS in Nestin-
Cre; Tor1a<sup>Tet<sup>flx</sup></sup> β-actin tTS (“Nes-Tet(TorA)”), in which Cre is expressed throughout the CNS. 
DOX administration from gestation in Nes-Tet(TorA) mice maintained normal levels of whole 
brain torsinA protein levels (Figure 1C, Supplemental Figure 1C). These data demonstrate that 
the Tet(TorA) allele expresses normally and can be regulated throughout the CNS.

*TorsinA is essential during a critical period of vulnerability.* Dysfunction of corticostriatal circuits 
is strongly implicated in dystonia pathophysiology in human (8, 32, 33) and rodent studies (10-12, 
34, 35). Dlx-CKO mice exhibit dystonic-like limb clasping and hyperactivity that emerges during 
the third postnatal week (12).
To explore whether these phenotypes depend upon torsinA LOF during a critical period of vulnerability, we compared the behavioral and histopathological effects of initiating suppression of torsinA expression in either developing or adult animals. We first tested if initiating torsinA suppression prenatally replicates established Dlx-CKO phenotypes including motor dysfunction during tail suspension and increased locomotor activity (12). tTS-mediated suppression of torsinA in Dlx5/6-Cre+ neurons starting in utero recapitulated the limb clasping and hyperactivity characteristic of Dlx-CKO mice (Figure 2B-C). These data provide additional evidence that tTS suppresses torsinA levels to a similar degree as the conditional null allele (Supplemental Figure 1A-B). To test the effect of initiating torsinA loss-of-function in adulthood, we administered DOX to Dlx-Tet(TorA) mice from conception (in mother’s chow) to maintain normal torsinA expression until P70. After P70, we withdrew DOX, allowing tTS to suppress the Tet(TorA) allele (Figure 2D). Western blots of striatal lysates confirmed the expected levels of torsinA protein (Figure 1B, Supplemental Figure 2). In marked contrast to initiating torsinA suppression in utero, adult suppression caused no behavioral abnormalities in any measures tested. Adult suppressed mice did not exhibit limb clasping or trunk twisting during tail suspension at any point during 6 months of longitudinal testing (Figure 2E). During this time, we observed no significant effects on locomotor activity (Figure 2F) or weight (Supplemental Figure 3A). We also assessed motor learning using the accelerating rotarod. Adult suppressed mice did not differ from their non-suppressed littermate controls in the rate at which they learned the task or the time they were able to remain on the rotarod (Supplemental Figure 3B). We confirmed that DOX itself did not exert an effect by administering DOX to Dlx-CKO mice (which lack any tetracycline-dependent alleles) from E0 to 6 months. DOX administration had no effect on the severity of limb clasping in Dlx-CKO mice (Supplemental Figure 4A).

Selective vulnerability during CNS development was similarly observed in histopathological studies of these animals. Suppressing torsinA in utero (Figure 3A) recapitulated the extent and pattern of ChI loss previously observed in Dlx-CKO mice (12, 36) (Figure 3B-C). In utero suppression also recapitulated the abnormal nuclear pore complex (NPC) clustering characteristic of Dlx-CKO mice (Figure 3D, Supplemental Figure 5A; (21)). In striking contrast, adult torsinA suppression caused no discernible histopathological abnormalities. Despite lacking torsinA expression for nearly 6 months, the cortex and striatum of these animals were indistinguishable from their littermate controls (Supplemental Figure 6A, C-D). There was no evidence of reactive astrogliosis (Supplemental Figure 6B), and the number of striatal neurons (Supplemental Figure 6E) did not differ from littermate controls. Similarly, there were no changes in ChI number or NPC distribution (Figure 3F-H, Supplemental Figure 5B). We also confirmed that DOX itself did not exert an effect by administering DOX to Dlx-CKO mice (which lack any tetracycline-dependent alleles) from E0 to 6 months. DOX treated Dlx-CKO mice exhibited the same severity of ChI degeneration as littermate Dlx-CKO mice fed regular chow (Supplemental Figure 4B). Considered together with the behavioral data, these observations demonstrate that the developing forebrain exhibits a critical period of susceptibility to torsinA loss-of-function.

To explore whether the critical period demonstrated for the forebrain generalizes to other torsinA-sensitive brain regions, we used Nestin-Cre to modulate torsinA expression in the entire CNS. We first confirmed that suppression of the tetracycline-responsive allele in the Nestin-Cre field replicated the phenotypes established for conditional CNS deletion of the Tor1a allele using Nestin-Cre (“Nes-CKO;” (23)). Nes-Tet(TorA) mice never administered DOX replicated all Nes-
CKO phenotypes previously described, including early postnatal lethality (Figure 4B-C). Nes-Tet(TorA) mice also exhibit abnormal postures similar to those reported for Nes-CKO mice (Supplemental Figure 7A; (23)). In striking contrast, Nes-Tet(TorA) mice that received DOX starting in utero were indistinguishable from their littermate controls. These animals exhibited normal viability (data not shown) and weight (Supplemental Figure 7A-B), performed normally in all behavioral assays, and showed no evidence of any abnormal twisting movements (Supplemental Figure 7A, data not shown). To test the effect of initiating pan-CNS torsinA loss-of-function in adulthood, we administered DOX to Nes-Tet(TorA) mice from conception (in mother’s chow) to maintain normal torsinA expression until P70. After P70, we withdrew DOX, allowing tTS to suppress the Nes-Tet(TorA) allele (Figure 4D). We confirmed that DOX removal eliminates torsinA protein expression (Supplemental Figure 8A-B). In striking contrast to the effects observed when torsinA suppression was initiated in utero, initiating torsinA suppression in adulthood had no discernible effect on any measure examined, despite the fact that we observed the animals for up to 6 months following DOX removal. These animals exhibited normal weight (Figure 4E) and viability (all mice survived; data not shown), and performed similarly to controls in all behavioral tests, including locomotor activity in the open field, rotarod motor learning (Figure 4F-G) and tail suspension (Figure 4H). We also tested for postural phenotypes reported in other torsinA LOF mouse models including abnormal spinal curvature and overt dystonia (e.g. disrupted gait, falling over) in the open field (36, 37). No abnormal postural phenotypes were observed (Figure 4H). We also confirmed that whole-life DOX treatment itself had no effect on the behavioral phenotypes (including early lethality) of Nes-CKO mice (which lack any tetracycline-dependent alleles) (Supplemental Figure 9), eliminating the possibility that torsinA-independent effects of DOX impact DYT1 phenotypes.

Neuropathological assessments of the brains of Nes-Tet(TorA) mice paralleled the behavioral findings. Initiating torsinA suppression in utero recapitulated all expected neuropathological phenotypes, whereas we observed no abnormalities when suppression was initiated in adulthood. The brains of Nes-Tet(TorA) mice that never received DOX (i.e., torsinA expression suppressed) exhibited reduced size (Figure 5A, Supplemental Figure 10A-C) and astrogliosis in sensorimotor regions described previously for conditional CNS mutants (Figure 5B-C, Supplemental Figure 10D; (23)). Initiating DOX from conception (supporting torsinA expression) completely rescued these abnormalities (Supplemental Figure 7C-D). Initiating torsinA suppression (by removing DOX) in adulthood (P70) did not cause any discernible neuropathologic abnormalities. Six months following adult DOX withdrawal, the brains of these mice exhibited normal size and cortical thickness, and no evidence of gliosis or neurodegeneration (Figure 5E-H and Supplemental Figure 11A-C). These results demonstrate that all brain regions previously identified as susceptible to torsinA loss-of-function exhibit a similar temporal torsinA requirement limited to early brain development. Considered together, our behavioral and histologic assessment of mice with prenatal versus adult removal of torsinA from the entire CNS highlight a selective developmental susceptibility to torsinA LOF. Prenatal torsinA removal causes early lethality, impairs postnatal growth, and induces widespread neurodegeneration. On the other hand, extensive behavioral and histologic analysis of mice with adult torsinA removal from the same structures reveals no detectable phenotype.

*TorsinA restoration defines a therapeutic critical period for DYT1 dystonia.* A specific temporal requirement for torsinA implies that to be effective, torsinA restoration strategies may similarly
need to be administered during a neurodevelopmental window. We tested for such a therapeutic critical period by initiating torsinA expression (by DOX administration) at different ages in mice that developed in the absence of torsinA (i.e., had never previously received DOX). We pursued these studies in Dlx-Tet(TorA) mice that, in the absence of DOX, develop abnormal twisting movements during the 3rd postnatal week (Figure 2B; (12)). To model intervention during early disease we induced torsinA expression at P21, approximately 1 week after the onset of abnormal limb clasping (Figure 6A). To model intervention in chronic disease, we induced torsinA expression at P70, approximately 7 weeks after the onset of motor abnormalities (Figure 6B). DOX administration efficiently activated torsinA expression at both time points (Supplemental Figure 12A-D). TorsinA restoration at P21 significantly reduced the duration of abnormal limb clasping by ~75% (Figure 6C; assessed at P70). In contrast, activating torsinA at P70 had no significant effect on the duration of limb clasping at any subsequent age tested, up to P168 (Figure 6D). We pursued histopathological analyses to determine whether ChI degeneration, which is linked to abnormal twisting behavior (12), paralleled the behavioral findings. TorsinA restoration at P21 significantly attenuated ChI loss, whereas P70 restoration produced no significant effect (Figure 6E-F). The significant (but partial) rescue of neurodegeneration selectively following P21 expression of torsinA is consistent with the established timeline of ChI loss, which begins at ~P12 and is complete by P70 or earlier (12). As with Dlx-CKO mice (21), Dlx-Tet(TorA) mice not treated with DOX exhibited nuclear pore clustering in SST+ cortical GABAergic interneurons. This phenotype was not reversed by induction of torsinA expression at either P21 or P70 (Figure 6G-H). These results demonstrate that early torsinA augmentation halts ongoing loss of ChI and rescues motor abnormalities, strengthening the correlation between ChI dysfunction and abnormal twisting (12). These findings also establish a therapeutic critical period during CNS maturation.

The lack of benefit from torsinA augmentation in adulthood suggests that, following early rescue, continued torsinA expression may not be required to maintain improved motor function and ChI integrity (Figures 6C, E). We tested this possibility by comparing four experimental groups: 1. Dlx-Tet(TorA) mice with continuous torsinA suppression (Dlx-Tet(TorA)^OFF), 2. Dlx-Tet(TorA) mice with torsinA activated from P21 until the end of the study (Dlx-Tet(TorA)^ON21-168), 3. Dlx-Tet(TorA) mice with torsinA expressed from P21-P70, then suppressed until the end of the study (Dlx-Tet(TorA)^ON21-70), and 4. Cre controls (Figure 7A). At P168, striatal lysates from Dlx-Tet(TorA)^OFF and Dlx-Tet(TorA)^ON21-70 mice show similarly suppressed levels of torsinA, while Dlx-Tet(TorA)^ON21-168 mice with torsinA activated through the end of the study exhibit normal striatal torsinA expression (Supplemental Figure 13A-B).

We performed behavioral testing from P70 to P168 followed by histologic assessment. Tail suspension testing at P70 confirmed our earlier finding that torsinA activation from P21 to P70 significantly reverses motor symptoms (Figure 7B, first time point; compare with Figure 6C). Within the same group of mice (Dlx-Tet(TorA)^ON21-70 group; Figure 7B), this significant reduction in the duration of limb clasping persisted even 14 weeks after DOX cessation (torsinA suppressed). Indeed, there was no significant difference in the duration of clasping between Dlx-Tet(TorA)^ON21-70 and Dlx-Tet(TorA)^ON21-168 (average time clasping: 12.3 s vs. 11.5 s), highlighting the lack of additional behavioral benefit from torsinA expression beyond P70. TorsinA activation at P21 also reversed hyperactivity at P168 whether or not torsinA expression was supported after P70 (Figure 7C). Stereological assessment of ChIs further supported the link between these cells and motor dysfunction. ChI numbers were rescued to a similar extent in both Dlx-Tet(TorA)^ON21-168 and Dlx-
Tet(TorA)\textsuperscript{ON21-70} groups (mean of control group = 17082 cells; Dlx-Tet(TorA)\textsuperscript{OFF} = 11363 cells; Dlx-Tet(TorA)\textsuperscript{ON21-168} = 14330 cells; Dlx-Tet(TorA)\textsuperscript{ON21-70} = 14049 cells; Figure 7D). Considered together, these data demonstrate that torsinA expression is required exclusively during a critical period before P70 to reverse DYT1 associated phenotypes. TorsinA supplementation beyond P70 is of no benefit either behaviorally or histopathologically.
DISCUSSION

Our studies are the first to establish a neurodevelopmental critical period during which the CNS is uniquely sensitive to torsinA function. Employing a novel genetic system to regulate expression of the endogenous Tor1a allele, we demonstrate an essential neurodevelopmental requirement for torsinA in supporting normal CNS structure and motor function that is dispensable in adult animals. We also demonstrate an analogous therapeutic critical period during which torsinA function must be restored to rescue the behavioral and neuropathological phenotypes caused by torsinA hypofunction. Our findings support a two-stage model of disease pathogenesis. Stage 1 events are directly or closely related to torsinA LOF and reversible by torsinA restoration. Stage 2 events, by contrast, are downstream molecular or circuit changes that are independent of torsinA function. This model has broad implications for defining key molecular events relevant to neurodevelopmental disease pathogenesis and for the timing and nature of effective therapeutic strategies.

Modeling DYT1 dystonia is challenging, as “construct valid” (Tor1aΔE+) mice do not exhibit clear behavioral or neuropathologic phenotypes (17, 20). Based on extensive biochemical, cell biological, and genetic evidence that the DYT1 mutation impairs torsinA function, we modeled the disease by deleting torsinA fully from corticostriatal circuits implicated in dystonia pathophysiology. Two caveats attend this approach. It almost certainly creates less torsinA enzymatic activity than the human DYT1 genotype, similar to how a transgenic modeling approach amplifies the effect of gain-of-function mutations. This approach also focuses exclusively on modeling torsinA LOF forebrain dysfunction (27). While dysfunction of corticostriatal circuits is strongly implicated in dystonia, this approach omits potential contribution from the wider motor circuit, including cerebellum, thalamus, and other regions (23, 37-41). Despite these caveats, our results demonstrate the necessity of torsinA function during a critical developmental period in all brain areas assessed (i.e. using both Nestin-Cre and Dlx5/6-Cre). This developmentally selectivity is similar to the childhood-onset critical period in DYT1 dystonia subjects (15), supporting the disease relevance of these findings. These findings are also relevant to recessive loss of torsinA function recently linked to arthrogryposis (42, 43).

We demonstrate that torsinA restoration in juvenile (P21) mice that have been symptomatic for ~1 week (one-third of their lives) rescues motor symptoms and ChI degeneration. In contrast, gene replacement in stably symptomatic adult mice has no apparent effect. These data indicate that, over time, circuit dysfunction causing motor symptoms becomes torsinA-independent. This idea is consistent with the finding that in P21-rescued Dlx-Tet(TorA) mice, torsinA expression beyond P70 confers no further benefit. Changes not amenable to torsinA repletion likely include ChI degeneration, as initiating torsinA replacement after ChI loss is ineffective. An association between prevention of ChI degeneration and behavioral rescue has been demonstrated in torsinA LOF models (44, 45), further supporting this connection. Striatal dysfunction secondary to ChI loss and dysfunction likely causes additional abnormalities of connectivity and function within and beyond the striatum, and failure to restore torsinA in these other neural elements may in part account for the incomplete motor rescue we observe. An important future direction for this work is to more resolutely define the time course and anatomic requirements for effective versus ineffective therapy.
Our studies of Dlx-Tet(TorA) mice add to a growing literature demonstrating striatal cholinergic abnormalities in dystonia. Striatal Chls exhibit morphologic, neurochemical, and electrophysiologic changes in DYT1 dystonia mouse models (10, 12, 34, 36, 46, 47), and are linked to deficits in corticostratial plasticity thought to contribute to the expression of motor symptoms (48, 49). In the Dlx-CKO model, Chls are uniquely vulnerable to torsinA LOF (12). We find that torsinA activation early enough to prevent Chl degeneration rescues motor abnormalities, while torsinA activation in adult mice, after Chl degeneration is complete, does not improve the motor phenotype. These findings are consistent with another recent study demonstrating an association between Chl survival and motor symptoms (44), further strengthening the relationship between striatal cholinergic dysfunction and dystonic-like movements. However, other cells lack torsinA in Dlx-CKO mice, and selective degeneration of Chls does not imply that they are the only key player. Other neuron populations in the Cre field, including cortical inhibitory interneurons and striatal fast-spiking interneurons have been implicated in dystonia pathophysiology (28-30). Future work will be needed to establish a causal link between Chl dysfunction and abnormal behavior and to explore whether dysfunction other cell types contributes to motor dysfunction.

TorsinA LOF may dysregulate developmental plasticity through interactions with diverse pathways in which it has been implicated, including elf2α signaling (50-52), secretory processing (53, 54) and nucleocytoplasmic transport (21, 55, 56). Manipulation of elf2α signaling restores normal corticostratial plasticity in brain slices from Tor1a<sup>AE/+</sup> knock-in mice (52). The secreted neurotrophic factor BDNF has also been linked to aberrant plasticity in Tor1a<sup>AE/+</sup> mice (35). Pharmacologic manipulation of BDNF signaling rescues plasticity deficits in juvenile mice (P26) but not adult mice, consistent with a therapeutic critical period for this intervention.

The biology of the torsin gene family provides clues to the mechanisms dictating a critical period of vulnerability in DYT1 dystonia. The torsinA paralog torsinB is developmentally regulated and strongly influences the severity of torsinA LOF phenotypes (22, 44, 57). Abnormal NE budding occurs when torsinB expression is relatively low in the developing brain. This abnormal phenotype resolves as torsinB levels rise during maturation, but conversely persists and worsens when torsinA and torsinB are both ablated (22). TorsinB overexpression prevents torsinA LOF-related motor phenotypes and neurodegeneration (44). Studies of torsinB expression in humans are limited and inconclusive (58, 59), representing an important area of future investigation.

TorsinA action at the nuclear envelope may also regulate critical period timing. The number of NPCs in neurons increases rapidly during development before plateauing (60), and turnover of NPCs is exceedingly low (61, 62). TorsinA LOF results in abnormal NE budding in neurons (17), and this phenomenon appears related to interphase nuclear pore biogenesis. Nuclear pore components have been observed within NE buds and the spatial characteristics of NE buds resemble those of nuclear pore complex intermediates (63). Further, torsinA deficient neurons exhibit nuclear pore structures that appear incomplete, as they contain early NPC components but lack later added nucleoporins present in mature nuclear pores (21, 64). This discrete developmental period of upregulated interphase nuclear pore insertion may be sensitive to torsinA function.

A report of motor abnormalities following shRNA-mediated torsinA knockdown in the cerebellum (37) of adult, but not early postnatal animals, differs from our finding of an early critical period for torsinA depletion (including for cerebellum; Figure 4 and Figure 5). In contrast to the specific
targeted approach employed here, the potential for off-target effects arising from multiple mechanisms in RNAi experiments may impact the shRNA findings (65-69). Indeed, our results are consistent with the natural history of the human disease (15), the multiple developmental processes described above in which torsinA has been implicated, and the presence of early motor abnormalities in multiple gene-targeted DYT1 and DYT6 models (12, 23, 70).

While it is important to bear in mind the many differences between mouse models and human disease, our observation of a therapeutic critical period for torsinA restoration has significant translational implications. Our results suggest that for maximal efficacy, such therapies may need to be initiated early in pathogenesis. The ideal scenario is to identify and treat mutation carriers prior to symptom onset. This approach is not currently viable for DYT1 dystonia because the mutation is incompletely penetrant and there are no reliable predictors of which carriers will develop disease. In contrast, a torsinA-independent circuit-based approach will likely be required for patients with long established symptoms. Current symptomatic treatments include pharmacologic agents (e.g., antimuscarinics), botulinum toxin injection for focal symptoms, and deep brain stimulation (71). An alternative idea, suggested by our work, is modulating the torsinA critical period to impede disease pathophysiology or to make circuits more receptive to torsinA restoration. Several experimental interventions have been shown to extend critical periods or reactivate juvenile-like plasticity in the adulthood (72, 73). Because DYT1 symptoms emerge during juvenile CNS maturation, critical period lengthening could worsen symptoms. Consistent with this possibility, aberrant plasticity is believed to be a core feature of dystonia pathophysiology (74-77). These considerations indicate that blocking defined plasticity pathways is also worthy of future study.

The identification of a therapeutic critical period for gene replacement is consistent with findings in mouse models of some but not all neurodevelopmental disorders. Early disruption or restoration of gene function is necessary to model or rescue behavioral phenotypes in mouse models of Angelman syndrome (1, 6). In contrast, the Rett syndrome protein MeCP2 plays an essential role in maintenance of normal adult nervous system function (5, 78). Juvenile and adult MeCP2 restoration ameliorate neurological dysfunction in Rett syndrome models with similar effectiveness (7). Similarly, in a Shank3 mouse model of autism, adult replacement of the gene is sufficient to reverse synaptic deficits and improves autism-related behaviors (79). Neurodevelopmental diseases therefore differ in terms of the extent that the pathogenic process is uniquely required to occur during CNS maturation.

This is the first report establishing developmental and therapeutic critical periods in DYT1 dystonia. Our findings emphasize that future studies of torsinA pathways relevant to dystonia pathogenesis should focus on events that are unique to or strongly upregulated in maturing neurons. These experiments also suggest that torsinA-based therapeutic strategies can be effective even after symptoms have emerged, but likely need to be administered very early in the course of disease.
METHODS

Mice
The Tet(TorA) mouse line was generated with Biocytogen using CRISPR/Extreme Genome Editing™ technology. A floxed stop cassette and TetO sequence were inserted upstream of Tor1a exons 1-5. For more information, please refer to the Supplemental Methods section.

Western blotting
PVDF membranes were probed with rabbit anti-torsinA (Abcam ab34540; 1:10,000) and rabbit anti-calnexin (Enzo Life Sciences SPA-860; 1:20,000) primary antibodies. The secondary antibody was an HRP-conjugated anti-rabbit antibody (Cell Signaling 7074; 1:20,000). Bands were visualized using chemiluminescent substrate and exposure to x-ray film. Protein levels were quantified in ImageJ. For more information, please refer to the Supplemental Methods section.

Immunohistochemistry
For immunofluorescence, 40 um free-floating brain sections were incubated with rabbit anti-GFAP (Dako Z0334; 1:2,000), rabbit anti-SST (Abcam ab103790; 1:500), and/or mouse anti-nuclear pore complex (“mAb414”; Abcam ab24609; 1:800) primary antibodies. This was followed by incubation with donkey anti-rabbit Ax555 secondary antibody (Invitrogen A-31572; 1:800), donkey anti-mouse Ax488 secondary antibody (Invitrogen A-21206), Hoechst (Thermo Scientific 62249; 1:10,000), and/or NeuroTrace green fluorescent Nissl stain (Invitrogen N21380). Sections were mounted on glass slides and coverslipped with ProLong Gold mounting medium (Invitrogen P36930). For DAB staining, 40 um frozen sections were stained with goat anti-ChAT (Millipore AB144P; 1:800) primary antibody, followed by incubation with biotinylated donkey anti-goat secondary antibody (Jackson Immunoresearch 705-06547; 1:800), ABC-HRP kit (Vector Laboratories PK-6100), and DAB substrate (Sigma-Aldrich D4293; 3,3’-diaminobenzidine). For traditional Nissl staining, 40 um fixed brain sections were mounted on glass slides, rehydrated, incubated in Cresyl violet for 3 min, and quenched in water. After Nissl or DAB staining, sections were dehydrated in ascending ethanol, cleared in xylene, and coverslipped with Permount mounting medium (Fisher SP15). For more information, please refer to the Supplemental Methods section.

Cell counting and image analysis
Stereology
ChIs, striatal neurons, 7N neurons, and medial DCN neurons were quantified with unbiased stereology using the optical fractionator probe in Stereoinvestigator (MBF Bioscience). 40 mm-thick serial sections were observed using a Zeiss Axioimager M2 microscope. Counting frame and sampling grid parameters were determined in pilot studies to reach a Gundersen coefficient of error of less than 0.1 for each cell type/region. For more information, please refer to the Supplemental Methods section

Morphological analysis
Cortical thickness and striatal volume were measured as previously described (12). Brain area was measured in sagittal sections by creating a contour around the brain in the section corresponding to ML +1.44 mm (80) and measuring the traced area in Stereoinvestigator.
Image analysis
GFAP fluorescence intensity was measured in ImageJ by creating a region-of-interest around the specific brain regions and quantifying fluorescence intensity in the GFAP channel. Nuclear pore complex clustering was scored (yes or no) in SST+ interneurons in motor cortex by a reviewer blinded to genotype.

Behavioral testing
Tail suspension
Mice were suspended by the tail and recorded for 60 s. 2 scorers blinded to genotype graded the presence of abnormal clasping and twisting movements as well as the duration of abnormal movements.

Locomotor activity
Mice were placed individually into a plastic mouse cage (18 cm x 28 cm) with a thin layer of bedding. The cage was placed into a dark plexiglass box, and locomotor activity was measured by photobeams (Photobeam Activity System, San Diego Instruments, San Diego, CA). Horizontal beam breaks were recorded for 1 hour.

Rotarod
Mice were tested on an accelerating rotarod with the speed of rotation increasing from 4 to 40 rpm over 5 min. Latency to fall was recorded with a cutoff of 5 min. 5 trials were conducted per day for 2 consecutive days, with 2 min rest between trials.

Kyphosis
Mice were observed in an empty cage for 2 min. Presence or absence of abnormal spinal kyphosis was noted by a reviewer blinded to the experimental group of the mouse.

Video tracking
Mice were recorded for 10 min by a camera above an open field environment (44 x 44 cm, walls 30 cm high). Two reviewers blinded to the experimental group of each mouse scored videos for presence or absence of overt dystonic postures observed in other dystonia models (37, 81, 82). These include difficulty maintaining balance, erratic gait, and other dystonic postures.

Statistics
Statistical testing and graph generation was performed using GraphPad Prism software. Data are represented as mean ± SEM. Descriptions of statistical tests and sample sizes are located in figure legends.

Study Approval
Animal work described in this manuscript has been approved and conducted under the oversight of the UT Southwestern and University of Michigan Institutional Animal Care and Use Committees.

Author Contributions
JL, SSP, and WTD designed research studies. JL, DSL, and AJK conducted experiments, acquired data, and analyzed data. JL, SSP, and WTD wrote and edited the manuscript.
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Figure 1. Spatiotemporal control of the endogenous Tor1a locus. (A) Design of the Tet(TorA) allele. Triangles denote loxP sites. (i) A “floxed-stop” cassette and TetO are inserted upstream of the start site of the Tor1a gene. (ii) Cre recombination removes the stop cassette, rendering the allele active specifically within the Cre expression field, unless suppressed by tTS. (iii) Doxycycline de-represses the allele in the Cre expression field by preventing tTS binding to TetO, allowing transcription. (B) Western blot analysis of torsinA expression in striatal lysates from Dlx-Tet(TorA) mice. Light gray (ON) bars represent ages when torsinA is expressed and dark gray (OFF) areas represent ages when torsinA is suppressed. TorsinA is expressed in Dlx-Tet(TorA) mice fed doxycycline chow, but is suppressed in Dlx-Tet(TorA) mice switched to regular chow at P70 (compare lanes 3 and 4, with and without DOX). (C) Western blot analysis of torsinA expression in whole brain lysates from Nes-Tet(TorA) mice treated with doxycycline for their whole lives. Doxycycline relieves tTS suppression, resulting in physiologic levels of torsinA expression.
Figure 2. Forebrain torsinA depletion causes abnormal limb clasping behavior only when initiated during CNS development. (A) Schematic of experimental design for prenatal torsinA suppression in the Dlx5/6-Cre field. Light gray (ON) bars represent ages when torsinA is expressed and dark gray (OFF) areas represent ages when torsinA is suppressed. Each color corresponds to an experimental group in subsequent graphs. (B) Proportion of Dlx-Tet(TorA) mice exhibiting tail suspension-induced limb clasping (P17 to P70) following prenatal torsinA suppression. n = 9-10 per group. (C) Locomotor activity of P70 Dlx-Tet(TorA) mice following prenatal torsinA suppression. These animals exhibit locomotor hyperactivity. n = 5-7 per group. (D) Schematic of experimental design for adult torsinA suppression in the Dlx5/6-Cre field. Light gray (ON) bars represent ages when torsinA is expressed and dark gray (OFF) areas represent ages when torsinA is suppressed. Each color corresponds to an experimental group in subsequent graphs. TorsinA expression was suppressed by doxycycline withdrawal at P70. (E) Proportion of Dlx-Tet(TorA) mice exhibiting tail suspension-induced limb clasping following adult suppression of torsinA. Adult removal of torsinA in the forebrain does not cause limb clasping. n = 10-12 per group. (F) Locomotor activity of Dlx-Tet(TorA) mice following adult suppression of torsinA. Adult removal of torsinA from the forebrain does not significantly alter locomotor activity. n = 9 per group. Data analyzed by χ² test (B, E), one-way ANOVA (C, F) and Dunnett’s multiple comparisons test (C). * p < 0.05, *** p = 0.0004, **** p < 0.0001.
Figure 3. Forebrain torsinA depletion causes neuropathology only when initiated during CNS development. (A) Schematic of experimental design for prenatal torsinA suppression in the Dlx5/6-Cre field. Light gray (ON) areas of bars represent ages when torsinA is expressed and dark gray (OFF) areas represent ages when torsinA is suppressed. Each color corresponds to an experimental group in subsequent graphs. (B) Chl density analysis in four quadrants of caudate putamen (DL: dorsolateral, DM: dorsomedial, VL: ventrolateral, VM: ventromedial) in P70 Dlx-Tet(TorA) mice following prenatal torsinA suppression. Consistent with Dlx-CKO findings, Dlx-Tet(TorA) mice with torsinA removed at embryonic age exhibit Chl loss in dorsolateral and dorsomedial quadrants of caudate putamen. n = 6-8 per group. (C) Representative image of ChAT-stained striatum in Dlx-Tet(TorA) mice following prenatal torsinA suppression. Scale bar
represents 250 µm. (D) Percentage of SST+ neurons in sensorimotor cortex with clustered nuclear pore complexes in P70 Dlx-Tet(TorA) mice following prenatal torsinA suppression. n = 4 per group. (E) Schematic of experimental design for adult torsinA suppression in the Dlx5/6-Cre field. Light gray (ON) areas of bars represent ages when torsinA is expressed and dark gray (OFF) areas represent ages when torsinA is suppressed. Each color corresponds to an experimental group in subsequent graphs. TorsinA expression was suppressed by doxycycline withdrawal at P70. (F) Striatal ChI counts in Dlx-Tet(TorA) mice following adult suppression of torsinA. Adult forebrain suppression of torsinA does not cause ChI degeneration. (G) Representative image of ChAT-stained striatum in Dlx-Tet(TorA) mice following adult suppression of torsinA. Scale bar represents 250 µm. (H) Percentage of SST+ neurons with abnormally clustered nuclear pore complexes in sensorimotor cortex of Dlx-Tet(TorA) mice following adult suppression of torsinA. Forebrain suppression of torsinA starting at P70 does not cause abnormal nuclear pore clustering. n = 4 per group. Data analyzed by one-way ANOVA (B, D), Dunnett’s multiple comparisons test (B, D), and two-way ANOVA (F, H). **** p < 0.0001.
Figure 4. Whole CNS torsinA depletion causes abnormal twisting behavior only when initiated during CNS development. (A) Schematic of experimental design for prenatal suppression of torsinA in the Nestin-Cre field. Light gray (ON) bars represent ages when torsinA is expressed and dark gray (OFF) areas represent ages when torsinA is suppressed. Each color corresponds to an experimental group in subsequent graphs. (B) Growth curves of Nes-Tet(TorA) mice following prenatal suppression of torsinA. Embryonic torsinA removal impairs growth (interaction of age and experimental group: p = 0.0026). n = 4-5 per group. (C) Survival curves of Nes-Tet(TorA) mice following prenatal suppression of torsinA. Embryonic torsinA removal causes early lethality (p < 0.0001). n = 5 per group. (D) Schematic of experimental design for adult suppression of torsinA in the Nestin-Cre field. Light gray (ON) bars represent ages when torsinA is expressed and dark gray (OFF) areas represent ages when torsinA is suppressed. Each color corresponds to an experimental group in subsequent graphs. TorsinA expression was suppressed by doxycycline withdrawal at P70. (E) Weight of Nes-Tet(TorA) mice following adult suppression of torsinA. Adult suppression of torsinA does not alter locomotor activity. n = 9 per group. (F) Locomotor activity of Nes-Tet(TorA) mice following adult suppression of torsinA. Adult forebrain suppression of torsinA does not impair motor learning. n = 10-12 per group. (G) Rotarod performance of Nes-Tet(TorA) mice following adult suppression of torsinA. Adult forebrain suppression of torsinA does not elicit torsinA-LOF associated behavioral phenotypes such as limb clasping, kyphosis, and overt dystonic symptoms. n = 10-12 per group for limb clasping and kyphosis. n = 8 per group for analysis of overt dystonic symptoms. Data analyzed by mixed-effects model (B), Gehan-Breslow-Wilcoxon test (C), two-way ANOVA (E, F, G), and χ² test (H).
Figure 5. Whole CNS torsinA depletion causes neuropathology only when initiated during CNS development. (A) Representative Nissl stained sagittal sections from P8 Nes-Tet(TorA) mice following prenatal suppression of torsinA. Scale bar represents 500 µm. (B) Representative sagittal sections from P8 Nes-Tet(TorA) mice following prenatal suppression of torsinA immunostained with an antibody targeted to GFAP. Arrows indicate cortical gliosis and the circle outlines gliosis in thalamus. Scale bar represents 500 µm. (C) GFAP fluorescence intensity analysis of P8 Nes-Tet(TorA) mice following prenatal suppression of torsinA. GFAP intensity in increased in DCN, 7N, RN, thalamus, and cortex. n = 3 per group. (D) Schematic of experimental design for adult suppression of torsinA in the Nestin-Cre field. Light gray (ON) bars represent ages when torsinA is expressed and dark gray (OFF) areas represent ages when torsinA is
suppressed. Each color corresponds to an experimental group in subsequent graphs. (E) Representative Nissl and GFAP co-stained sagittal sections from Nes-Tet(TorA) mice following adult suppression of torsinA. Scale bar represents 1 mm. (F) GFAP fluorescence intensity analysis of P250 Nes-Tet(TorA) mice following adult suppression of torsinA. GFAP intensity is unchanged by adult torsinA suppression in all brain regions examined. n = 5 per group. (G) Cell counts of medial DCN neurons in P250 Nes-Tet(TorA) mice following adult suppression of torsinA. n = 5 per group. (H) Cell counts of 7N neurons in P250 Nes-Tet(TorA) mice following adult suppression of torsinA. n = 5 per group. Data analyzed by unpaired t-test (C) and two-way ANOVA (F, G, H). * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure 6. TorsinA restoration is uniquely effective during a neurodevelopmental therapeutic critical period. (A) Schematic of experimental design for Dlx5/6-Cre juvenile torsinA restoration study. Light gray (ON) bars represent ages when torsinA is expressed and dark gray (OFF) areas represent ages when torsinA is suppressed. Each color corresponds to an experimental group in subsequent graphs. TorsinA expression was restored in early symptomatic Dlx-Tet(TorA) mice at P21. (B) Schematic of experimental design for Dlx-Tet(TorA) adult torsinA restoration study. Light gray (ON) bars represent ages when torsinA is expressed and dark gray (OFF) areas represent ages when torsinA is suppressed. Each color corresponds to an experimental group in subsequent graphs. TorsinA expression was restored in late symptomatic Dlx-Tet(TorA) mice at P70. (C) Duration of abnormal movements during one minute of tail suspension in Dlx-Tet(TorA) juvenile torsinA restoration mice. n = 9 per group. (D) Duration of abnormal movements during one minute of tail suspension in Dlx-Tet(TorA) adult torsinA restoration mice. n = 6 per group. (E) Striatal ChI counts in Dlx-Tet(TorA) juvenile torsinA restoration mice. TorsinA activation in juvenile mice partially prevents ChI degeneration. n = 5 per group. (F) Striatal ChI counts in Dlx-Tet(TorA) adult torsinA restoration mice. TorsinA activation in adult mice does not prevent ChI degeneration. n = 4 per group. (G) Percent of SST+ neurons with abnormally clustered nuclear pore complexes...
in sensorimotor cortex of Dlx-Tet(TorA) juvenile torsinA restoration mice. Juvenile torsinA activation does not rescue abnormal nuclear pore clustering. (H) Percent of SST+ neurons with abnormally clustered nuclear pore complexes in sensorimotor cortex of Dlx-Tet(TorA) adult torsinA restoration mice. Adult torsinA activation does not rescue abnormal nuclear pore clustering. n = 3 per group. Data analyzed by two-way ANOVA (C, D, F, G, H) with Sidak’s multiple comparisons test (C, F, G, H), and one-way ANOVA with Tukey’s multiple comparisons test (E). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
Figure 7. TorsinA expression is not required after P70 to maintain early therapeutic rescue. (A) Schematic of experimental design for Dlx-Tet(TorA) therapeutic critical period study. Light gray (ON) bars represent ages when torsinA is expressed and dark gray (OFF) areas represent ages when torsinA is suppressed. Each color corresponds to an experimental group in subsequent graphs. To determine if ongoing torsinA expression in adulthood is necessary for persistent symptom amelioration, we compared Dlx-Tet(TorA) mice in which torsinA was not expressed (red; Dlx-Tet(TorA)[OFF]), expressed from P21 to the end of the study (blue; Dlx-Tet(TorA)[ON21-168]), and expressed only from P21-P70 (magenta; Dlx-Tet(TorA)[ON21-70]) then suppressed from P70 to the end of the study at P168. (B) Duration of abnormal movements during one minute of tail suspension in Dlx-Tet(TorA) mice following torsinA repletion during a critical therapeutic period. n = 8-12 per group. (C) Locomotor activity in Dlx-Tet(TorA) mice following torsinA repletion during a critical therapeutic period. Reduction of hyperactivity in torsinA rescued mice persists to at least P168 even without ongoing adult torsinA expression. n = 8-11 per group. (D) Striatal ChI counts in Dlx-Tet(TorA) mice following torsinA repletion during a critical therapeutic period. TorsinA activation at P21 prevents striatal ChI degeneration, and no further degeneration occurs even when torsinA is inactivated at P70. n = 7 per group. Data analyzed by two-way ANOVA with Sidak’s multiple comparisons test (B) and one-way ANOVA with Tukey’s multiple comparisons test (C, D). * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
REFERENCES


