

Inducible Knockout of Mouse *Zfhx3* Emphasizes Its Key Role in Setting the Pace and Amplitude of the Adult Circadian Clock

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Abstract The transcription factor zinc finger homeobox 3 (ZFHX3) plays a key role in coupling intracellular transcriptional-translational oscillations with intercellular synchrony in mouse suprachiasmatic nucleus (SCN). However, like many key players in central nervous system function, ZFHX3 serves an important role in neurulation and neuronal terminal differentiation while retaining discrete additional functions in the adult SCN. Recently, using a dominant missense mutation in mouse *Zfhx3*, we established that this gene can modify circadian period and sleep in adult animals. Nevertheless, we were still concerned that the neurodevelopmental consequences of ZFHX3 dysfunction in this mutant may interfere with, or confound, its critical adult-specific roles in SCN circadian function. To circumvent the developmental consequences of *Zfhx3* deletion, we crossed a conditional null *Zfhx3* mutant to an inducible, ubiquitously expressed Cre line (B6.Cg-Tg(UBC-cre/ERT2)1Ejb/J). This enabled us to assess circadian behavior in the same adult animals both before and after Cre-mediated excision of the critical *Zfhx3* exons using tamoxifen treatment. Remarkably, we found a strong and significant alteration in circadian behavior in tamoxifen-treated homozygous animals with no phenotypic changes in heterozygous or control animals. Cre-mediated excision of *Zfhx3* critical exons in adult animals resulted in shortening of the period of wheel-running in constant darkness by more than 1 h in the majority of homozygotes while, in 30% of animals, excision resulted in complete behavioral arrhythmicity. In addition, we found that homozygous animals reentrain almost immediately to 6-h phase advances in the light-dark cycle. No additional overt phenotypic changes were evident in treated homozygous animals. These findings confirm a sustained and significant role for ZFHX3 in maintaining rhythmicity in the adult mammalian circadian system.

Keywords conditional mutagenesis, suprachiasmatic nucleus, intracellular coupling, period, neuropeptide

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In mammals, circadian oscillations are evident in all tissues where these oscillations are believed to be directed by a well-characterized, cell autonomous transcription-translation feedback loop (TTFL). Briefly, this comprises the period (PER) and cryptochrome (CRY) proteins that inhibit their own transcription via inhibition of regulatory CLOCK and BMAL1 proteins. This TTFL also directly regulates the expression of numerous additional genes that are cell- or tissue-specific and mediates tissue-specific clock-relevant functions (Mohawk et al., 2012). The ability of the whole organism to synchronize all of these cell or tissue oscillations is not fully understood, but the master oscillator residing in the suprachiasmatic nucleus (SCN) of the hypothalamus is known to play a crucial role. The SCN maintains an intercellular synchrony of cellular oscillations through the coordinated activities of numerous neurotransmitters acting as coupling factors, including vasoactive intestinal peptide (VIP), arginine vasopressin (AVP), and γ -aminobutyric acid (GABA) (Herzog et al., 2017). Consequently, robust signals are relayed to other cells and tissues through orchestrated neural and humoral signals. These activities and signals are believed to be aligned to the SCN intracellular TTFL, and numerous candidate mechanisms have been proposed.

Recent studies (Parsons et al., 2015) have highlighted the key role of the transcription factor zinc finger homeobox 3 (*Zfhlx3*) in coupling intracellular TTFL oscillations with intercellular synchrony in mouse SCN. Although *Zfhlx3* is not expressed at high levels throughout adult brain, *Zfhlx3* expression is highly enriched in adult SCN. Moreover, a dominant missense mutation in *Zfhlx3* (short circuit, *Sci*) shortens circadian wheel-running period by almost 1 h and SCN molecular oscillations by a similar margin. ZFH3 regulates gene expression in SCN through its interaction with conserved AT motifs in promoters of target genes including clock-related genes and multiple SCN-enriched neuropeptides and neuropeptide receptors. In *Zfhlx3^{Sci/+}* mutants, expression of many of these genes is affected. However, we wanted to establish further whether null mutations in *Zfhlx3* had a similar effect on circadian function in vivo and whether, by circumventing developmental effects of *Zfhlx3* function, we could still establish an effect on the circadian system.

In determining the significance of circadian phenotypes in adult mutant mice, it is important to consider whether phenotypes might be secondary consequences of primary developmental anomalies. ZFH3 plays important roles in multiple systems in developing and adult tissues, and the pleiotropic nature of mutations in *Zfhlx3* testifies to this. The *Sci* mutation is homozygous perinatal lethal (Parsons et al., 2015), while a constitutive knockout of *Zfhlx3*

can result in preweaning sublethality in heterozygotes (Sun et al., 2012). These observations confirm the nature of the *Sci* allele, already suggested (Parsons et al., 2015), as a gain of function, most probably dominant negative, allele. Moreover, the sublethality in heterozygous constitutive null mice suggests a haploinsufficiency in knockout mice. ZFH3 plays vital roles in development and in other systems including mammary gland (Zhao et al., 2016) and the nervous system (Jung et al., 2005). Additionally, the transcription factor is known to play a role in tumorigenesis (Cho et al., 2007; Sun et al., 2015) and has been consistently associated with atrial fibrillation (Huang et al., 2015). Moreover, *Zfhlx3* is expressed in developing mouse SCN from at least E13.5 and may be important in the terminal differentiation of its peptidergic neurons (VanDunk et al., 2011). In comparison, it is notable that other transcription factors expressed in developing SCN have arguable adult circadian regulatory roles. Notably, deletion of the Lim homeodomain transcription factor (*Lhx1*) has contrasting effects on terminal differentiation and adult circadian function depending on when the gene is deleted in the maturing SCN (Bedont et al., 2014; Hatori et al., 2014).

To circumvent early developmental changes associated with *Zfhlx3* deletion and to assess its role specifically in adult mice, we used a tamoxifen-inducible transgenic line expressing Cre from the human ubiquitin C promoter (UBC CreERT2) (Ruzankina et al., 2007). Using this approach we demonstrate that ZFH3 maintains an active role in setting the pace of the circadian clock in adult mice, thus sustaining a key role in the maintenance of stable circadian rhythms independent of any earlier developmental disruptions.

MATERIALS AND METHODS

Mice

All animal studies were performed under the guidance issued by the Medical Research Council in Responsibility in the Use of Animals for Medical Research (July 1993) and Home Office Project License 30/3206, with local ethical approval. When not being tested, mice were group housed in individually ventilated cages under 12:12 h light-dark conditions with food and water available ad libitum. Floxed *Zfhlx3* mice, *Zfhlx3^{lml.1}td*, were kindly provided by Dr. Jintang Dong (Emory University) (Sun et al., 2012). These mice arrived on a mixed background of 129S6 and C57BL/6J and were subsequently backcrossed onto C57BL/6J to congenicity before being used for experimental crosses. An inducible Cre line

expressing UBC-cre (B6.Cg-Tg(UBC-cre/ERT2)1Ejb/J) was obtained from the Jackson Laboratory (Ruzankina et al., 2007). Mice were genotyped for *Zfhlx3* using a quantitative PCR (qPCR) copy count Taqman assay. Primers were as follows: WT primers: AAGAAGCGA TAAGCTAACACCAGG and ACGCCAAAGGTTGA GGAGAATG, probe sequence: TTAAAGGAATTCAC GGGGTTAGGGC; mutant primers: GCCATAACTTC GTATAATGTATGCTATACG and ACGCCAAAGGTT GAGGAGAATG, probe sequence: TTATAAGCTTAC GGGGTTAGGGCTGT. The mutant assay was designed to target the floxed region of the gene to distinguish between allele types while the copy count assay confirmed that the gene was not deleted prior to tamoxifen dosing. For the UBC-cre line, the same copy count assay was used with the following primers: GGGCTGCAGGTCGACTCT and TCGTTGCATC GACCGTAATG, probe sequence: AGAGGATCCAG TTAACCTCGAGGGC.

Tamoxifen Dosing

Tamoxifen was administered via oral gavage for 5 days, and a 7-day recovery period was allowed before further testing. A stock concentration of 20 mg/mL tamoxifen solution was prepared by dissolving tamoxifen (Cambridge Bioscience, Cambridge, UK) in corn oil containing 1% ethanol (MP Biomedicals, Leicester, UK). At 8 weeks, mice were dosed daily according to their weights for 5 days; the total dose given over the 5 days was 1 g/kg.

Circadian Phenotyping

Wheel-running activity was performed as described previously (Banks and Nolan, 2011). Briefly, mice were singly housed in cages containing running wheels placed in light-controlled chambers, and wheel-running activity was monitored via ClockLab (Actimetrics, Wilmette, IL). Default settings were used for analysis in ClockLab. Animals were monitored for 5 days in a 12-h light-dark cycle (100-lux light intensity) followed by 12 days in constant darkness. For the jet lag protocol, mice were maintained for 3 days in standard light-dark conditions, after which light onset was abruptly advanced by 6 h, and the new LD schedule was maintained for 14 days. Light onset was then delayed by 6 h, and mice were again maintained for 14 days under these new conditions. Reentrainment to the altered LD cycle was assessed by measuring the difference between lights-off and activity onset. An onset of sustained activity within 30 min following lights-off was used as the cutoff for an individual mouse to be deemed entrained.

Statistics

All data were analyzed by repeated-measures, mixed-model parametric analysis with Bonferroni correction and Dunnett test for multiple testing corrections using InVivoStat (Clark et al., 2012).

Immunofluorescence

Mouse brains were dissected and immediately embedded in molds containing OCT compound (VWR International, Radnor, PA); molds were then floated in isopentane on dry ice to freeze the samples. Next, 12- μ m sections were cut on a cryostat and collected onto slides. Slides were fixed in 4% paraformaldehyde for 4 h at 4 °C, washed in phosphate-buffered saline (PBS), and then incubated in 3% hydrogen peroxide in methanol for 30 min at 4 °C before washing with PBS again and then blocking in 5% normal goat serum made up in 0.5% PBS-Triton. Anti-ZFH3 raised in rabbit (Parsons et al., 2015) was diluted 1:1000 in the blocking solution, and primary incubation was 48 h at 4 °C. Anti-rabbit 488 raised in goat (Abcam, Cambridge, UK) was diluted 1:200 in PBS, and secondary incubation was 2 h at room temperature, following a PBS wash. Slides were coverslipped using ProLong Gold Antifade mountant with DAPI (Life Technologies, Carlsbad, CA). Sections were imaged using an inverted confocal microscope running Zen software; settings were reused between images, and the “best fit” function was applied to all captured images.

RESULTS

Adult Specific Knockout of *Zfhlx3* Using an Inducible Cre Line

To generate inducible knockout *Zfhlx3* animals, tamoxifen inducible cre mice (UBC-Cre) were crossed to *Zfhlx3* floxed (*Zfhlx3^{Flox}*) mice to produce an initial stock of *Zfhlx3^{Flox/+}*;UBC-Cre⁺ mice. These animals were subsequently crossed to *Zfhlx3^{Flox/+}* mice to generate experimental cohorts (Sun et al., 2012). This resulted in progeny of 6 possible genotype combinations: animals that carried neither the Cre nor *Zfhlx3^{Flox}* alleles (*Zfhlx3^{+/+}*;UBC-Cre⁻), animals heterozygous or homozygous for the *Zfhlx3^{Flox}* allele but not carrying the Cre allele (*Zfhlx3^{Flox/+}*;UBC-Cre⁻ or *Zfhlx3^{Flox/Flox}*;UBC-Cre⁻), animals that were hemizygous for the Cre allele but did not carry the *Zfhlx3* allele (*Zfhlx3^{+/+}*;UBC-Cre⁺), and animals that were hemizygous for the Cre allele and were either heterozygous or homozygous for the *Zfhlx3^{Flox}* allele (*Zfhlx3^{Flox/+}*;UBC-Cre⁺ or *Zfhlx3^{Flox/}*

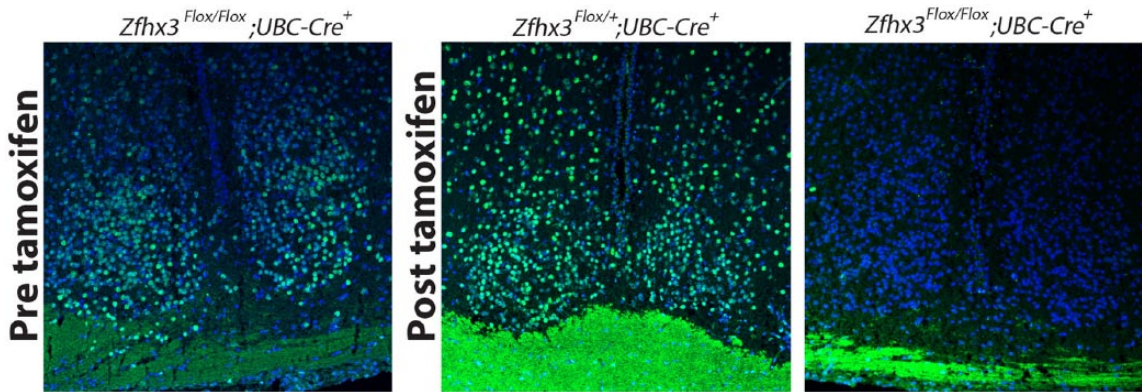


Figure 1. ZFHX3 immunofluorescence in representative SCN sections ($n = 3$ for each genotype); although detectable prior to tamoxifen treatment, ZFHX3 immunopositive cells are undetectable following treatment in $Zfhx3^{Flox/Flox};UBC-Cre^+$ mice, while staining remains robust in SCN of $Zfhx3^{Flox/+};UBC-Cre^+$, used here as a representative control. Blue, DAPI; green, ZFHX3.

$Flox;UBC-Cre^+$). The final group would result in heterozygous or homozygous deletion of critical floxed $Zfhx3$ exons following tamoxifen administration, thus creating a temporal null allele.

Immunofluorescence was undertaken on SCN sections from these animals to validate protein loss following tamoxifen treatment (Fig. 1). Prior to tamoxifen treatment, there was no obvious decrease in ZFHX3 staining in homozygous mutants compared with control SCN. Following treatment, however, ZFHX3 staining was effectively absent in SCN. SCN sections from control genotypes showed no evident decrease in ZFHX3 staining following tamoxifen treatment.

Tamoxifen-mediated Deletion of $Zfhx3$ in Adult Mice Shortens τ_{DD}

The effect of tamoxifen treatment on all groups was assessed using repeated-measures parametric analysis with tamoxifen treatment as a repeated-measures variable. All genotypes underwent circadian analysis (data shown in Table 1); however, $Zfhx3^{Flox/Flox};UBC-Cre^-$ mice have been used as the representative “control” group in figures for uniformity and clarity. Ten $Zfhx3^{Flox/Flox};UBC-Cre^+$ mice and all controls were screened for changes in circadian parameters both before and after tamoxifen treatment. We found no significant group differences in any circadian parameters analyzed prior to tamoxifen treatment (Table 1). Following tamoxifen treatment, 7 out of 10 $Zfhx3^{Flox/Flox};UBC-Cre^+$ mice displayed a significant shortening of τ_{DD} ($F_{4,41} = 28.4$, $p < 0.0001$). Multiple test correction of pairwise comparisons between the experimental groups confirmed a significant shortening of τ_{DD} in treated $Zfhx3^{Flox/Flox};UBC-Cre^+$ mice compared with all other genotypes ($p < 0.005$ for $Zfhx3^{Flox/Flox};UBC-Cre^+$ vs. all other genotypes). Furthermore, only $Zfhx3^{Flox/Flox};UBC-Cre^+$

animals displayed a significant change in τ_{DD} following tamoxifen treatment ($Zfhx3^{Flox/Flox};UBC-Cre^+$ circadian period: before vs. after tamoxifen treatment; $p = 0.0045$). Percentage activity in the light phase for $Zfhx3^{Flox/Flox};UBC-Cre^+$ animals (Fig. 2D) showed an increase following tamoxifen treatment. Amplitude was also decreased in $Zfhx3^{Flox/Flox};UBC-Cre^+$ animals (Fig. 2E); however, neither of these parameters remained statistically significant following multiple testing correction.

In addition to the period shortening seen in the 7 homozygous mutants described, 3 $Zfhx3^{Flox/Flox};UBC-Cre^+$ mice became arrhythmic in constant darkness (DD) following tamoxifen treatment (Fig. 2, B and C) and so could not be included in the statistical analysis of τ_{DD} . A comparison of chi-square periodograms in these animals before and after tamoxifen treatment showed a loss of significant peak amplitude after tamoxifen (Fig. 2C). $Zfhx3^{Flox/+};UBC-Cre^+$ mice and all control genotypes screened showed no significant changes in other circadian parameters following tamoxifen treatment. The interaction between genotype and tamoxifen treatment showed no significant differences in the phase angle of entrainment (ϕ) ($F_{4,45} = 1.9$, $p = 0.1266$) or in alpha (activity duration) in LD (α_{LD}) and DD (α_{DD}) (LD: $F_{4,45} = 0.43$, $p = 0.7878$; DD: $F_{4,41} = 1.39$, $p = 0.2558$), suggesting that these parameters were unaffected by disruption of $Zfhx3$. To confirm this statistical analysis, a Dunnett test for multiple corrections was used to compare $Zfhx3^{Flox/Flox};UBC-Cre^+$ mice against all other genotypes. This analysis found no additional significant differences.

Activity Pattern Relative to LD Is Altered in $Zfhx3^{Flox/Flox};UBC-Cre^+$ Mutants

To analyze whether the daily pattern of activity over 24 h differed in tamoxifen-treated $Zfhx3^{Flox/}$

Table 1. Circadian behavioral data for all genotypes tested.

Circadian Parameter and Genotype	<i>n</i>	Before Tamoxifen	After Tamoxifen	<i>p</i> Value	Adjusted <i>p</i> Value
τDD (hours)					
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁺	7	23.72 ± 0.09	22.66 ± 0.15	0.0001	0.0045
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁺	11	23.76 ± 0.03	23.74 ± 0.05	0.7968	1.0000
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁻	8	23.75 ± 0.02	23.73 ± 0.11	0.8102	1.0000
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁻	10	23.76 ± 0.04	23.78 ± 0.04	0.8155	1.0000
<i>Zfhx3</i> ^{+/+} ;UBC-Cre ⁺	10	23.69 ± 0.09	23.74 ± 0.04	0.4852	1.0000
Amplitude (AU)					
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁺	7	5272 ± 636	3658 ± 646	0.0034	0.1530
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁺	11	5284 ± 361	5568 ± 541	0.4964	1.0000
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁻	8	5405 ± 517	6165 ± 614	0.1459	1.0000
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁻	10	4218 ± 320	4830 ± 320	0.1666	1.0000
<i>Zfhx3</i> ^{+/+} ;UBC-Cre ⁺	10	4571 ± 423	5789 ± 656	0.0076	0.3420
Activity in light phase (%)					
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁺	10	6.79 ± 1.02	11.32 ± 2.01	0.0135	0.6075
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁺	11	4.51 ± 1.04	5.25 ± 1.50	0.8215	1.0000
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁻	10	8.81 ± 1.99	5.98 ± 1.67	0.1141	1.0000
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁻	10	10.17 ± 1.99	6.10 ± 1.03	0.0253	1.0000
<i>Zfhx3</i> ^{+/+} ;UBC-Cre ⁺	10	6.46 ± 1.32	3.69 ± 0.98	0.1236	1.0000
φ (degrees)					
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁺	10	179 ± 0.9	169 ± 7.1	0.0055	0.2475
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁺	11	175 ± 3.7	172 ± 7.5	0.3587	1.0000
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁻	9	175 ± 5.1	176 ± 4.0	0.9868	1.0000
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁻	10	179 ± 0.7	181 ± 0.5	0.6193	1.0000
<i>Zfhx3</i> ^{+/+} ;UBC-Cre ⁺	10	180 ± 0.7	181 ± 0.6	0.8466	1.0000
α_{LD} (hours)					
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁺	10	12.09 ± 0.14	11.59 ± 0.52	0.0796	1.0000
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁺	11	12.10 ± 0.16	11.85 ± 0.14	0.3470	1.0000
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁻	9	12.22 ± 0.22	12.03 ± 0.13	0.4906	1.0000
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁻	10	12.11 ± 0.17	12.07 ± 0.09	0.8707	1.0000
<i>Zfhx3</i> ^{+/+} ;UBC-Cre ⁺	10	11.93 ± 0.12	11.86 ± 0.10	0.8508	1.0000
α_{DD} (hours)					
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁺	10	12.20 ± 0.82	10.73 ± 1.26	0.0229	1.0000
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁺	11	12.84 ± 0.27	13.22 ± 0.23	0.5089	1.0000
<i>Zfhx3</i> ^{Flox/Flox} ;UBC-Cre ⁻	8	13.64 ± 0.39	13.08 ± 0.39	0.5556	1.0000
<i>Zfhx3</i> ^{Flox/+} ;UBC-Cre ⁻	10	12.67 ± 0.24	12.77 ± 0.21	0.8686	1.0000
<i>Zfhx3</i> ^{+/+} ;UBC-Cre ⁺	10	12.68 ± 0.19	12.27 ± 0.18	0.4978	1.0000

τDD = period in constant darkness; AU = arbitrary units; φ = phase angle of entrainment in light-dark (180° denotes a phase angle consistent with a ZT12 activity onset); α_{LD} = activity duration in light-dark; α_{DD} = activity duration in constant darkness. Both unadjusted and adjusted *p* values are shown for pairwise comparisons of group data before and after tamoxifen treatment; Bonferroni adjustment was used for correction of multiple comparisons. Units of measurement for parameters refer to measurements before and after tamoxifen administration, expressed as mean ± standard error of the mean.

Zfhx3^{Flox/+};UBC-Cre⁺ animals compared with controls, average wheel-running activity was measured in 6-min time bins over the 24-h LD cycle. Following tamoxifen treatment, all *Zfhx3*^{Flox/Flox};UBC-Cre⁺ mice showed an increased proportion of activity in the light phase of a standard 12:12 LD cycle. The interaction between this and tamoxifen treatment was found to be significant ($F_{4,446} = 3.91$, $p = 0.0081$); however, significant changes did not survive multiple testing correction. Investigation of total activity over 24 h (Fig. 3A) revealed that tamoxifen-treated *Zfhx3*^{Flox/Flox};UBC-Cre⁺ animals showed a significantly different average

pattern of activity compared with controls ($F_{240,4079} = 2.03$, $p = 0.0001$). This difference was clear at 2 stages of the dark phase: First, during the early period of the dark phase, *Zfhx3*^{Flox/Flox};UBC-Cre⁺ animals showed an elevated activity compared with controls; second, during the late period of the dark phase, *Zfhx3*^{Flox/Flox};UBC-Cre⁺ animals showed decreased activity compared with controls. Moreover, although not identified as a significant phase angle of entrainment effect, mutants appeared to show some increase in activity relative to the onset of the dark phase compared with controls.

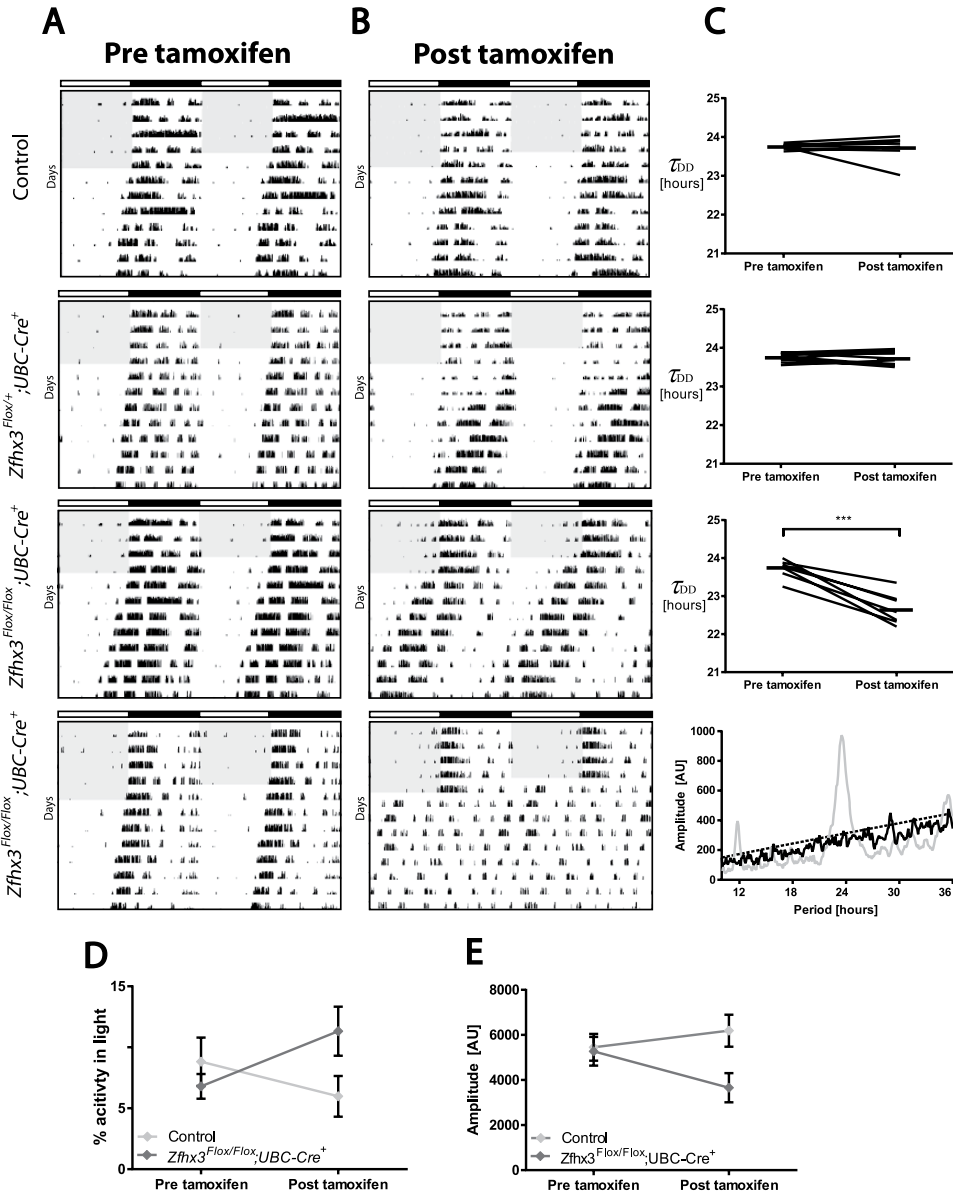


Figure 2. Representative double-plotted actograms for *Zfhx3*^{Flox/Flox};UBC-Cre⁺, *Zfhx3*^{Flox/+};UBC-Cre⁺, and control (*Zfhx3*^{Flox/Flox};UBC-Cre⁻) mice (A) before and (B) after tamoxifen treatment; shading denotes lights-on. Animals are in constant darkness from day 5. (C) Change in period in constant darkness following tamoxifen treatment for genotypes shown (control, *n* = 8; *Zfhx3*^{Flox/+};UBC-Cre⁺, *n* = 11; *Zfhx3*^{Flox/Flox};UBC-Cre⁺, *n* = 7) (***) *p* < 0.001). A representative chi-square periodogram in constant darkness is shown for *Zfhx3*^{Flox/Flox};UBC-Cre⁺ mice that became arrhythmic following tamoxifen treatment (*n* = 3); gray line denotes periodogram before tamoxifen, black line denotes periodogram after tamoxifen treatment, and dashed line denotes chi-square line of significance. (D) Change in percentage activity in the light phase following tamoxifen treatment for control (*n* = 8) and *Zfhx3*^{Flox/Flox};UBC-Cre⁺ mice (*n* = 7). (E) Change in amplitude following tamoxifen treatment for control (*n* = 8) and *Zfhx3*^{Flox/Flox};UBC-Cre⁺ mice (*n* = 7).

***Zfhx3*^{Flox/Flox};UBC-Cre⁺ Mutants Show Rapid Reentrainment to an Advance in the LD cycle**

Upon release into DD, all tamoxifen-treated *Zfhx3*^{Flox/Flox};UBC-Cre⁺ mice that maintained stable rhythms showed an initial advance in activity onset compared with this measurement prior to tamoxifen treatment (*p* = 0.01) or in controls (*p* = 0.002). This advance in activity was, on average, around 4 h

greater than the modest advances typically seen upon release into DD from LD in controls (Fig. 3, B and C). This apparent strong advance in activity onset prompted us to investigate the behavior of these mutants in jet lag protocols. To analyze reentrainment to a shift in the LD cycle, animals were subjected to an abrupt 6-h advance, and also delay, in lights-on. The number of days taken to reentrain to the new LD cycle was measured for all groups fol-

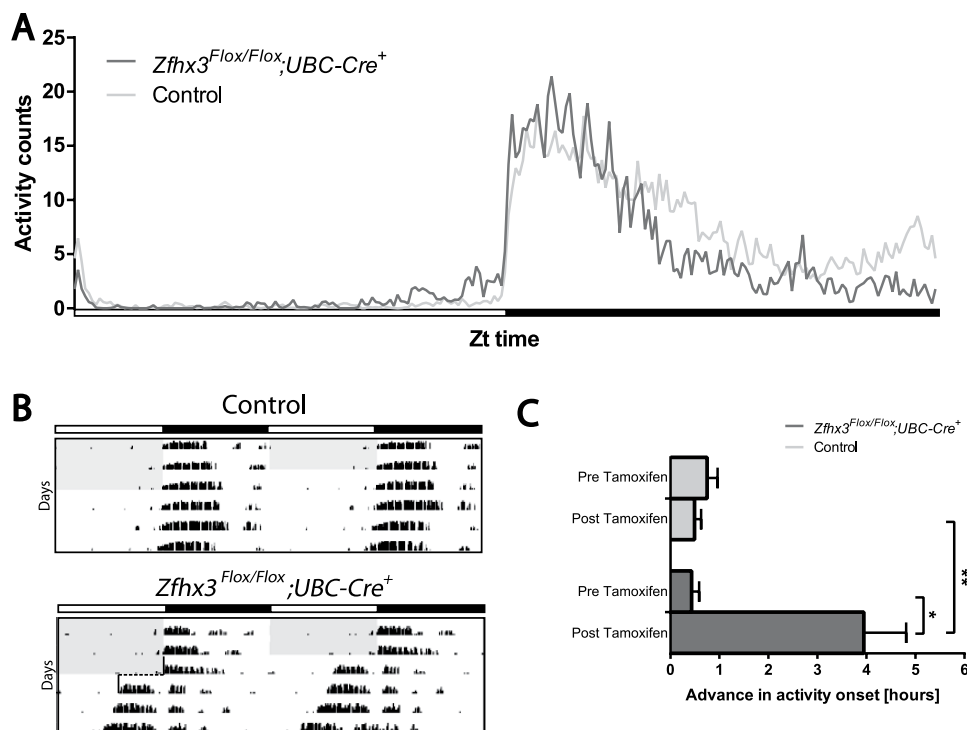


Figure 3. (A) Average activity profile for $Zfhx3^{Flox/Flox};UBC-Cre^+$ mice (dark gray line) and control ($Zfhx3^{Flox/Flox};UBC-Cre^-$) mice (light gray line) after tamoxifen treatment over 12:12 LD cycle ($n = 10$). (B) Representative actograms showing advance in activity onset upon release into DD for control and $Zfhx3^{Flox/Flox};UBC-Cre$ mice after tamoxifen treatment. (C) Advance in activity onset upon release into DD conditions. Difference in activity onset from last day of LD before (upper bars) and after (lower bars) tamoxifen for $Zfhx3^{Flox/Flox};UBC-Cre^+$ mice (dark gray) and control mice (light gray) ($n = 6$). * $p < 0.05$, ** $p < 0.01$.

lowing tamoxifen treatment (data shown in Table 2). Since a defective clock may cause entrainment effects that are difficult to interpret, $Zfhx3^{Flox/Flox};UBC-Cre^+$ mice that were arrhythmic in constant darkness were excluded from this analysis. However, it is notable that when tested, these arrhythmic animals did resynchronize to the new LD cycle in a similar manner to rhythmic animals (number of days to reentrain or resynchronize: rhythmic animals = 3.0 ± 0.52 ; arrhythmic animals = 2.0 ± 0.57 ; $p = 0.28$).

No significant difference was noted in response of activity onsets to a 6-h delay in the LD cycle between genotypes ($F_{4,214} = 0.37$, $p = 0.825$). Similarly, changes in activity offsets were not significantly different. However, there was a marked, significant difference between $Zfhx3^{Flox/Flox};UBC-Cre^+$ mice and controls in response to a 6-h advance ($F_{4,39} = 3.94$, $p = 0.0088$), with mutants reentraining on average twice as fast as control mice (Fig. 4). Average activity onsets in these mice advanced by around 4 h on day 1 and then by almost a further 2 h on day 2. Pairwise comparisons following multiple test correction demonstrated that $Zfhx3^{Flox/Flox};UBC-Cre^+$ animals had an earlier onset of activity for the first 2 days following the phase advance ($p < 0.05$ and $p < 0.01$ for $Zfhx3^{Flox/Flox};UBC-Cre^+$ for days 1 and 2, respectively, following phase

advance). There were no other significant differences between genotypes throughout the time course of the experiment. Since the reduction in reentrainment time demonstrated by $Zfhx3^{Flox/Flox};UBC-Cre^+$ animals could be due to the combined effects of a faster running clock and of negative masking, we compared the advances in activity onsets that occurred on the first 2 days of release into DD with those that occurred on the first 2 days of the phase advance protocol. These advances were comparable in $Zfhx3^{Flox/Flox};UBC-Cre^+$ animals, suggesting that the effects of negative masking upon release into constant darkness can fully account for differences in reentrainment in the phase advance protocol.

DISCUSSION

The transcription factor ZFH3 has a range of crucial functions in cell cycle regulation, development, and cellular differentiation (Ishii et al., 2003; Jung et al., 2005; Sun et al., 2012). In addition, recent studies have demonstrated that the protein modulates circadian activity in mice by regulating the transcription of many key genes in the SCN, including neuropeptides and their receptors (Parsons et al., 2015). In

Table 2. Days to reentrain in jet lag protocol for all genotypes tested.

Protocol and Genotype	<i>n</i>	Days to Reentrain	<i>p</i> value
Advancing LD			
<i>Zfhlx3</i> ^{Flox/Flox} ;UBC-Cre ⁺	6	3.0 ± 0.5	n/a
<i>Zfhlx3</i> ^{Flox/+} ;UBC-Cre ⁺	10	5.6 ± 0.5	0.0471
<i>Zfhlx3</i> ^{Flox/Flox} ;UBC-Cre ⁻	9	4.9 ± 0.7	0.097
<i>Zfhlx3</i> ^{Flox/+} ;UBC-Cre ⁻	10	6.6 ± 0.5	0.0109
<i>Zfhlx3</i> ^{+/+} ;UBC-Cre ⁺	10	6.3 ± 0.7	0.0146
Delaying LD			
<i>Zfhlx3</i> ^{Flox/Flox} ;UBC-Cre ⁺	5	3.0 ± 0.8	n/a
<i>Zfhlx3</i> ^{Flox/+} ;UBC-Cre ⁺	6	2.8 ± 0.2	1.0000
<i>Zfhlx3</i> ^{Flox/Flox} ;UBC-Cre ⁻	4	3.8 ± 0.6	1.0000
<i>Zfhlx3</i> ^{Flox/+} ;UBC-Cre ⁻	8	3.1 ± 0.3	1.0000
<i>Zfhlx3</i> ^{+/+} ;UBC-Cre ⁺	4	3.3 ± 0.5	1.0000

The table provides the number of days (mean ± standard error of the mean) taken to reentrain to a 6-h advance and a 6-h delay in the light-dark cycle for all genotypes tested in the jet lag protocol. All data shown are for animals after tamoxifen treatment.

P values shown are for that genotype compared with *Zfhlx3*^{Flox/Flox};UBC-Cre⁺ and Bonferroni corrected for multiple comparison.

instances where circadian gene mutations have pleiotropic effects, it is often difficult to establish which mutant phenotypes are consequences of developmental defects and which are specifically due to circadian rhythm disruption in the adult. By using a tamoxifen-inducible Cre line, we have confirmed that ZFH3 has a sustained function in regulating adult circadian rhythms that is distinct from any of its developmental functions.

Genetic pleiotropy is evident in many clock components, affecting diverse functions from neural development and differentiation to metabolic homeostasis and cell cycle regulation. Inducible and conditional knockout tools enable one to study specific gene effects in the adult while still maintaining the gene's developmental function. Surprisingly, such techniques are not routinely used in the circadian field. Constitutive mutations in one of the core circadian genes, *Rora*, for example, result in ataxia and perinatal lethality (Brown and Moore, 2012; Gold et al., 2007), while conditional mutagenesis has not been used to establish this gene's role in adult rhythm maintenance. In a study contrasting developmental and adult functions of *Bmal1* using inducible conditional knockout mice, many of the phenotypes previously attributed to circadian disruption of *Bmal1* could be reassigned to developmental functions of the gene (Yang et al., 2016). Such studies demonstrate the usefulness of tamoxifen-inducible conditional knockout approaches in dissecting circadian function. Similar studies could be undertaken for other core clock genes such as *Per*, *Cry*, and *Clock*, whose roles in SCN development may have been underestimated or not distinguished from adult circadian rhythm maintenance. Moreover, with inducible Cre

driver lines, the ability to use the same animal for data collection before and after tamoxifen treatment allows each animal to act as its own control, eliminating some of the natural variability in circadian parameters observed between animals.

The experimental data reported here reinforce a pivotal function for *Zfhlx3* in maintaining normal circadian rhythms and a robust circadian clock. It is particularly notable that 30% of the animals displayed behavioral arrhythmicity in DD conditions following adult knockout of *Zfhlx3*. Single gene knockouts capable of inducing arrhythmia are rare (Baggs et al., 2009), with *Bmal1* currently being the only known example sufficient to cause arrhythmia in constant conditions (Bunger et al., 2000). The remaining 70% of *Zfhlx3* mutants described all showed shortening of τ_{DD} by around an hour, which is comparable to the effect of the heterozygous *Sci* mutation in *Zfhlx3* previously reported (Parsons et al., 2015). These varied effects of gene knockout on behavioral rhythms reflect elements of those seen in both *Lhx1* (Bedont et al., 2014; Hatori et al., 2014) and *Vip* mutants (Aton et al., 2005; Colwell et al., 2003) and may represent combined effects of the mutation on the robustness of SCN intracellular clocks and on maintenance of SCN intercellular synchrony. It is also notable that heterozygous knockout of *Zfhlx3* in adult mice in this study results in no detectable phenotype. This is in contrast to the reported effects of the *Sci*/+ mutation and of localized *Zfhlx3* gene knock-down in SCN (Parsons et al., 2015). Differences here could be attributable to the as-yet undefined, integrated effects of *Zfhlx3* knockout in other hypothalamic and thalamic brain nuclei, while they might also be related to differential effects of *Zfhlx3* knockout on dorsal AVP-expressing and ventral VIP-expressing SCN neurons. Similar inconsistencies in SCN-neural and whole-animal behavioral rhythms have been reported in circadian period aftereffects following the entrainment of animals to light-dark cycles that are longer or shorter than 24 h (Aton et al., 2004; Azzi et al., 2017; Molyneux et al., 2008). Further investigations are required to clarify these effects in *Zfhlx3* mutants. Furthermore, as the Cre driver used in this study has strong expression in all tissues, effects of gene deletion on peripheral clocks would be possible. Despite significant changes to period, other key circadian parameters (such as changes in alpha and phase angle of entrainment) were unaffected following the loss of *Zfhlx3*. While we found no significant effect of genotype and tamoxifen treatment on the phase angle of entrainment, we did see an apparent increase in activity immediately prior to the onset of darkness in adult *Zfhlx3* knockout animals. Although noticeable, the changes show no significant effect using ClockLab algorithms to determine phase angle of entrainment.

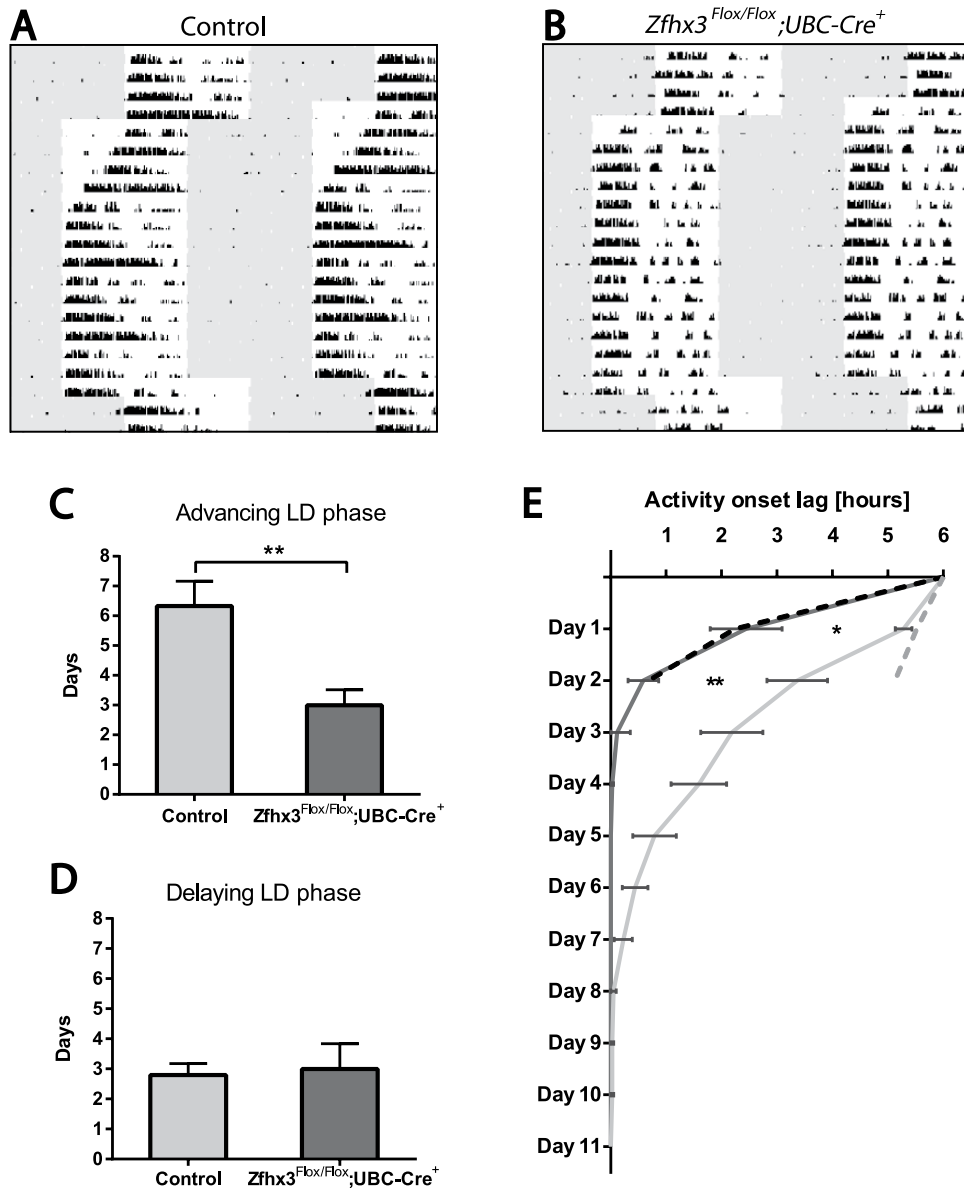


Figure 4. Representative double-plotted actograms showing reentrainment of wheel-running activity in response to 6-h phase advances or phase delays for (A) control (*Zfhx3*^{Flox/Flox};UBC-Cre⁻) mice and (B) *Zfhx3*^{Flox/Flox};UBC-Cre⁺ mice. (C, D) Average number of days taken for control (light gray) and *Zfhx3*^{Flox/Flox};UBC-Cre⁺ (dark gray) mice to reentrain to (C) a 6-h advance in LD and (D) a 6-h delay in LD. (E) Average activity onset for control (light gray, *n* = 9) and *Zfhx3*^{Flox/Flox};UBC-Cre⁺ (dark gray, *n* = 6) mice following an advance in LD cycle of 6 h. All jet lag experiments were undertaken after tamoxifen treatment. For reference, dashed lines show respective advances in activity onset over the first 2 days following release into DD for control (gray) and mutant (black) mice. **p* < 0.05, ***p* < 0.01.

In addition to changes in free-running period, rapid reentrainment to advancing LD cycles was observed in adult *Zfhx3* knockout mice. Consistent with the apparent increases in activity in mutants in LD, this effect can be explained by the negative masking in adult *Zfhx3* knockout mice. The effect is also reflected in the 4-h phase advances when mutants are released into constant darkness. Conversely, however, no differences were detected in time taken to reentrain to 6-h phase delays as reflected in changes to both activity onsets and offsets. Phase-resetting

phenotypes have previously been shown in mice lacking the vasopressin receptors V1a and V1b (Yamaguchi et al., 2013). Our previous data demonstrating that *Zfhx3* modulates the expression of a number of neuropeptides, including vasopressin (Parsons et al., 2015), suggested that similar disturbances would be observed in adult *Zfhx3* knockout mice. Notably, however, in contrast to adult *Zfhx3* knockout mice, V1a and V1b knockout animals showed no changes in circadian period (Yamaguchi et al., 2013). This indicates the complexity of

the phenotype when *Zfhlx3* is deleted in the adult, indicating that its transcriptional effects are mediated through an integration of numerous downstream effector systems and are not restricted to neuropeptidergic effects. This complexity may also underlie why, in contrast with adult *Zfhlx3* knockout mice, V1a and V1b knockout animals also reentrain faster to a phase-delaying light-dark cycle. It remains to be determined whether combined effects on AVP and VIP peptidergic systems can explain the full effect of the *Zfhlx3* mutant deficits or whether additional downstream effectors might contribute to this. It is also notable that similar complex effects have been achieved by knocking out the transcription factor *Eif4ebp1* (which also modulates neuropeptide expression). Knockout of this transcription factor can lead to a greater resistance to light-induced disruption (Cao et al., 2013).

This study confirms that ZFHX3 plays an active role in maintaining circadian period in adult mice in contrast to its well-characterized developmental functions. Based on aforementioned published data (Parsons et al., 2015) and the new data presented in this publication, it is likely that *Zfhlx3* is acting as a master regulator of coupling agents in the circadian system (Mohawk et al., 2012) to maintain synchrony in the SCN. Nevertheless, we are only part of the way in resolving ZFHX3's multiple roles in developing and adult SCN. This is further confounded by the fact that the constitutive null mutant is subviable even in the heterozygous state. To investigate ZFHX3 function further, it will be necessary to investigate the consequences of its limited deletion using a number of spatially restricted and inducible Cre driver lines. Similar approaches have been used in further defining the roles of clock genes and SCN-enriched genes in circadian regulatory processes (Bedont et al., 2014; Hatori et al., 2014; Lee et al., 2015; Mieda et al., 2015; Smyllie et al., 2016). The use of hypothalamic Cre driver lines expressed in differentiating SCN should help to clarify how *Zfhlx3* disruption from early development might affect circadian behavior in the adult, while the use of inducible Cre lines that specify particular neuronal classes in adult SCN will help to establish how acute ablation of *Zfhlx3* in specific adult SCN cells can disrupt the clock. Such work will assist in defining whether the mechanisms of ZFHX3-mediated circadian disruption are similar in differentiating and adult SCN neurons.

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CONFLICT OF INTEREST STATEMENT

The author(s) have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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