Cholinergic neuron circadian clock mediates RNA-binding protein function and contributes to ALS disease phenotypes

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ABSTRACT

Circadian clocks are encoded by a transcription-translation feedback loop that aligns physiological processes with the solar cycle. Previous work linking the circadian clock to the regulation of RNA-binding proteins (RBPs) and alternative splicing provides a foundation for the vital examination of their mechanistic connections in the context of amyotrophic lateral sclerosis (ALS)—a fatal neurodegenerative disease characterized by disrupted RBP function. Here, we reveal enrichment of genes associated with ALS and other neurodegenerative diseases in the spinal cord cholinergic neuron rhythmic transcriptome. We demonstrate that there is circadian regulation of ALS-linked RBPs and rhythmic alternative splicing of genes involved in intracellular transport (*Aftph* and *Mvb12a*), microtubule cytoskeleton organization (*Limch1* and *Drc3*), and synaptic function (*Sipa1l2*) in this neuronal sub-type. Further, we show that the cholinergic neuron clock regulates sporadic ALS-associated changes in cytoskeleton and neuromuscular junction synapse gene expression. Finally, we report that cell-type-specific *Bmal1*-deletion *(i)* increases lumbar spinal cord motor neuron loss and sciatic nerve axon degeneration, *(ii)* drives alternative splicing of genes encoding ALS-linked RBPs (*Matr3* and *Srsf7*), and *(iii)* drives alternative splicing of genes associated with microtubule transport and postsynaptic organization. Our results establish a role for the cholinergic neuron circadian clock in RBP function and ALS disease phenotypes.

INTRODUCTION

The circadian system acts as a biological timekeeper that aligns physiological energetic cycles with the day/night cycle. In mammals, the molecular clock is encoded by a transcription-translation feedback loop comprised of activators (CLOCK and BMAL1) that induce the transcription of repressors (PERs and CRYs), which feedback to inhibit the forward limb in a cycle that repeats itself every ~24 hours, with a stabilizing loop consisting of REV-ERB and ROR transcription factors that modulate BMAL1 expression (Bass 2012). In recent decades, clock transcriptional regulation has been characterized in diverse mammalian tissues and is estimated to drive the rhythmic expression of 10-15% of all mRNAs within a given tissue (Lowrey and Takahashi 2004; Panda et al. 2002; Vollmers et al. 2009). Intriguingly, recent studies in liver have demonstrated that most of the genes that exhibit rhythmic mRNA expression do not exhibit rhythmic expression of the corresponding intron-containing pre-mRNA, suggesting that post-transcriptional modifications play a major role in the regulation of circadian gene expression (Green 2018; Koike et al. 2012; Kojima et al. 2011; Menet et al. 2012).

Emerging evidence points to a connection between the circadian clock and RNA-processing events, such as methylation, polyadenylation, and alternative splicing (Fustin et al. 2013; Kojima et al. 2012; Marcheva et al. 2020). Time-of-day-dependent production of alternatively spliced transcripts has been previously reported in plants, flies, and mice, primarily in neuronal cell-types (Hughes et al. 2012; Marcheva et al. 2020; McGlincy et al. 2012; Sanchez et al. 2010; Wang et al. 2018; Yang et al. 2020). Alternative splicing allows a single gene to produce multiple distinct mRNA transcripts in a tissue- and cell-type specific manner, which contributes to transcriptomic diversity and enables cells to perform complex and specialized functions (Chen and Manley 2009; Young et al. 1981). This process is strongly regulated by RNA-binding proteins (RBPs), which bind to specific regulatory sequence motifs in pre-mRNA and either enhance or suppress spliceosome recruitment to the splice sites (Ule and Blencowe 2019). While RBPs are ubiquitously expressed, cells and tissue within the central nervous system (CNS) demonstrate greater disease vulnerability to RBP dysregulation due to their higher frequency of alternative splicing and more intricate transcriptomes (Pan et al. 2008; Yeo et al. 2004).

Disrupted RBP homeostasis is a hallmark of many neurological diseases, including amyotrophic lateral sclerosis (ALS), which is a progressive neurodegenerative disease that originates in upper and lower motor neurons in the brain and spinal cord (Nussbacher et al. 2019; Prashad and Gopal 2021). Genetic studies have revealed associations between ALS and mutations in genes encoding a number of RBPs, including TDP-43, FUS, ATXN2, TAF15, EWSR1, hnRNPA1, hnRNPA2B1, MATR3, and TIA1 (Kapeli et al. 2017; Zhao et al. 2018). These RBPs are key regulators of RNA metabolism and neuronal health, and ALS-linked mutations have been shown to vastly disrupt RNA processes, such as alternative splicing, mRNA transport, liquid phase transitions, and local translation (Kapeli et al. 2017; Nagano and Araki 2021). For example, many of the proteins that co-purify with TDP-43 are associated with RNA splicing and translation (Freibaum et al. 2010), and ALS-linked mutations in TDP-43 were shown to impair mRNA axonal transport and alter the fluid properties of TDP-43-positive liquid droplets (Alami et al. 2014; Schmidt and Rohatgi 2016).

Previous work linking the circadian clock to RBP function provides a foundation for the critical examination of their mechanistic connections in the context of ALS. The role of the lower motor neuron clock in disrupted RBP function and alternative splicing as it relates to ALS disease pathophysiology is not known. Here, we perform transcriptomic analysis of spinal cord cholinergic neurons to elucidate the time-of-day-dependent effects of cell-type-specific clock disruption. We report that the cholinergic neuron rhythmic transcriptome is enriched for genes associated with ALS and other neurodegenerative diseases. We demonstrate that there is circadian regulation of ALS-linked RBPs and rhythmic alternative splicing of genes involved in intracellular transport, microtubule cytoskeleton organization, and synaptic function. Moreover, we establish that the cholinergic neuron clock regulates sporadic ALS (sALS)-associated changes in cytoskeleton and neuromuscular junction (NMJ) synapse gene expression. Finally, we show that cholinergic neuron-specific *Bmal1*-deletion *(i)* increases lumbar spinal cord motor neuron loss and sciatic nerve axon degeneration, *(ii)* drives alternative splicing of genes encoding ALS-linked RBPs, and *(iii)* drives alternative splicing of genes associated with microtubule transport and postsynaptic organization. Thus, we propose that disruption of the cholinergic neuron circadian clock alters RBP function and contributes to ALS disease phenotypes.

RESULTS

Rhythmic transcriptome profiling of spinal cord cholinergic neurons reveals enrichment for ALS and other neurodegenerative diseases

To elucidate the cholinergic neuron transcriptome, we utilized *ChAT-Cre*;*RiboTag*$^{fx/+}$ mice, which possess a ribosomal hemagglutinin (HA) tag to enable the isolation and characterization of mRNAs specifically bound to cholinergic neuron ribosomes. We isolated RNA from spinal cord cholinergic neurons every 4 hours across the 24-hr day for RNA sequencing (RNA-seq) (Fig. 1A and Fig. S1A-B). We identified 2,391 oscillating transcripts (~12% of genes) by Jonckheere-Terpstra-Kendall (JTK) Cycle analysis and observed that most of the rhythmic transcripts reached maximal abundance at Zeitgeber Time (ZT) 20 and 22 in the dark (active) period, while a smaller cluster of genes was also found to peak at ZT8 in the light (rest) period (Fig. 1B-C; Table S1A). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed overall enrichment of genes associated with *(i)* neurodegenerative diseases (ALS, Parkinson's disease, Huntington's disease, prion disease, and spinocerebellar ataxia), *(ii)* protein synthesis and degradation (spliceosome, ribosome, protein processing in the endoplasmic reticulum, proteasome, and ubiquitin-mediated proteolysis), *(iii)* metabolism (oxidative phosphorylation, TCA cycle, and metabolic pathways), and *(iv)* synaptic transmission (GABAergic synapse, dopaminergic synapse, and long-term potentiation) (Fig. 1D).

There were 711 rhythmic genes that had peak abundance during the light period (ZT0-12), and Gene Ontology (GO) enrichment analysis revealed over-representation for metabolic and biosynthetic processes, including translation and RNA processing (Fig. S2). In contrast, 1,680 rhythmic genes exhibited peak abundance during the dark period (ZT12-24), and GO analysis revealed enrichment for pathways associated with protein localization and transport (Fig. S2). Notably, we found that 89 of the oscillating transcripts overlapped with KEGG pathway genes associated with ALS, including genes related to oxidative phosphorylation (e.g., *Ndufa5*, *Cox5a*, and *Atp5f1c*), autophagy (e.g., *Sqstm1*, *Atg101*, and *Atg14*), and the oxidative stress response (e.g., *Atf4*, *Pink1*, and *Als2*) (Fig. 1E; Table S1B). Additionally, 15 of the rhythmically expressed ALS genes were associated with the GO term "cytoskeleton organization," including *Pfn1*, *Dctn2*, and *Nefm* (Fig. 1F; Table S1B).

Figure 1

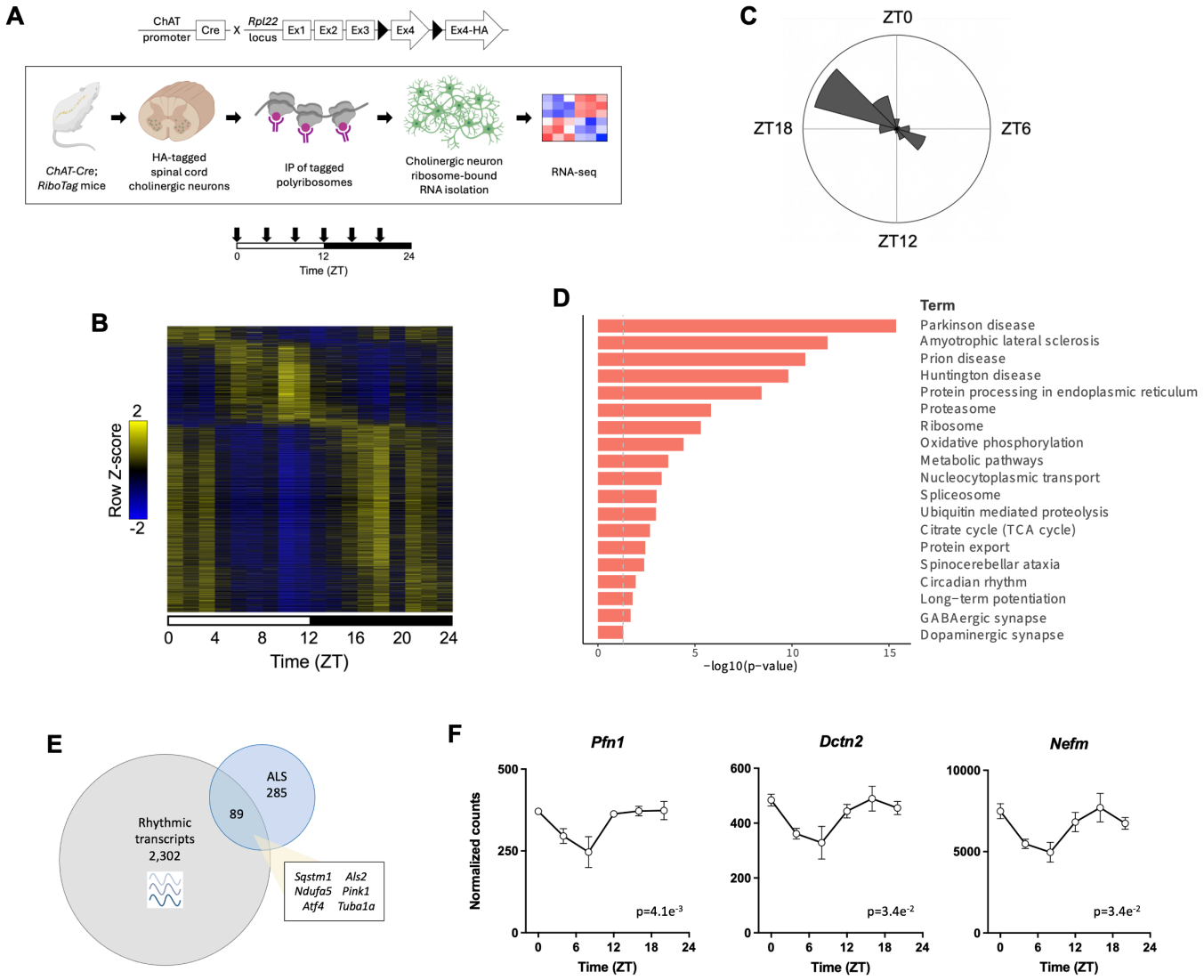

Figure 1. Rhythmic transcriptome profiling of spinal cord cholinergic neurons reveals enrichment for ALS and other neurodegenerative diseases. **(A)** Schematic showing RNA isolation in spinal cord cholinergic neurons from *ChAT-Cre*;*RiboTag*$^{fx/+}$ mice every 4 hours across the 24-hr day (ZT0, 4, 8, 12, 16, and 20) for RNA-seq. Created using BioRender. **(B)** Heatmap showing rhythmic cholinergic neuron mRNA expression (n = 3 per time point). **(C)** Radial histogram showing the number of oscillating genes that have peak expression within each 2-hr window. **(D)** KEGG pathway analysis of all oscillating genes in cholinergic neurons. See also Figure S2. **(E)** Overlap of rhythmic transcripts and KEGG pathway ALS genes. **(F)** Examples of time-of-day-dependent expression of ALS-linked genes that regulate cytoskeleton dynamics, shown as normalized counts identified through DESeq2 (n = 3 per time point). Data are represented as mean ± SEM. Rhythmicity was determined by JTK_Cycle analysis (adjusted p-value shown).

Circadian clock regulates alternative splicing in spinal cord cholinergic neurons

To determine whether alternative pre-mRNA splicing in cholinergic neurons is influenced by circadian time, we used replicate multivariate analysis of transcript splicing (rMATS) to identify alternative splicing events in wild-type (WT) cholinergic neurons collected at 4-hr intervals (Fig. 1A), including skipped exons (SE), mutually exclusive exons (MXE), and alternative 3' and 5' splice sites (A3SS and A5SS). We calculated the percent-spliced in (psi, Ψ) value for all common alternative splicing events in each of the three replicate samples at each time point and performed JTK_Cycle analysis to identify statistically significant oscillations in Ψ. This analysis revealed 11 rhythmic alternative splicing events, consisting of 8 SE, 1 MXE, 1 A3SS, and 1 A5SS (Fig. 2A), with most Ψ values peaking at ZT12 (Fig. 2B; Table S2A-D). Sashimi plots, which illustrate exon inclusion levels by mapping reads across splice junctions, are shown throughout the circadian day to demonstrate rhythmic splicing of *Aftph* (SE), which encodes Aftiphilin, a factor that enables clathrin binding and intracellular transport as part of the AP-1 adaptor complex (Fig. 2C) (Burman et al. 2005). We found that *Aftph* was rhythmically spliced by both SE and A5SS (Fig. 2A; Tables S2A and S2D).

Limch1 and *Drc3* have roles in microtubule and cytoskeleton organization and also displayed rhythmic altered splicing by SE (Fig. 2A; Table S2A). Of note, *Limch1* promotes actin stress fiber assembly and focal adhesion, and alternative splicing of *Limch1* is associated with muscle weakness and calcium-handling deficits (Lin et al. 2017; Penna et al. 2023). Further, Rap GTPase-activating protein (RapGAP) encoding gene *Sipa1l2* regulates synaptic function and vesicle trafficking at presynaptic terminals, and we found that *Sipa1l2* was rhythmically spliced by SE (Fig. 2A; Table S2A) (Andres-Alonso et al. 2019). Two genes exhibited both rhythmic mRNA expression and rhythmic splicing: *Mvb12a*, which regulates vesicle trafficking by enabling ubiquitin and lipid binding, and *Pdcd2l*, which is involved in apoptosis as well as embryonic development (Fig. 2D) (Gao et al. 2022; Tsunematsu et al. 2010). We establish here that genes involved in intracellular transport, cytoskeleton organization, and synaptic function are rhythmically spliced in cholinergic neurons. Notably, a previous report demonstrated that rhythmically spliced genes in pancreatic islets were similarly enriched for trafficking and synaptic regulation (Marcheva et al. 2020).

Figure 2

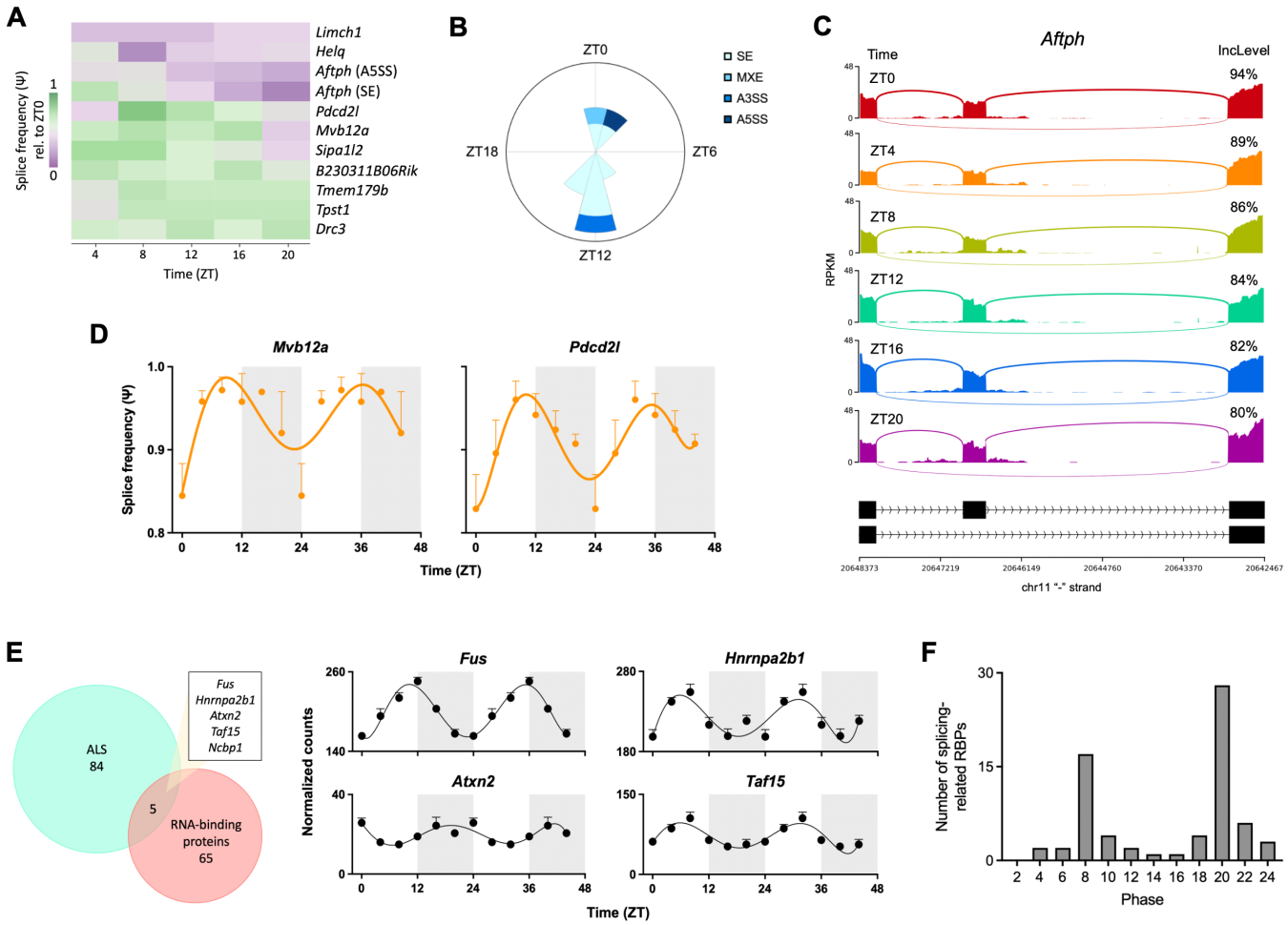

Figure 2. Circadian clock regulates alternative splicing in spinal cord cholinergic neurons. **(A)** Heatmap showing rhythmically spliced genes in WT cholinergic neurons obtained using rMATS splicing analysis, represented as splice frequency (Ψ) relative to ZT0 (n = 3 per time point). **(B)** Radial histogram showing the number of each type of splicing event with peak Ψ occurring within each 2-hr window. Radius corresponds to 4 splicing events. SE, skipped exon; MXE, mutually exclusive exon; A3SS and A5SS, alternative 3' and 5' splice sites. **(C)** Sashimi plots showing time-of-day-dependent alternative splicing of *Aftph* by SE every 4 hours across the 24-hr day, with the exon inclusion level indicated at each time point. **(D and E)** Rhythmic splice frequency graphs of the genes that are both rhythmically expressed and rhythmically spliced in WT cholinergic neurons (D) and rhythmically expressed ALS-linked genes that encode RNA-binding proteins (RBPs), shown as normalized counts identified through DESeq2 (E). N = 3 per time point. Data are represented as mean ± SEM and double-plotted for better visualization. Rhythmicity was determined by JTK_Cycle analysis (adjusted p-value < 0.05). The shaded regions denote the dark (active) time periods. **(F)** Peak phase in expression of oscillating RBPs within the GO term "RNA splicing."

Because alternative splicing results from interactions between RBPs and splicing regulatory elements in pre-mRNA, we next investigated RBP expression in the cholinergic neuron rhythmic

transcriptome. Clues to the rhythmic expression of genes encoding ALS-linked RBPs in neuronal sub-types originated from earlier studies demonstrating oscillatory RBP abundance in various mouse CNS regions, including brain stem, SCN, hypothalamus, and pituitary (Fig. S3) (Pizarro et al. 2013). Here, we identified 70 oscillating transcripts that encode splicing-related RBPs in cholinergic neurons (Table S1C), including 5 that overlap with rhythmically expressed ALS genes—*Fus*, *Hnrnpa2b1*, *Atxn2*, *Taf15*, and *Ncbp1* (Fig. 2E). This indicates that there may be circadian regulation of ALS-linked RBPs and RNA metabolism in spinal cord cholinergic neurons, the cell-type affected in ALS pathophysiology. Interestingly, none of the rhythmically expressed RBPs were differentially expressed or differentially spliced in cholinergic neurons with *Bmal1*-deletion at ZT8 or ZT20, which suggests that their mRNA expression is not under direct clock control. We observed a bimodal distribution in peak phases of mRNA abundance of the oscillating splicing-associated RBPs 12 hours apart at ZT8 and ZT20 (Fig. 2F). While not aligned with the peak phases of RBP expression, peak Ψ values also displayed a bimodal distribution 12 hours apart (at ZT0-2 and ZT12-14) (Fig. 2B). Taken together, these data demonstrate time-of-day-dependent expression of ALS-associated RBPs and establish for the first time that the molecular clock regulates alternative splicing in cholinergic neurons.

Cholinergic neuron circadian clock regulates sALS-associated cytoskeleton, ECM, and NMJ gene expression

Given the involvement of the cholinergic neuron clock in neurodegenerative processes, we sought to investigate the transcriptomic consequences of clock disruption through cholinergic neuron-specific deletion of clock activator BMAL1 in the light (ZT8) and dark (ZT20) periods (Fig. 3A and Fig. S1C). We found 48 upregulated genes and 21 downregulated genes in mouse cholinergic neurons with *Bmal1* knockout, or *ChAT-BKO* (*ChAT-Cre;RiboTag*$^{fx/+}$*;Bmal1*$^{fx/fx}$), compared to controls (*ChAT-Cre;RiboTag*$^{fx/+}$) at ZT8 (Fig. 3B; Table S3A). KEGG, GO, and Reactome functional pathway analyses revealed over-representation of genes associated with cell adhesion and movement, cell signaling, development, and Th17 cell differentiation among the upregulated genes, and blood vessel development among the downregulated genes (Fig. 3C). At the opposite phase, we found 74 upregulated genes and 12

downregulated genes in *ChAT-BKO* cholinergic neurons at ZT20 (Fig. 3B; Table S3B). Functional pathway analyses revealed over-representation of genes related to cell adhesion and movement (similar to at ZT8), immune response, and muscle systems among the upregulated genes, and negative regulation of inflammatory response among the downregulated genes (Fig. 3C). Enrichment for circadian rhythms appeared in the up- and down- regulated genes at both time points, as BMAL1 is a component of the core clock molecular feedback loop and its dysregulation influences the abundance of other circadian genes (e.g., *Per2*, *Cry1*, *Cry2*, *Rorc*, *Nr1d1*, and *Nr1d2*) (Table S3C).

Figure 3

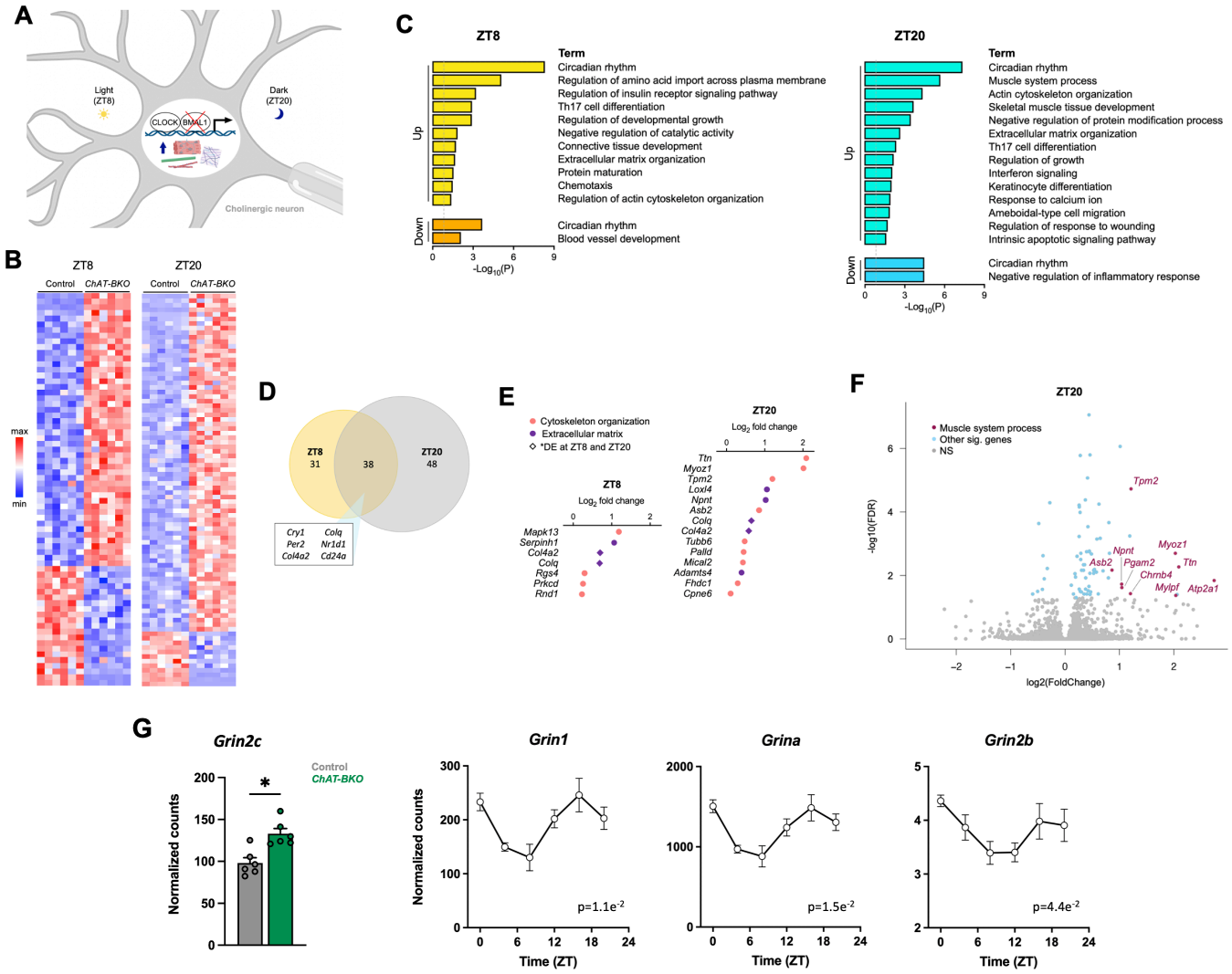

Figure 3. Cholinergic neuron circadian clock regulates cytoskeleton, ECM, and NMJ gene expression. **(A)** Model of cholinergic neuron-specific deletion of BMAL1 during the light (ZT8) and dark (ZT20) periods to induce the expression of cytoskeleton, extracellular matrix (ECM), and neuromuscular junction (NMJ) genes. Created using BioRender. **(B)** Heatmaps showing differentially expressed genes between cholinergic neurons isolated from control (*ChAT-Cre;RiboTag*$^{fx/+}$) and *ChAT-BKO* (*ChAT-Cre;RiboTag*$^{fx/+}$*;Bmal1*$^{fx/fx}$) mice at ZT8 and ZT20 (n = 6 per condition). **(C)** KEGG, GO, and Reactome functional pathway analyses of genes that are up- or down- regulated in *ChAT-BKO* cholinergic neurons vs. controls at ZT8 and ZT20. **(D)** Overlap of differentially expressed genes in *ChAT-BKO* cholinergic neurons at ZT8 and ZT20. **(E)** Increased expression of GO or literature-defined genes associated with cytoskeleton organization and ECM in *ChAT-BKO* cholinergic neurons at ZT8 and ZT20. **(F)** Volcano plot showing induction of NMJ genes within the GO term "Muscle system process" in *ChAT-BKO* cholinergic neurons at ZT20. **(G)** Induction of glutamate receptor NMDA subunit *Grin2c* in *ChAT-BKO* cholinergic neurons at ZT20 (n = 6 per condition) and time-of-day-dependent expression of genes encoding other NMDA subunits (n = 3 per time point), shown as normalized counts identified through DESeq2. Data are represented as mean ± SEM. Statistical significance was calculated using the Wald test with Benjamini-Hochberg correction for multiple comparisons (*adjusted $p < 0.05$) and rhythmicity was determined by JTK_Cycle analysis (adjusted p-value shown).

Taking a closer look at the genes involved, there were 38 overlapping genes between the differentially expressed genes in *ChAT-BKO* cholinergic neurons at ZT8 and ZT20, including the circadian genes previously mentioned, genes involved in ECM organization (*Col4a2* and *Colq*), and *Cd24a*, which functions as a cell adhesion molecule and is also involved in immune cell signaling (Fig. 3D). *Cd24a* was rhythmically expressed in WT cholinergic neurons and downregulated with loss of *Bmal1* at both time points, suggesting that it is under direct clock control. Several genes associated with ECM and cytoskeleton organization were upregulated with loss of *Bmal1* at ZT8 (e.g., *Mapk13* and *Serpinh1*) and at ZT20 (e.g., *Tubb6*, *Palld*, and *Loxl4*) (Fig. 3E). The cytoskeleton plays an important role in axonal transport and synaptic integrity, and cytoskeletal defects are prominent in ALS patients and disease models (Clark et al. 2016; Gall et al. 2020). Furthermore, ECM and cytoskeleton crosstalk is crucial for normal cell adhesion and migration processes, and both transcriptomic and proteomic studies have noted dysregulation of ECM biology in ALS samples (Geiger et al. 2001; Lin et al. 2020; Milioto et al. 2024; Qiu et al. 2014). The prevalence of cytoskeleton and ECM-associated genes within the differentially expressed genes in *Bmal1*-deficient cholinergic neurons suggests that clock disruption drives a restructuring that may contribute to deficits in axonal transport, synaptic integrity, and cell adhesion.

At ZT20, deletion of *Bmal1* induced the expression of NMJ genes within the GO term “muscle system process,” including *Chrnb4*, *Tpm2*, *Myoz1*, *Ttn*, and *Atp2a1* (Fig. 3F). Moreover, our data

revealed an increase in gene expression of the glutamate receptor *N*-methyl-D-aspartate (NMDA) subunit *Grin2c* in *ChAT-BKO* cholinergic neurons at ZT20, along with time-of-day-dependent expression of genes encoding other NMDA subunits (*Grin1*, *Grina*, and *Grin2b*) (Fig. 3G). Lower motor neurons directly innervate skeletal muscle at the NMJ synapse, where they release neurotransmitters that bind to receptors on the muscle fiber to trigger an action potential and muscle contraction (Khalil et al. 2025). In ALS, degeneration of lower motor neurons leads to muscle weakness and atrophy as a result of muscle denervation (Kubat and Picone 2024). Glutamate is the most abundant excitatory neurotransmitter in the CNS and glutamate excitotoxicity, or excess glutamate, is linked to motor neuron death in ALS and other neurodegenerative diseases (Bosch et al. 2006; Foran and Trotti 2009). While acetylcholine is the primary neurotransmitter at mammalian NMJs, glutamate can also be released in a manner that modulates synaptic transmission and plasticity (Brunelli et al. 2005; Colombo and Francolini 2019; Nishimaru et al. 2005). Our data indicate that there is circadian regulation of glutamatergic signaling at the NMJ and suggest that clock disruption during the dark/active period may increase glutamate excitotoxicity.

To further determine whether transcriptomic findings in mouse cholinergic neurons can be translated to humans, we performed *in silico* analysis of RNA-seq data from ALS patients and found that gene expression profiles altered in disease correspond with rhythmic gene networks. In analyzing lumbar spinal cord motor neurons from 13 sALS patients and 8 healthy controls (Krach et al. 2018), we found 1,270 upregulated genes and 318 downregulated genes (Fig. 4A; Table S4A). KEGG, GO, and Reactome functional pathway analyses revealed over-representation of genes associated with ECM, cytoskeleton organization, and immune response among the upregulated genes (Fig. S4A), and enrichment of genes broadly linked to cytoskeleton dynamics and synaptic activation among the downregulated genes (Fig. S4B). Gene set enrichment analysis (GSEA) revealed that the upregulated genes in *ChAT-BKO* mice at both ZT8 and ZT20 tended to be significantly upregulated in sALS patients vs. controls as well (Fig. 4B). Through hypergeometric testing, we found several genes that were upregulated both in *Bmal1* cholinergic neuron knockout mice and sALS patients, including ECM organization genes (*Col4a2* and *Colq*), actin

cytoskeleton gene *Palld*, core clock repressor gene *Cry1*, and fatty acid elongation factor *Elovl7* (Fig. 4C; Table S4B).

Figure 4

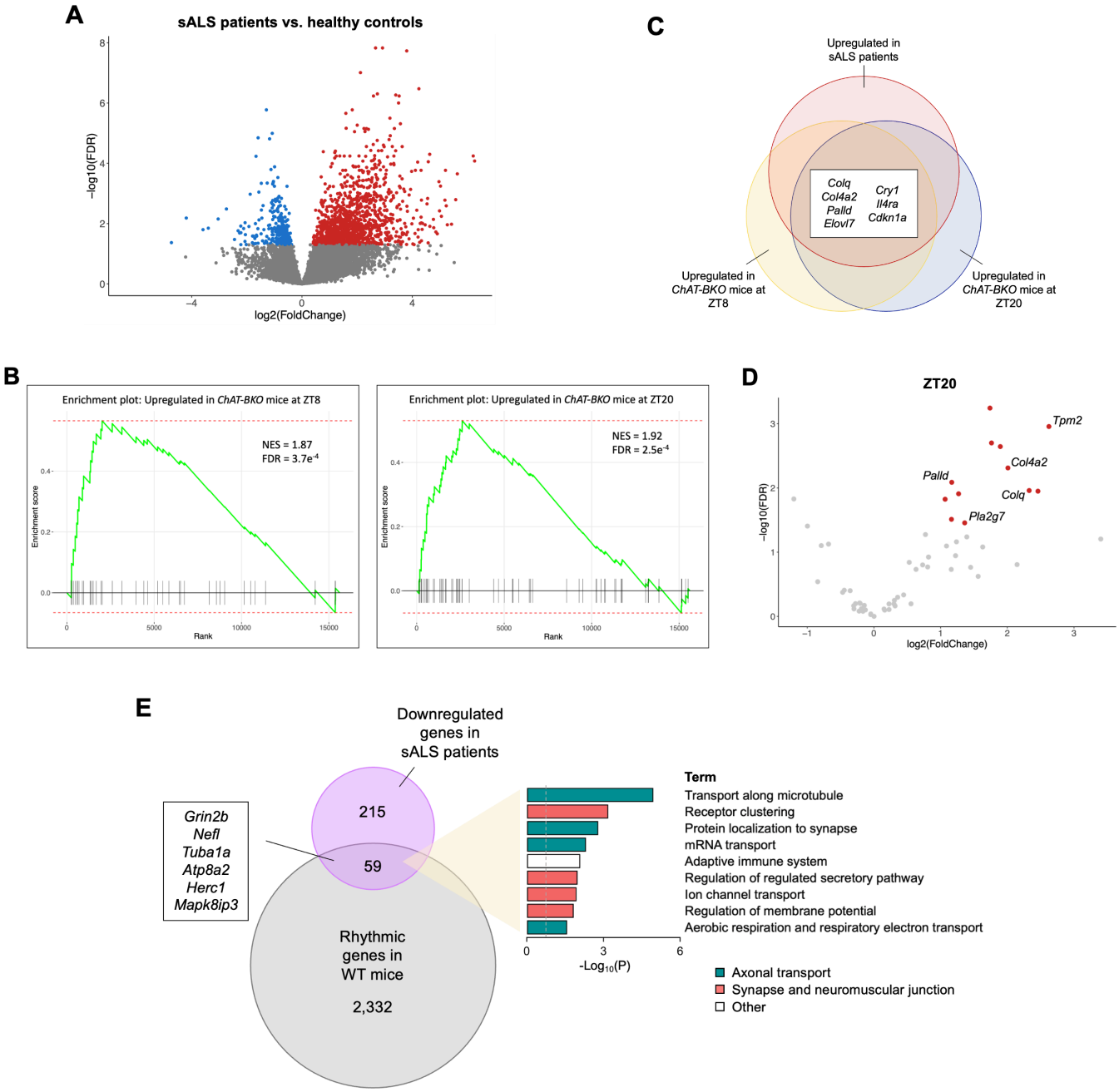

Figure 4. Cholinergic neuron circadian clock regulates sALS-associated cytoskeleton, ECM, and NMJ gene expression. **(A)** Volcano plot showing differentially expressed genes between sporadic ALS (sALS) patient lumbar spinal cord motor neurons (*n* = 13) and healthy controls (*n* = 8). Re-analysis of data set from Krach et al. 2018. See also Figure S3. **(B)** GSEA plots showing

enrichment of upregulated genes in *ChAT-BKO* (*ChAT-Cre;RiboTag*$^{fx/+}$*;Bmal1*$^{fx/fx}$) mouse cholinergic neurons vs. controls (*ChAT-Cre;RiboTag*$^{fx/+}$) at ZT8 and ZT20 [data from Figure 3] among rank-ordered genes in sALS patient motor neurons vs. controls (normalized enrichment score [NES] and false discovery rate [FDR]-adjusted *p*-value shown). **(C)** Upregulated genes in *ChAT-BKO* mice at ZT8 and ZT20 that are over-represented among upregulated genes in sALS patients (adjusted $p < 0.01$ in hypergeometric tests at both time points). **(D)** Volcano plot of upregulated genes in *ChAT-BKO* mice at ZT20 that are over-represented among upregulated genes in sALS patients. **(E)** Enrichment of rhythmic genes in WT mouse cholinergic neurons among sALS patient downregulated genes involved in axonal transport and NMJ (adjusted $p < 0.01$ in hypergeometric test).

Importantly, *Colq* is the collagenic tail of acetylcholinesterase (AChE) responsible for anchoring it to the synaptic space of the NMJ (Rotundo 2003). AChE is an enzyme that breaks down the neurotransmitter acetylcholine, which has been found to be decreased in ALS patients (Campanari et al. 2016). The upregulation of *Colq* in both mouse *ChAT-BKO* neurons and sALS patient lower motor neurons suggests that there may be compensatory induction of this factor following increased degradation of acetylcholine in both clock-disrupted and sALS neurons. Our analysis also revealed upregulation of actin filament stabilization gene *Tpm2* and cytoskeleton-regulating gene *Pla2g7* with *Bmal1*-loss at ZT20 (Lehtinen et al. 2017), and enrichment of these genes among the upregulated genes in sALS patients (Fig. 4D). Finally, we found that 59 of the rhythmic genes in WT mouse cholinergic neurons were significantly enriched among downregulated genes in sALS patients associated with axonal transport (e.g., *Nefl*, *Tuba1a*, and *Mapk8ip3*) and NMJ synaptic regulation (e.g., *Grin2b*, *Atp8a2*, and *Herc1*) (Fig. 4E; Table S4C). Collectively, these findings indicate that there is circadian regulation of sALS-associated processes on the mRNA level and support a role for the cholinergic neuron clock in ALS disease phenotypes.

Cholinergic neuron-specific *Bmal1*-deletion drives ALS-associated changes in lumbar spinal cord and sciatic nerve but does not alter mouse metabolic indices

To elucidate the histological changes resulting from cholinergic neuron-specific deletion of *Bmal1*, we performed Nissl staining on lumbar spinal cord sections from mice with *Bmal1*-loss (*ChAT-Cre;Bmal1*$^{fx/fx}$) and controls (*Bmal1*$^{fx/fx}$). We found a decrease in the number of motor neurons in spinal cord sections with *Bmal1*-deletion (Fig. 5A-G), which suggests that *Bmal1* is important for cholinergic neuron integrity

and survival. Of note, extensive loss of lower motor neurons in the spinal cord and brain stem is a key neuropathological feature of ALS (Grad et al. 2017).

Figure 5

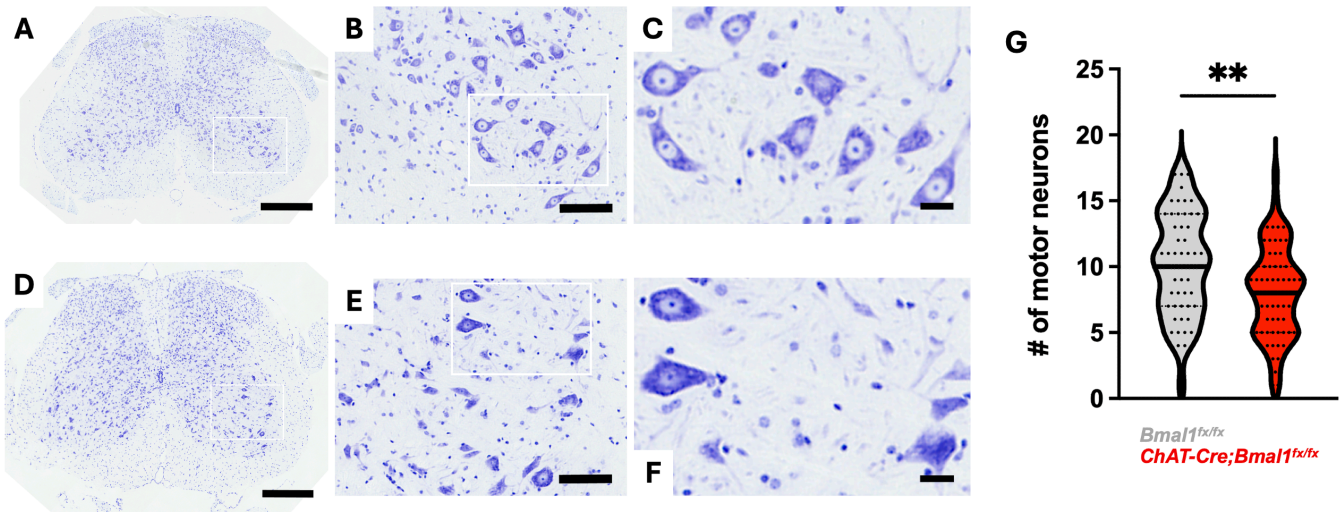

Figure 5. Cholinergic neuron-specific *Bmal1*-deletion increases lumbar spinal cord motor neuron loss. **(A-F)** Representative images of Nissl-stained lumbar spinal cord sections from control *Bmal1*$^{fx/fx}$ mice (A-C) and *ChAT-Cre;Bmal1*$^{fx/fx}$ mice (D-F). Scale bars represent 400μm (A and D), 100μm (B and E), and 25μm (C and F). **(G)** Quantification of motor neurons per lumbar spinal cord section in *ChAT-Cre;Bmal1*$^{fx/fx}$ vs. control mice (n = 5-6). Statistical significance was calculated by unpaired two-tailed Student's t-test (*$p < 0.05$, **$p < 0.01$).

We also performed transmission electron microscopy (TEM) to visualize the sciatic nerve, which enables cholinergic neuron innervation of hindlimb skeletal muscle at the NMJ. In mice with *Bmal1*-loss, we observed an increase in axon degeneration, as characterized by expansion of the myelin sheath and axoplasmic abnormalities, such as shrinkage of the axoplasm (Fig. 6A-D) (Anderson et al. 2016; Essawy et al. 2011; Muzio et al. 2020). Sciatic nerve axon damage is associated with muscle denervation and ALS disease progression (Gordon et al. 2009; Moloney et al. 2014). In line with our transcriptomic analysis of *Bmal1*-deficient cholinergic neurons, we demonstrate morphological changes to sciatic nerve axons that may contribute to altered NMJ dynamics.

Figure 6

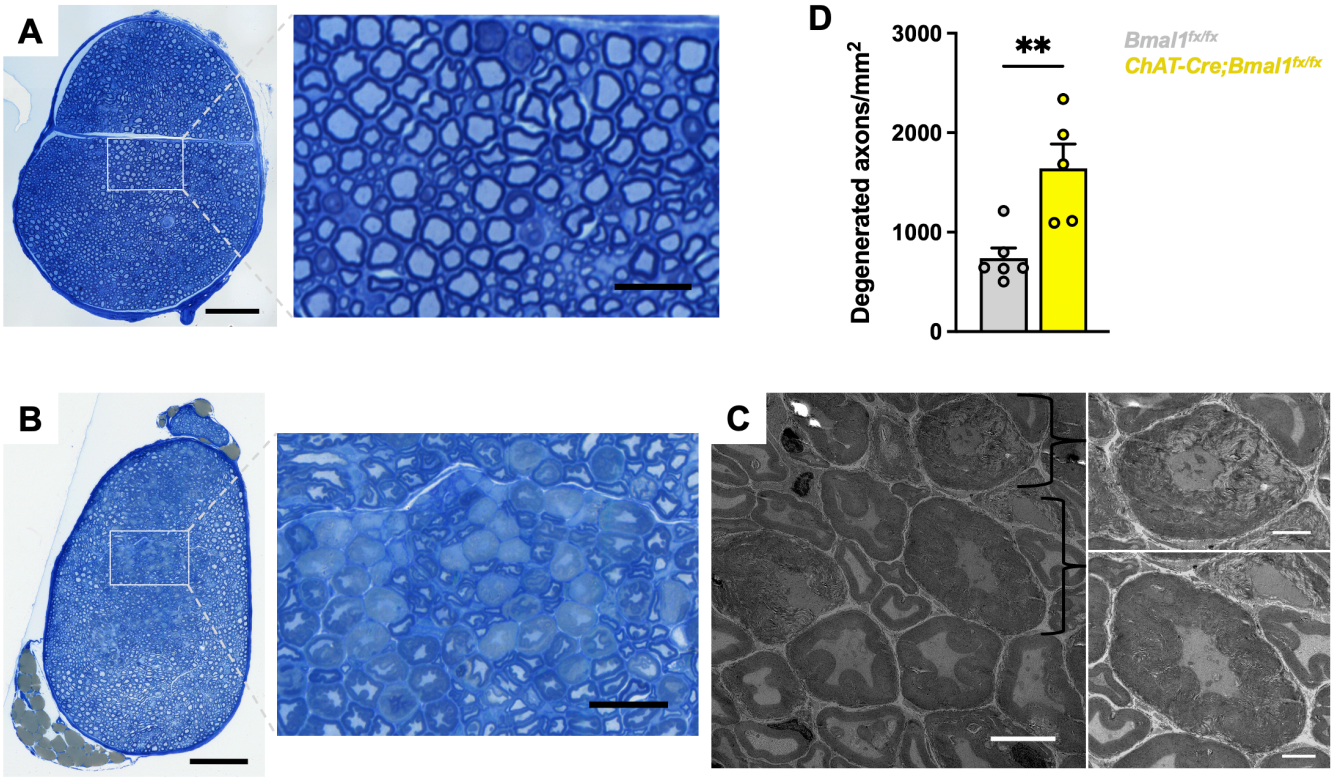

Figure 6. Cholinergic neuron-specific *Bmal1*-deletion increases axon degeneration in sciatic nerve. **(A and B)** Representative images of toluidine blue-stained sciatic nerve sections from control *Bmal1*$^{fx/fx}$ mice (A) and *ChAT-Cre;Bmal1*$^{fx/fx}$ mice (B). Scale bars represent 100μm and 25μm (magnified view). **(C)** Transmission electron microscopy (TEM) images of degenerated axons shown in (B). Scale bars represent 5μm and 2μm (magnified view). **(D)** Quantification of degenerated axons per sciatic nerve section (measured in mm^2) in *ChAT-Cre;Bmal1*$^{fx/fx}$ vs. control mice (n = 5-6). Data are represented as mean ± SEM. Statistical significance was calculated by unpaired two-tailed Student's t-test (*$p < 0.05$, **$p < 0.01$).

Circadian behavior includes rhythmic patterns of locomotor activity, respiration, and feeding (Bass and Takahashi 2010), yet none of these parameters appeared to be altered with cholinergic neuron-specific *Bmal1*-deletion (Fig. S5A-C). Therefore, we postulate that the cellular pathologies of cholinergic neuron clock disruption arise due to cell-autonomous defects. Further, we did not observe changes in mouse whole-animal body or spinal cord weights (Fig. S5D-E).

Cholinergic neuron-specific *Bmal1*-deletion drives alternative splicing of genes encoding ALS-linked RBPs and genes associated with microtubule transport and postsynaptic organization

Since we observed enrichment of differentially expressed genes in *Bmal1* cholinergic neuron knockout mice and sALS patients, we applied rMATS to determine whether these factors likewise undergo alternative splicing. Our analysis of *ChAT-BKO* cholinergic neurons harvested at ZT8 and ZT20 revealed 60 alternative splicing events across 49 unique genes during the light period, consisting of 34 SE, 5 MXE, 12 A3SS, and 9 A5SS (Fig. 7A and 7C; Table S5A-D), and 50 alternative splicing events across 41 unique genes during the dark period, consisting of 37 SE, 1 MXE, 4 A3SS, and 8 A5SS (Fig. 7A and 7C; Table S6A-D). We uncovered differentially spliced genes with multiple transcript variants in *ChAT-BKO* cholinergic neurons, including ALS-associated genes *Stk16* (at ZT8) and *Atg10* (at ZT20), both of which underwent multiple exon skipping events (Fig. 7B) (García-García et al. 2021; Lee et al. 2015; Wang et al. 2019; Zhao et al. 2017). Microtubule-associated protein gene *Ccdc74a* and histone variant encoding gene *H2az2* were the only overlapping alternatively spliced genes between the two time points (Fig. 7C).

Functional pathway analyses of all the genes that were differentially spliced with *ChAT-BKO* at ZT8 revealed enrichment for protein transport, postsynaptic organization, and DNA and RNA stability (Fig. 7D). Notably, deletion of *Bmal1* at ZT8 resulted in altered splicing of several key genes related to microtubule dynamics and transport (*Dync2h1*, *Brsk2*, *Kif3a*, *Tubgcp4*, *Camsap3*, and *Ccdc61*) as well as postsynaptic organization (*Srcin1*, *Arhgef15*, and *Nrcam*) (Fig. 7E). *Kif3a* encodes a subunit of the heterotrimeric KIF3 motor complex, which is crucial for neurite elongation and found to be decreased in sALS patient motor cortex (Hirokawa et al. 2009; Pantelidou et al. 2007). Taken together, this suggests that alternative splicing may alter cytoskeleton and NMJ dynamics during the light/rest period. Intriguingly, the genes that were differentially spliced with *ChAT-BKO* at ZT20 were enriched for a different set of pathways, including RNA splicing, ubiquitin-dependent protein catabolic process, stress response, and purine compound biosynthesis and metabolism (Fig. 7D). This demonstrates that there are time-of-day-dependent differences in clock-mediated regulation of alternative splicing in cholinergic neurons.

An early study comparing sALS lumbar spinal cord motor neurons to healthy controls noted little overlap between the differentially expressed and differentially spliced genes, positing that these cohorts of genes were largely distinct from one another (Rabin et al. 2009). Consistent with this finding, we did not see any overlap between the differentially expressed and differentially spliced genes in *ChAT-BKO*

Figure 7

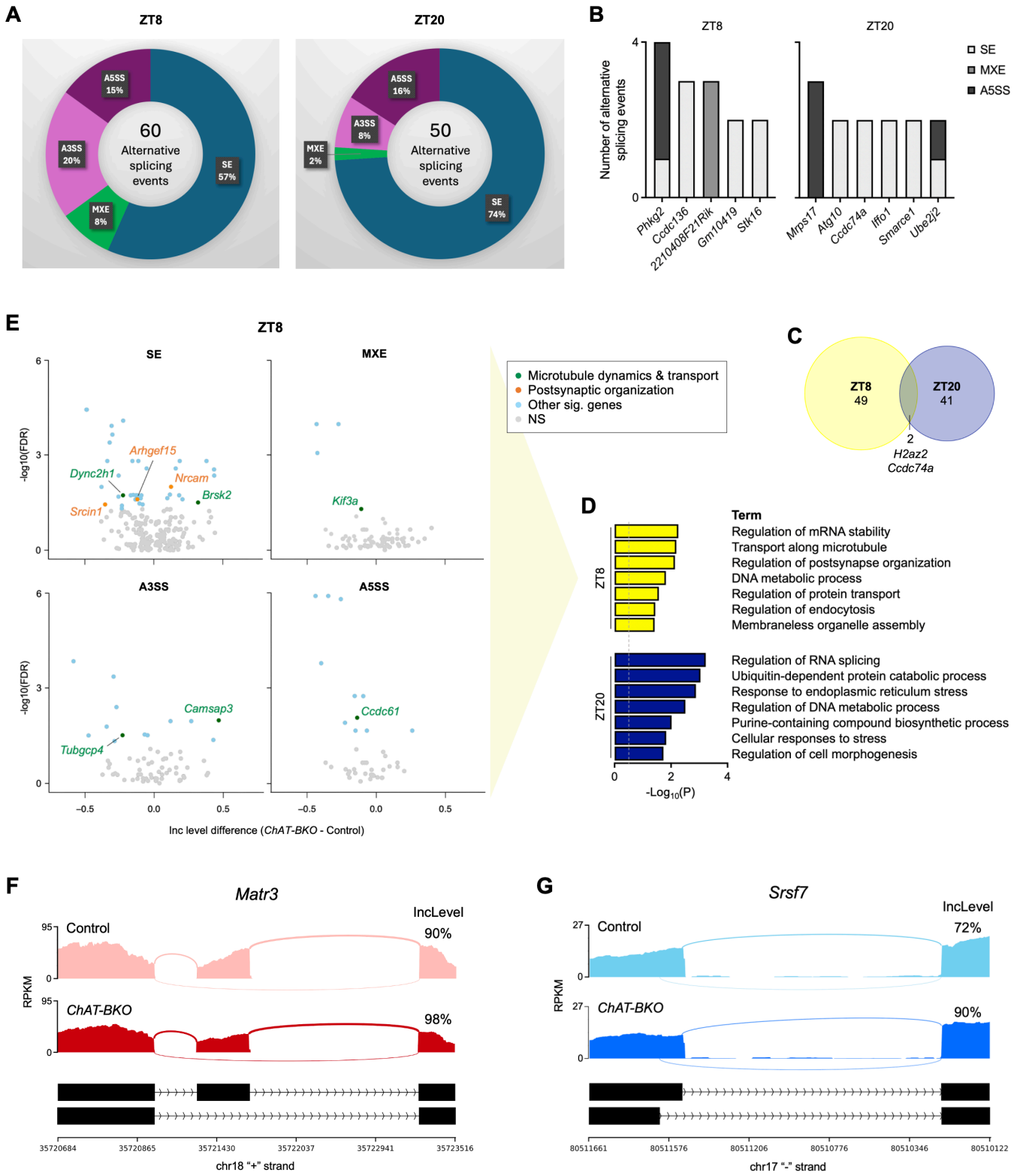
A
ZT8
ZT20
60
Alternative splicing events
A5SS 15%
A3SS 20%
MXE 8%
SE 57%
50
Alternative splicing events
A5SS 16%
A3SS 8%
MXE 2%
SE 74%
B
ZT8
ZT20
Number of alternative splicing events
SE
MXE
A5SS
Phkg2
Ccdc136
2210408F21Rik
Gm10419
Stk16
Mrps17
Atg10
Ccdc74a
Iffo1
Smarce1
Ube2j2
E
ZT8
SE
MXE
A3SS
A5SS
-log10(FDR)
Arhgef15
Dync2h1
Nrcam
Brsk2
Srcin1
Kif3a
Camsap3
Tubgcp4
Ccdc61
Microtubule dynamics & transport
Postsynaptic organization
Other sig. genes
NS
Inc level difference (ChAT-BKO - Control)
C
ZT8
49
ZT20
41
2
H2az2
Ccdc74a
D
Term
ZT8
Regulation of mRNA stability
Transport along microtubule
Regulation of postsynapse organization
DNA metabolic process
Regulation of protein transport
Regulation of endocytosis
Membraneless organelle assembly
ZT20
Regulation of RNA splicing
Ubiquitin-dependent protein catabolic process
Response to endoplasmic reticulum stress
Regulation of DNA metabolic process
Purine-containing compound biosynthetic process
Cellular responses to stress
Regulation of cell morphogenesis
-Log10(P)
F
Matr3
IncLevel
Control
90%
ChAT-BKO
98%
RPKM
chr18 "+" strand
G
Srsf7
IncLevel
Control
72%
ChAT-BKO
90%
RPKM
chr17 "-" strand

Figure 7. Cholinergic neuron-specific *Bmal1*-deletion drives alternative splicing of genes encoding ALS-linked RBPs and genes associated with microtubule transport and postsynaptic organization. **(A)** Alternative splicing events in *ChAT-BKO* (*ChAT-Cre;RiboTag*$^{fx/+}$*;Bmal1*$^{fx/fx}$) cholinergic neurons vs. controls (*ChAT-Cre;RiboTag*$^{fx/+}$) at ZT8 and ZT20 determined by rMATS analysis (n = 6 per condition). SE, skipped exon; MXE, mutually exclusive exon; A3SS and A5SS, alternative 3' and 5' splice sites. **(B)** Differentially spliced genes with multiple transcript variants in *ChAT-BKO* cholinergic neurons at ZT8 and ZT20. **(C)** Overlap of alternatively spliced genes in *ChAT-BKO* cholinergic neurons at ZT8 and ZT20. **(D)** KEGG, GO, and Reactome functional pathway analyses of alternatively spliced genes in *ChAT-BKO* cholinergic neurons at ZT8 and ZT20. **(E)** Volcano plots of alternatively spliced genes by SE, MXE, A3SS, and A5SS in *ChAT-BKO* cholinergic neurons at ZT8, including several associated with microtubule dynamics and postsynaptic organization. **(F and G)** Sashimi plots showing altered splicing of ALS-linked RBPs *Matr3* by SE in *ChAT-BKO* cholinergic neurons at ZT8 (F) and *Srsf7* by A5SS in *ChAT-BKO* cholinergic neurons at ZT20 (G), with exon inclusion levels indicated.

cholinergic neurons at either time point. Moreover, another report found minimal overlap between differentially expressed and differentially spliced genes (0.6% and 4% overlap, respectively) in clock-disrupted pancreatic β-cells (Marcheva et al. 2020). These findings support that changes in splicing and mRNA abundance are regulated by distinct mechanisms that are downstream of the molecular clock.

Of note, our data revealed altered splicing of RBP-encoding genes *Matr3*, *Syncrip*, and *Thoc7* in *ChAT-BKO* cholinergic neurons compared to controls at ZT8, and *Srsf7*, *Snrnp70*, and *U2af2* at ZT20. We found that ALS-linked DNA- and RNA- binding protein *Matr3* was alternatively spliced by SE in cholinergic neurons with *Bmal1*-deletion during the light period, with an increase in exon inclusion levels from 90% to 98% (Fig. 7F). MATR3 is involved in processes such as chromatin organization, RNA processing, and mediating NMDA-induced neuronal cell death, and mutations have been associated with the inhibition of mRNA nuclear export as well as increased cryptic exon inclusion (Boehringer et al. 2017; Giordano et al. 2005; Johnson et al. 2014b; Khan et al. 2024; Salton et al. 2011). Furthermore, MATR3 has been found to interact with TDP-43, and a subset of sALS patient TDP-43-positive cytoplasmic inclusions were also immunoreactive for MATR3 (Iradi et al. 2018; Tada et al. 2018). Our analysis further revealed that ALS-linked RBP *Srsf7* was alternatively spliced by A5SS in cholinergic neurons with *Bmal1*-deletion during the dark period, with an increase in exon inclusion levels from 72% to 90% (Fig. 7G). SRSF7 is a serine/arginine splicing factor whose depletion was recently found to decrease the abundance of STMN2, which is an ALS-associated microtubule binding protein involved in axon maintenance and regeneration following injury (Klim et al. 2019; Wang et al. 2025).

We establish that there is circadian clock regulation of the splicing of RBPs themselves—including RBPs associated with ALS—and that, because the alternatively spliced RBPs at ZT8 and ZT20 are distinct, this process is time-of-day-dependent. Importantly, we demonstrate that ALS-linked RBPs are rhythmically expressed in WT cholinergic neurons and that, with *Bmal1*-deletion, ALS-linked RBPs are differentially spliced. Given that RBP mutations are a hallmark of ALS and other neurological diseases, it would be interesting to consider how clock-dependent gene expression and splice variants of these RBPs contribute to their own dysregulation as well as their control of alternative splicing in health and disease.

DISCUSSION

Alternative splicing plays a crucial role in the generation of transcriptomic and proteomic diversity, especially in neuronal cell-types, whose complex and specialized functions render them particularly vulnerable to disruption. The circadian clock adds another layer of complexity to this process by modulating post-transcriptional events based on the time-of-day-specific energetic demands of each cell. In addition to elucidating the role of the clock in regulating RBP splice mediators, insight into the spinal cord cholinergic neuron rhythmic transcriptome provides a window into the 24-hr physiological processes occurring within lower motor neurons, the cell-type of ALS disease pathogenesis and fatality. Given the length of motor neuron axons (up to ~1 meter) and the extensive distances RNA cargo have to travel, motor neurons are also uniquely susceptible to disruptions in axonal transport and synaptic transmission. As observed in ALS patients and disease models, this dysregulation often results from disrupted RBP homeostasis.

Mutations in genes encoding a number of RBPs have been linked to ALS. In healthy cells, RBP-bound mRNA is packaged into ribonucleoprotein (RNP) granules that are transported by motor proteins (e.g., dyneins and kinesins) along microtubules to axon terminals for local translation (Maday et al. 2014). Local translation of targeted mRNAs enables the production of specific proteins when and where they are required, such as at synapses, where this process is critical for maintaining synaptic integrity and neurotransmission (Das et al. 2021; Jung et al. 2020). Translation is also mediated by cellular stress, and

RBP mutations have been shown to promote the formation of aberrant RNP stress granules, which are membraneless organelles that undergo phase transitions from transient liquid droplets to irreversible, often pathological, solid states (Kiebler and Bassell 2006; Lin et al. 2015). For example, ALS-linked FUS mutations were found to alter FUS regulation of a motor neuron survival factor SMN and inhibit its transport to axons (Groen et al. 2013) as well as impair RNP granule function by inducing FUS liquid phase transitions (Murakami et al. 2015; Patel et al. 2015). A recent report found that polyglutamine (polyQ) expansions in ATXN2 inhibited mRNA transport and local translation through abnormal sequestration of TDP-43 in RNP granules (Wijegunawardana et al. 2025). Further, ALS-linked mutations in TAF15 were found to increase the number of TAF15-positive axonal and dendritic stress granules (Couthouis et al. 2011), and inhibition of TAF15 methylation resulted in the accumulation of TAF15 in TIA1-positive stress granules (Jobert et al. 2009).

Our investigation into the link between two central evolutionarily conserved processes, the circadian clock transcriptional program and alternative splicing, yields several key findings in the context of ALS pathophysiology. Our data support that disruption of the cholinergic neuron clock alters RBP function and contributes to ALS disease phenotypes, although functional analyses would be needed to inform causal relationships. Notably, our findings reveal an increase in gene expression of the ionotropic glutamate receptor NMDA subunit *Grin2c* in cholinergic neurons with *Bmal1*-loss during the dark/active period, along with time-of-day-dependent expression of genes encoding other NMDA subunits (*Grin1*, *Grina*, and *Grin2b*). Glutamate excitotoxicity, or excess release of glutamate and overstimulation of glutamate receptors, is associated with motor neuron death in ALS and other neurodegenerative diseases (Bosch et al. 2006; Foran and Trotti 2009). Our data propose that *(i)* circadian clock disruption increases glutamate excitotoxicity and *(ii)* there is time-of-day-dependent release and/or uptake of glutamate at the NMJ. These findings may have important implications for the timing and dosage of ALS drugs, such as riluzole, which utilizes sodium channel blockade to inhibit glutamatergic neurotransmission and excitotoxicity (Arnold et al. 2024; Nagoshi et al. 2015).

Environmental disturbances such as jetlag, exposure to artificial light, changes in diet, and mistimed feeding have been shown to disrupt the alignment of the internal clock with the external 24-hr

light/dark cycle (Arble et al. 2009; Fonken et al. 2010; Roenneberg et al. 2012). Rhythmic splicing events are also driven by environmental triggers, such as body temperature cycles, which have been shown to stabilize cold-inducible RNA-binding protein (CIRBP) at low ambient temperatures, and drive rhythmic phosphorylation of serine-arginine (SR) proteins to modulate alternative splicing (Gotic et al. 2016; Morf et al. 2012; Preußner et al. 2017). The role of clock disruption and altered RBP function in ALS remains incompletely understood. Future studies should explore whether circadian disruption exacerbates ALS disease phenotypes and how the dysregulation of interconnected circadian and metabolic networks contributes to ALS-associated metabolic changes, both in relation to- and independent of- RBP function (Koletsou and Huppertz 2025; Ludolph et al. 2023; Nelson and Trotti 2022). One goal here would be to apply new insight to ALS patient care regimens in which circadian disruption due to environmental factors come into play.

MATERIALS & METHODS

Animals

All animal procedures were conducted in accordance with regulations of the Institutional Animal Care and Use Committee at Northwestern University. All experiments were conducted using C57BL/6J mice maintained on a 12:12 light/dark cycle. WT cholinergic neuron isolation was achieved by crossing *ChAT-Cre* mice (Rossi et al. 2011; JAX #006410; gift from Dr. Loukia Parisiadou at Northwestern University) with *RiboTag*$^{fx/fx}$ mice (Sanz et al. 2009; obtained from Jackson Laboratories #029977) to generate *ChAT-Cre;RiboTag*$^{fx/+}$ mice. These experiments were performed using 10-week-old male mice. Cholinergic neuron-specific deletion of *Bmal1* was achieved by crossing *Bmal1*$^{fx/fx}$ mice (Johnson et al. 2014a) with *ChAT-Cre* or *RiboTag*$^{fx/fx}$ mice to ultimately generate *ChAT-Cre;Bmal1*$^{fx/fx}$ mice (along with *Bmal1*$^{fx/fx}$ littermate controls) and *ChAT-Cre;RiboTag*$^{fx/+}$*;Bmal1*$^{fx/fx}$ mice (along with *ChAT-Cre;RiboTag*$^{fx/+}$ controls). These experiments were performed using 5–7-month-old male mice to assess the added insult of older age on phenotype and transcriptional program.

Nissl staining and morphometric quantification

Mice were anesthetized with intraperitoneal injection of Euthasol (390mg/mL pentobarbital sodium, 50mg/mL phenytoin sodium), and then transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.4M phosphate buffer (pH 7.4). Lumbar spinal cord was isolated, post-fixed in the same fixative overnight at 4°C, and paraffin embedded. 6μm thick sections were cut and mounted onto slides. Samples were then deparaffinized and hydrated, prior to staining with heated Cresyl Violet acetate (Sigma-Aldrich, C5042) solution for 10 min and differentiation in 90% ethanol for 20 min. Finally, samples were dehydrated in ethanol, cleared in xylene, and mounted with DPX Mountant (Sigma-Aldrich, 06522). Tile stitched images at 20X magnification were acquired using TissueFAXS PLUS (TissueGnostics) to obtain whole spinal cord cross sections. After images were blinded, motor neurons were counted in every sixth section in an average of 11 sections per animal. Criteria for motor neuron designation were neurons located in the ventral horn with cell bodies between 30-70μm in diameter, the presence of a multi-polar structure, and a visible nucleolus.

Transmission electron microscopy and morphometric quantification

Mice were anesthetized with intraperitoneal injection of Euthasol (390mg/mL pentobarbital sodium, 50mg/mL phenytoin sodium), and then transcardially perfused with PBS followed by 4% PFA in 0.4M phosphate buffer (pH 7.4). Sciatic nerve was isolated and immersion-fixed in 0.1M sodium cacodylate buffer (pH 7.35) containing 2.5% glutaraldehyde and 2% PFA overnight at 4°C. Tissue was post-fixed in 2% osmium tetroxide, en bloc stained with 3% uranyl acetate, and dehydrated in ascending grades of ethanol followed by propylene oxide. Samples were then embedded in EMbed 812 Resin (Electron Microscopy Sciences, 14900) and cured overnight in a 60°C oven. 1μm thick sections were cut using the EM UC6 Ultramicrotome (Leica Microsystems) and stained with Toluidine Blue O. Tile stitched images at 40X magnification were acquired using TissueFAXS PLUS (TissueGnostics) to obtain whole nerve cross sections. After images were blinded, severely degenerated axons were counted within each nerve section and divided by the total area of the section (in mm^2). For transmission electron microscopy (TEM), ultrathin 70nm sections were mounted onto mesh copper grids and stained with uranyl acetate and

Reynolds Lead Citrate. Images at 1,400X and 2,900X magnification levels were captured at 80 kV using Tecnai G2 Spirit (FEI Company) and Tecnai Imaging & Analysis Software.

Cholinergic neuron ribosome immunoprecipitation and RNA isolation

Spinal cord tissue was isolated and snap-frozen from 10-week-old *ChAT-Cre*;*RiboTag*$^{fx/+}$ mice at ZT0, 4, 8, 12, 16, and 20 or from 6-month-old *ChAT-BKO* (*ChAT-Cre;RiboTag*$^{fx/+}$*;Bmal1*$^{fx/fx}$) mice and controls (*ChAT-Cre;RiboTag*$^{fx/+}$) at ZT8 and ZT20. All samples for each experiment were processed on the same day. Homogenization buffer consisting of 1% sodium deoxycholate, 50mM Tris (pH 7.5), 100mM KCl, 12mM $MgCl_2$, 1% Tergitol (NP-40), 1mM dithiothreitol (DTT), 200U/mL RNasin Ribonuclease Inhibitor (Promega, N2115), 1mg/mL heparin, 100µg/mL cycloheximide, and Protease Inhibitor Cocktail (Sigma-Aldrich, P8340) was made fresh and pre-chilled. One spinal cord was placed in a 2mL Dounce homogenizer vessel on ice with 2mL homogenization buffer, and homogenized with 30 strokes of 'pestle A' followed by 30 strokes of 'pestle B.' The homogenate was then centrifuged at 10,000 RCF for 10 min at 4°C. TRIzol Reagent (Invitrogen, 15596026) was added to 80µL of the spun homogenate and frozen at -80°C as pooled Input (RNA from whole spinal cord). 800µL of the remaining non-pelleted homogenate was incubated with 4µL of anti-HA antibody (BioLegend, 901514) for 4 hours at 4°C with end-over-end rotation. Samples were then incubated with 200µL of pre-washed protein A/G magnetic beads (Thermo Scientific Pierce, 88803) overnight at 4°C with end-over-end rotation. High salt buffer consisting of 50mM Tris (pH 7.5), 300mM KCl, 12mM $MgCl_2$, 1% NP-40, 0.5mM DTT, and 100µg/mL cycloheximide was made fresh and pre-chilled. Samples were placed on a magnetic stand on ice and washed three times with high salt buffer (800µL per wash) to remove non-specific binding from the immunoprecipitate (IP) product. After careful removal of high salt buffer, samples were treated with 700µL of TRIzol Reagent and frozen at -80°C until RNA was extracted using the Direct-zol RNA MicroPrep kit (Zymo Research Corp., R2062) following the manufacturer's instructions.

Library preparation and RNA sequencing

Following RNA isolation, RNA quality was assessed by Bioanalyzer (Agilent) and those with an RNA Integrity Number (RIN) greater than 7 were used for library prep. Libraries were constructed from 100 or 300 ng of RNA (for rhythmic or differential analyses, respectively) using the Illumina Stranded mRNA Prep kit (Illumina, 20040534) according to the manufacturer's instructions. Libraries were quantified by both Bioanalyzer (Agilent) and qPCR-based quantification (Kapa Biosystems). 150bp paired-end sequencing was performed on a NovaSeq X Plus (Illumina) to a minimum depth of 30M reads. Sequences were aligned to the mm10 transcriptome with STAR (v2.7.5) using the –quantMode Transcriptome and GeneCounts options. Gene counts were obtained from mm10 annotations (Ensembl 98) using rsem-calculate-expression (v1.3.0).

Differential expression, differential splicing, and rhythmicity analyses

Differential mRNA expression analysis was performed using DESeq2 (v1.32.0) after removal of genes with < 10 assigned counts. Differentially expressed transcripts were defined as having a false discovery rate (FDR)-adjusted p-value < 0.05. Alternative splicing analysis was performed using rMATS (v4.0.2) (Shen et al. 2014) to quantify reads across annotated splice junctions present in the mm10 assembly. Differentially spliced transcripts were defined as having an FDR-adjusted p-value < 0.05 and a minimum exon inclusion level difference > 10%. Rhythmic mRNA expression and rhythmic splice frequency throughout the day were assessed by JTK_Cycle analysis (Hughes et al. 2010), allowing for period lengths of 20, 24, or 28 hours to account for 4-hr sampling frequency. Rhythmicity was determined by an FDR-adjusted p-value < 0.05. KEGG, GO, and Reactome pathway enrichment analyses were performed using limma and biomaRt (v2.48.2), ShinyGO (v0.77) (Ge et al. 2019), Metascape (Zhou et al. 2019), and Cytoscape (Shannon et al. 2003). Certain plots were made in R (v4.0.3) using ggplot2 (v3.3.0), gplots (v3.1.1), pheatmap (v1.0.12), and fgsea (v1.16.0).

Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Unless otherwise indicated, statistical analysis was performed by unpaired two-tailed Student's t-test. One-way analysis of variance

(ANOVA) with Dunnett's correction for multiple comparisons was used for statistical analysis of qPCR data, and two-way ANOVA was used for statistical analysis of activity, RER, and feeding. Hypergeometric tests were used to determine enrichment of genes from one dataset within another. The Wald test with Benjamini-Hochberg correction for multiple comparisons was used for statistical analysis of normalized count data from DESeq2. *P*-values < 0.05 were considered statistically significant. Detailed information on genotype, sample size, and *p*-value can be found within individual figures and figure legends.

DATA ACCESS

All raw and processed sequencing data generated in this study have been submitted to the NCBI Gene Expression Omnibus under accession number GSE297457.

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