UNIVERSITY OF CALIFORNIA SAN DIEGO

Modeling Neuronal Circadian Rhythms in Major Depressive Disorder using Human Induced Pluripotent Stem Cells

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Anusuya Arjavalingam is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

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Anusuya Arjavalingam

Master of Science in Biology

University of California San Diego, 2022

Professor Michael McCarthy, Chair
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Major Depressive Disorder (MDD) is a neuropsychiatric disorder characterized by persistent episodes of depressed mood and loss of interest or pleasure in normal activities. MDD patients have disturbed patterns of mood, sleep, metabolism, all factors of which are linked to circadian rhythms. The connection between MDD and abnormalities in the circadian clock has been investigated through studies on human behavior, post-mortem human brains, and animal models. We hypothesize that the abnormalities in the circadian clock persist at the molecular level, and that we can model circadian rhythms in neurons associated with MDD using induced pluripotent stem cells (iPSCs).

The MDD cell line donors in this study were categorized into two groups by antidepressant response: remitters (responders) and non-remitters (non-responders) (Mrazek et al., 2014). Primary patient fibroblasts were reprogrammed into iPSCs (Vadodaria et al., 2019), which were differentiated into glutamatergic neurons, a cell type previously implicated in MDD. To measure circadian oscillatory activity, neurons were transfected with a lentivirus to deliver *Per2* promoter-activated lentiviral luciferase, and bioluminescence was recorded over 5+ days. We found that MDD non-remitters neurons had lower amplitude circadian rhythms. To better understand the potential mechanisms underlying this finding, we performed qRT-PCR gene expression analysis of nine core clock genes. We found that neurons from MDD SSRI non-remitters exhibited decreased gene expression of four core circadian clock genes, *PER2*, *PER1*, *CRY2*, and *CLOCK*. These findings suggest that MDD non-remitters have a weaker circadian clock at the transcriptional level. To understand the circadian clock in MDD at the protein level, we performed immunocytochemistry of PER2 and BMAL1. MDD remitter neurons had an elevated signal of PER2 and BMAL1. Together, we demonstrate abnormalities in the molecular clock that are specific to either MDD non-remitters or remitters.

INTRODUCTION

1.1 Major Depressive Disorder

Major depressive disorder (MDD) is a neuropsychiatric disorder characterized by persistent episodes of depressed mood, loss of interest or pleasure, weight gain or loss, fatigue, feelings of worthlessness or guilt, insomnia or hypersomnia, psychomotor retardation or agitation, an inability to concentrate, and recurring thoughts of death or suicide (American Psychiatric Association, 2013), causing impairment in important social and occupational activities. Major depression is also often accompanied by irritability and anxiety (Uher et al., 2014). About 10% of people in the United States currently experience major depression disorder (Kessler et al., 2012; Luo et al., 2020; Hasin et al., 2018). Lifetime prevalence is estimated to be 15-20% (Kessler et al., 2012; Hasin et al., 2018).

The causes of MDD are incompletely understood but are thought to have a biological basis, which interact with environmental factors, such as childhood abuse (Edwards et al., 2003), and stress (Gold et al., 2015). It has an estimated heritability of around 46% (Corfield et al., 2017). Biologically, MDD has been associated with reduced neurogenesis (Hanson et al, 2011), lower synaptic density (Holmes et al., 2019), alterations in the cortico-limbic system (Disner et al., 2011; Park et al., 2019), inflammation (Arteaga-Henríquez et al., 2019; Kitzbichler et al., 2021, Toenders et al., 2021), and changes in the hypothalamic–pituitary–adrenal axis (Iob et al., 2020).

MDD has also been linked to circadian rhythm disruption. Circadian rhythms regulate mood and positive affect (Murray et al., 2009), and many MDD patients have consistent daily patterns of mood symptoms. Often, the worst symptoms appear in early morning, a phenomenon

termed “diurnal variation” (Gordijn et al., 1994; Tolle and Goetze, 1987), with a minority showing the opposite, with worse symptoms in the evening, or “reversed diurnal variation” (Joyce et al., 2005). People who reversed diurnal variation may have a poorer response to a serotonergic antidepressant (Joyce et al., 2005). Having an evening chronotype (colloquially called being a “night owl”) has been associated with Major Depressive Disorder (Chan et al., 2014). The extent of phase delay is correlated with severity of depression (Emens et al., 2009), with people with extreme evening chronotypes having a greater risk for depression (Shirayama et al., 2003; Kitamura et al., 2010; Abe et al., 2011). In general, people with MDD generally prefer later schedules (Drennan et al., 1991; Chelminski et al., 1999). MDD patients also exhibit dysregulated sleep, attention/cognition and metabolism, which are regulated by circadian rhythms.

Disruptions in external time cues that help synchronize circadian rhythms can induce depressive symptoms in humans. Surveys of people who do shift work show that they experience increased rates of depression (Rosenberg et al., 2011). Seasonal affective disorder is a type of depression triggered by winter months with later dawn and shorter light phase (Edgar et al., 2013), and can be treated with bright light therapy, showing that changing external circadian regulators can alleviate depressive symptoms (Magnusson et al., 2003).

1.2 Major Depressive Disorder and SSRIs

Selective serotonin reuptake inhibitors (SSRIs) are the most common drug treatment for MDD in the U.S. (Luo et al., 2020). The serotonin transporter terminates the action of the neurotransmitter serotonin in the synaptic cleft and transports the serotonin back into the presynaptic terminal. SSRIs bind to serotonin transporters on the terminal axon and inhibit their

activity, therefore blocking the reuptake of serotonin into the pre-synaptic neuron and prolonging the activity of serotonin in synapses (Sangkuhl et al., 2009). This results in more serotonin bound to receptors and signaling (Taylor et al., 2005).

In clinical trials of MDD, response is typically defined as an improvement of at least 50% reduction in symptoms using the 16-item Quick Inventory of Depressive Symptomatology, Self-Report (QIDS-SR) (Trivedi et al., 2006). Remission is a higher level of improvement and typically defined by a score of ≤5 on the (QIDS-SR), indicating near-absence of mood symptoms. In the 2006 STAR*D study, 2,876 MDD patients treated with citalopram had a response rate of 47%, and remission rates of only 33% based on the QIDS-SR (Trivedi et al., 2006). Therefore, treatment with SSRIs is commonly incomplete and/or ineffective.

Treatment with SSRIs commonly takes 2–4 weeks to be effective, suggesting that long-term changes in neurotransmission and downstream neuronal processes underlie the therapeutic effects of the drugs (Sangkuhl et al., 2009). There is preliminary evidence to suggest that SSRIs work by inducing neurogenesis in the dentate gyrus (Hanson et al, 2011; Segi-Nishida, 2017). SSRIs' anti-inflammatory effects have also been hypothesized to contribute to its therapeutic effects (Tynan et al., 2012). SSRI-non-response has been associated with serotonin-induced hyperexcitability in iPSC-derived neurons (Vadodaria et al., 2019), increased levels of the serotonin receptors 5-HT2A and 5-HT7. One common feature of all of these processes is a connection with the circadian clock that organizes many different aspects of physiology across the varying demands of the 24 h light/dark cycle.

1.3 The Circadian Clock

An organism's circadian rhythm determines when recurring physiological and behavioral processes occur during the 24-hour day, such as sleep/wake cycles and metabolism (Porcu et al., 2019). In humans and most mammals, each cell has an autoregulated circadian rhythm that is governed by a transcriptional-translational negative feedback loop (reviewed by Takahashi et al., 2008). The proteins CLOCK and BMAL1 heterodimerize to form a transcription factor complex that binds to E-box elements in the DNA promoters of Period (PER) 1 and 2 and Cryptochrome (CRY) 1 and 2 genes (reviewed by Porcu et al., 2019). The resulting PER and CRY proteins heterodimerize in the cytoplasm and translocate into the nucleus. They inhibit the transcription of BMAL1 and CLOCK, and therefore inhibit their own expression. The PER-CRY complex degrades over time, and the cycle starts again (Takahashi et al., 2008).
A secondary, parallel negative feedback loop that includes ROR and REV-ERBα proteins has been shown to modulate amplitude (Porcu et al., 2019). ROR and REV-ERBα are upregulated by CLOCK-BMAL1 like the PERs and CRYs. REV-ERBα represses BMAL1 by binding to retinoic acid-related orphan receptor response elements (RREs) in the BMAL1 promoter, competing with the positive regulator ROR (Takahashi et al., 2008).

In humans, the 24-hour cycle is aligned to the day and night by the input of light to intrinsically photosensitive retinal ganglion cells. This information is projected to the hypothalamic suprachiasmatic nucleus (SCN) through the retinohypothalamic tract. The SCN is the master pacemaker of the human body (Takahashi et al., 2008), and controls other cells through a wide array of neural and endocrine pathways (Ko et al., 2006).

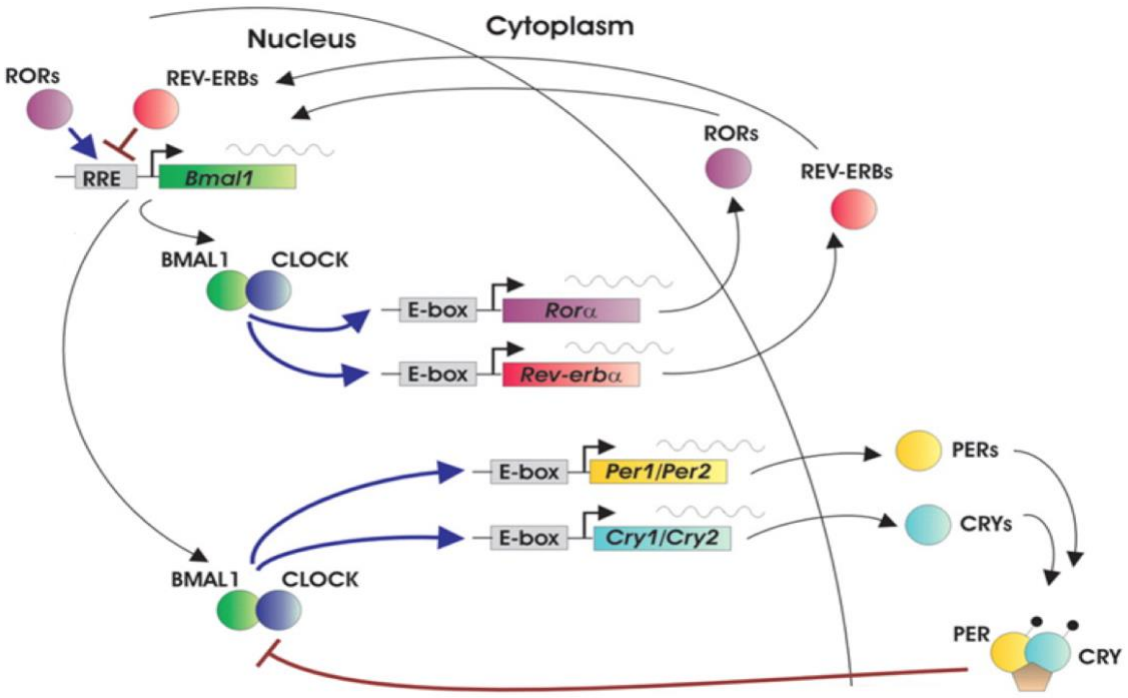


Figure 1.1: The molecular circadian clock. (Takahashi et al., 2008)

1.4 Circadian Rhythms Abnormalities are Associated with MDD

MDD patients have disturbed temporal patterns of mood, sleep, metabolism, all factors which are linked to circadian rhythms. Having an evening-type chronotype has been associated with depression (Shirayama et al., 2003; Kitamura et al., 2010; Abe et al., 2011). One such study which demonstrated this association took a cohort of 253 people and assessed them for chronotype, depressive symptoms, and insomnia of 253 patients; the study found that those with an evening chronotype tended to exhibit more depressive symptoms, independent of insomnia (Chan et al., 2014). The researchers then decided to focus on depression non-remission, Non-remission was defined as a score of 8 or higher in the HAM-D and the presence of a current major depressive episode. They found that higher eveningness was associated with a threefold increase in non-remission. A study of SSRI response found that people with an evening

chronotype have more depressive symptoms while on SSRIs (McGlashan et al., 2018). These findings indicate that circadian rhythms may influence the ability of MDD patients to respond to antidepressant treatment.

There seems to be a positive correlation between degree of eveningness and MDD risk. For instance, Emens and colleagues found a positive correlation between phase delay and MDD risk (Emens et al., 2009). Still, correlational studies have been difficult to interpret because of the difficulty of disentangling cause and effect. To address this issue, Daghlas and colleagues used Mendelian Randomization to demonstrate a protective association between high levels of morningness against the risk for MDD (Daghlas et al., 2021). Mendelian Randomization is a powerful method used to disentangle complex cause-and-effect relationships by making use of the fact that genetic variants that are typically "randomly assigned" in the population and their causal effects can be estimated as subjects with varying genetic risk are exposed to a modifiable risk factor. Using a GWAS analysis of chronotype data conducted in >750,000 people, they found 340 loci associated with high morningness. Using these markers, the authors could then calculate a genetic proxy of morning preference. The authors next used markers for depression using a meta-analysis of GWAS data from the Psychiatric Genomics Consortium and UK Biobank with a combined 500,199 individuals. They could then examine the influence of genetics both on morningness and MDD. This study found that for every 1-hour earlier sleep midpoint estimated by the genetic proxy marker, there was a 23% reduction in depressive symptoms at a given level of genetic risk for MDD. This compelling study with a very large sample, demonstrates a cause-and-effect relationship between circadian rhythms and MDD in humans, in which high morningness is protective against depression.

Others have studied circadian rhythms in MDD using other methods, such as by examining post-mortem brains. Li and colleagues examined post-mortem human brain tissue from 55 controls and 34 patients with MDD. They treated the independently sampled data points, one for each subject, as a pseudo-time series spanning one 24-h cycle based on their time of death. They found that cyclic patterns of circadian gene expression were much weaker in the brains of patients with MDD across 6 brain regions: the dorsolateral prefrontal cortex, amygdala, cerebellum, nucleus accumbens, anterior cingulate cortex, and the hippocampus (Li et al., 2013).

Studies using animal models also have shown the importance of circadian rhythms in mood. Knocking down *BMAL1* in the SCN of mice results in more helplessness, despair, and anxiety-indicating behavior, as well as increase in corticosterone and changes in the circadian expression of corticosterone (Landgraf et al, 2016). Mice who have undergone learned helplessness protocols experience long, free-running circadian periods (Stewart et al., 1990). *PER2* mutant mice show reduced depression and anxiety-like behaviors (Hampp et al., 2008).

Evidence across models clearly indicate a relationship between circadian rhythms and mood disorders. Furthermore, circadian rhythms are related to treatment response: eveningness is associated with MDD nonremission (Chan et al., 2014) and a worse response to SSRIs (McGlashan et al., 2018) and reversed diurnal expression is associated with less response to serotonergic antidepressants (Joyce et al., 2005). Both human and animal studies have shown the relationship between MDD and specific genes in the circadian clock. Still missing from this picture is a characterization of circadian clock gene expression in live neurons with patients.

1.5 Modeling Depression using iPSC-derived neurons

While the previous models used to link circadian rhythms and MDD have been vital for our current understanding, they also have their drawbacks, leaving a niche for induced pluripotent stem cell (iPSC)-derived neurons to contribute. We believe that iPSCs-derived neurons closely resemble neurons implicated in MDD, and that we can observe MDD neurons with differences in circadian clock expression

Observational studies on human behavior document how major depression affects the daily life of individuals, which is especially important since MDD diagnosis is solely based on behavioral symptoms; yet, these studies cannot explore underlying brain mechanisms. Human behavioral studies done in a lab setting allow more investigator control and can better determine cause-and-effect, but these studies are expensive and labor intensive. Standard techniques to study the behavioral effects of circadian rhythms, like forced desynchrony or constant routine protocols, require significant subject commitment and can create stress for patients already contending with depression (Saini et al., 2015). Taking biological samples, such as drawing blood or administering surveys across many timepoints – as is required to measure circadian oscillations in a patient's gene expression or behavior – is especially labor-intensive.

While fMRI and body temperature readings study live human subjects at a biological level without invasive procedures and fMRI in particular can allow insight into the workings of the brain but are costly and lack molecular resolution (Viswanath et al., 2015; Saini et al., 2015). Post-mortem brain tissue can be studied at the anatomical, cellular, and molecular level. While pseudo-time series can be created by treating each subject as one point on the circadian day based on their time of death (Li et al., 2013), the same sample cannot be studied over many

timepoints. Additionally, the brain could be affected by biological processes that occur after death.

Animal models, usually mice, can provide insight into behavior, anatomy, and cellular and molecular mechanisms. They allow manipulation of variables that cannot be performed in humans, such as knocking out genes, doing drug tests, and controlling light/dark environments for long periods of time. Animal studies cannot be completely translated to humans.

iPSC-derived neurons uniquely allow the study of the molecular inner-workings of live human neurons. Gene and protein expression analysis can be performed on these cells through methods like qPCR, immunohistochemistry, and bioluminescent reporters. The same neuron sample can be studied over time. For the patient, creating iPSCs requires a one-time skin biopsy, so the procedure is minimally invasive. iPSC-derived neurons are collected from already-characterized human patients. Previous studies reveal the importance of accounting for the heterogeneity of MDD, and that separating subjects based on treatment response is a rational approach to do this for a circadian study. Thus, this study includes patient lines from patients who are SSRI-responders (remitters) and SSRI non-responders (non-remitters). This will allow us to study circadian underpinnings of drug response.

iPSC-derived neurons have already been used to study mood disorders, including MDD (Vadodaria et al.) and bipolar disorder (Mishra et al., 2021). We hypothesize that iPSC-derived neurons and neuronal progenitor cells (NPCs) are close representations of the neurons in the living brains of patients.

We have chosen to use glutamatergic cortical neurons as our model for MDD. While the SCN is the master circadian pacemaker and is therefore often used in circadian studies, animal studies have shown that circadian clocks outside the SCN may be important for mood regulation

(McCarthy et al., 2012), and postmortem and magnetic resonance spectroscopy (MRS) studies have shown that frontal cortical levels of glutamate were elevated in BD and MDD individuals compared to controls (Jun et al., 2014; Hashimoto et al., 2007).

We examined the expression of circadian genes in the primary and secondary TTFL (*BMAL1*, *CLOCK*, *PER1/2/3*, *CRY1/2/3*, *RORA*, *REVERBα*). We examined whether differences in the expression of circadian genes over time could lead to differences in the overall circadian rhythms and is associated with phenotypic differences in MDD patients.

METHODS

2.1 Human Subjects

Cell line donors were MDD patients with established SSRI response histories. Clinical characterization of the donors was conducted through the Mayo-PGRN SSRI study, an SSRI clinical trial performed by the Mayo Clinic Rochester Department of Psychiatry and Psychology details of which have been published previously Pharmacogenomics Research Network Antidepressant Medication Pharmacogenomics Study (PGRN-AMPS, 1–004134-07) (Mrazek et al., 2014). In this study, 529 patients were taken off prior medication and started on SSRI either citalopram and escitalopram (10-20 mg) to determine remission/non-remission. Standard depression scales were used to track depressive symptoms at baseline and over the course of the 8-week trial. Scales included the Quick Inventory for Depressive Symptomology (QIDS) and Hamilton Depression Inventory (HAMD). From these patients, 3 patients with the most improvement in depression were chosen as representative of the SSRI-responsive group (MDD remitter) and 3 that did not go into remission were chosen to represent SSRI- non-remitters (MDD non-remitter). For each group, the most extreme cases were chosen. Remission was defined as a QIDS-C16 score of 5 or less.

Table 2.1: MDD patient information. (Vadodaria et al., 2019)
Patients were assessed before and after 8 weeks on SSRIs. Patients were age and sex matched. Remitters had on average about a 70% reduction in MDD symptoms, while non-remitters had only about 20% reduction in symptoms.

SSRI response state	Gender	Age	SSRI prescribed	% Reduction in QIDS score after SSRI	% Reduction in HAM-D s after SSRI
Remitter	F	46	Escitalopram	80	83
Remitter	F	48	Citalopram	73	79
Remitter	F	46	Citalopram	60	61
Nonremitter	F	34	Escitalopram	5.8	-10
Nonremitter	F	33	Citalopram	28	-3.2
Nonremitter	F	53	Citalopram	32	23

2.2 hiPSC Culture

Patient iPSCs were obtained from collaborators at the Salk Institute as described before (Vadodaria et al., 2019). Briefly, skin punch biopsies were performed on the patients to collect fibroblasts and cells were subsequently reprogrammed into iPSCs. Skin punch biopsies of patients and healthy controls were obtained under sterile conditions, in accordance with institutional regulations (Mayo Clinic IRB 10-006845). Fibroblasts from the skin punch biopsies were taken and cultured for reprogramming into iPSCs by viral vectors expressing reprogramming transcription factors from the Sendai virus within the CytoTune-iPS Sendai Reprogramming Kits following the manufacturer's instructions (Invitrogen, A13780-02, A16517, A16518) and manufactured by ReGen Theranostics (Rochester, MN). Karyotypically normal iPSC clones that passed quality control criteria and expressed pluripotency markers Nanog, cMyc, Oct3/4, Sox2, Dmnt3b, Tdgf1, and Rex1 were selected for functional experiments with one clone per individual donor. iPSCs were cultured on Matrigel-coated plastic plates (BD Biosciences) using a modified recipe of mTeSR1 medium (Stemcell Technologies). Full details of this procedure have been published previously (Vadodaria et al., 2019)

2.3 Embryoid Bodies and Neural Rosette Cell Culture

Patient iPSCs were differentiated into embryoid bodies (EBs) and neural rosettes by collaborators at the Salk Institute (Vadodaria et al., 2019). Briefly, floating EBs were generated by mechanical and enzymatic dissociation of iPSC colonies using collagenase and were transferred to low-attachment plates in neural induction media (NIM: DMEM/F12 containing N2 and B27 supplements, Thermo Fisher Scientific). EBs were maintained in NIM containing Noggin (100 ng/ml), LDN193189 (100 nM), and SB431542 (10 μM). After a week, EBs were

transferred to polyornithine and laminin-coated plates in NIM containing fibroblast growth factor-2 (20 ng/ml) and laminin (1 μg/ml). Rosette-forming EBs were selected and dissociated using Accutase (Chemicon) and plated on polyornithine- and laminin-coated plates to generate NPCs.

2.4 Neural Progenitor Cell Culture

NPCs were obtained in frozen cryovials from the Gage lab and differentiated into neurons at VASD for circadian rhythm studies. They were thawed by warming the cryovials briefly in a 30°C water bath until any liquid was visible. The contents of the cryovial were immediately transferred into 10 ml of DMEM F12 to dilute the DMSO used to prevent crystals from forming in the cryovial contents. The diluted cryovial contents were centrifuged at 1800 rpm for 2 minutes and the supernatant was aspirated, leaving the cell pellet. The cell pellet was then resuspended in 1.5 ml of NPM media in one well of a poly-L-ornithine/laminin coated 6 well plate.

NPCs were grown on monolayers on polyornithine- and laminin-coated plates with neural proliferation medium (NPM: DMEM/F-12 (Invitrogen) with N2 (Invitrogen), B27 without Vitamin A supplement (Invitrogen), 20ng/mL fibroblast growth factor 2 (Peprotech), and Anti-anti (Bio Core)), which was replaced every other day. NPCs were maintained at high confluence to prevent differentiation. Cells were propagated by passaging them when they reached a very high density, which happened about every 5-7 days. To passage the NPCs, the cells were washed with DMEM/F12 and dissociated using Accutase (Stem Cell Tech #7920), then centrifuged at 1800 rpm for 2 minutes. The supernatant was aspirated, and the cell pellet was resuspended in NPM and distributed in wells of polyornithine/laminin-coated 6-well plates at a

1:2 or 1:3 expansion ratio. NPCs were plated for experimentation in Matrigel coated plates at a density of $400x10^3$ cells/well, for qPCR time-course and Lumicycle experiments. NPCs and neurons were cultured in a Nuaire Water-Jacketed IR Autoflow CO2 incubator with 5% CO2 at 35°C.

2.5 Cryopreservation of NPCs

Cells were frozen for long-term preservation by resuspending the cell pellet in preservation media consisting of 90% NPM and 10% DMSO. This solution was immediately aliquoted into cryotubes, put into a freezing container with an isopropyl alchohol-filled chamber (Mr. Frosty, Thermofisher), and placed at -80°C. Freezing container slows freezing rate to -1°C/minute. DMSO prevents crystals from forming during freezing and fracturing the cells, but it is also toxic to the cells, which is why freezing immediately is crucial.

2.6 Neuron Cell Culture

NPCs were differentiated into cortical glutamatergic neurons by plating the NPCs on matrigel-coated 35mm dishes in NPM. Cells were plated at $400x10^3$ cells/well for circadian rhythms and gene expression analyses, and at $90x10^3$ cells/well for single cell experiments. For immunostaining, cells were plated in 96-well plates on coverslips coated in polyornithine/laminin at a density of $90x10^3$ cells/well. Cell density was measured by mixing the cell suspension with trypan blue at a 1:1 ratio and transferring the solution into a hemocytometer. Trypan blue-dyed cells were manually counted by viewing the hemocytometer under brightfield microscopy. The cell suspension was then diluted with NPM accordingly. After 2 days of culture in the Matrigel-coated dishes, the media was switched to neural differentiation medium (NDM:

DMEM/F-12 (Invitrogen) with N2 (Invitrogen), B27 (Invitrogen), 10 ng/ml brain derived neurotrophic factor (Peprotech), 10 ng/ml glial cell line-derived neurotrophic factor (Peprotech), 1 um dibutyryl-cyclic adenosine monophosphate (Sigma), and 1 uM ascorbic acid (Sigma)). Half media changes were conducted once or twice per week, according to when the media turned acidic, indicated when the phenol red pH indicator in the DMEM F12 turned yellow. Neurons developed extensive morphological arborization and neural circuitry over a six-week period.

2.7 Immunocytochemistry

Immunocytochemistry was performed to characterize and validate neuronal identity and measure clock proteins. Media was aspirated from the cell culture dishes, and cells were washed with DPBS and fixed with 4% paraformaldehyde. After 30 minutes in paraformaldehyde, cells were washed twice in DPBS. Cells were permeabilized using 0.2% TritonX-100 (Sigma #X100) diluted in DPBS. Coverslips were placed in a wet chamber and incubated in 5% donkey serum (Jackson ImmunoResearch #017-000-121) for one hour to block non-specific proteins. Primary antibodies (Table 2.1) were diluted in 2% donkey serum and added to the coverslips. Coverslips were incubated at 4°C overnight. The following day, coverslips were washed three times using 0.2% TritonX-100 diluted in DPBS and then incubated in secondary antibodies (1:500) diluted in 2% donkey serum for two hours at 21°C. Coverslips were washed in DPBS and incubated in DAPI 13 (ThermoFisher Sci #D1306) for five minutes, then washed in dH2O and mounted on slides using an aqueous mounting medium (Polysciences, Inc #18606-20). Quantification was conducted via fluorescence microscopy (Leica Microsystems #C2343-33722164). Image analysis was conducted using Fiji ImageJ. The regions of interest for cellular measurements were created using β-III tubulin staining. DAPI staining was used to visualize and quantify nuclei for

immunocytochemical protein measurements. Statistical analysis was performed using GraphPad Prism version 5. One-way analysis of variance (ANOVA) was performed to find differences in fluorescent intensity based on diagnosis.

Table 2.2: Primary antibodies for immunocytochemistry.

Target	Species	Company	Cat #	Dilution
TUJ-1	Mouse	Biolegend	801201	1:300
GFAP	Goat	Abcam	ab53554	1:600
VGLUT2	Mouse	Synaptic Systems	135 421	1:300
PER2	Rabbit	Abcam	ab179813	1:300
PER1	Rabbit	Abcam	ab136451	1:300
BMAL1	Mouse	Santa Cruz Biotechnology	sc-365645	1:300

2.8 Live Cell Circadian Rhythm Assays

We measured the circadian rhythms in the iPSC-derived neurons using a *Period2-luciferase* (*Per2-luc*) reporter. *PER2* was chosen because it is an essential component of the circadian clock TTL and likely reflects the cellular circadian rhythm as a whole. Circadian rhythms were recorded from neurons plated at 400 x 10^3 cells per well. Neurons were transduced with *Per2-Luc* bioluminescence reporter using the surfactant polybrene and a lentiviral *Per2-Luc* construct in NDM. After 48 h of incubation, media was removed and replaced with NDM with 10uM forskolin (Tocris) to synchronize rhythms. After two hours of forskolin treatment, cells were washed in DPBS and a recording media including luciferin was added to each dish (DMEM (Invitrogen 12100046, serum-free, 1.2g/L sodium bicarbonate, pH 7.41) 10mM HEPES, a bicarbonate buffer solution, 25 U/mL penicillin, 25 ug/mL streptomycin,

2%B-27 (GIBCO 17504-044), 1mM luciferin). A glass coverslip was adhered to the 35mm dishes with an airtight grease seal and placed inside a 32-channel LumiCycle luminometer (Actimetrics) housed inside a dry incubator without CO_2. Bioluminescence recording occurred for 70 seconds every 10 minutes for five to seven days. Cells were entrained to a 24 h rhythm using a temperature cycle, in which the temperature within the LumiCycle luminometer alternated between 35°C for 12 h and 37.5°C for 12 hours. Using LumiCycle analysis software, raw counts were fit to a damped sine curve using the least squares method to calculate the rhythm parameters period and phase, and an un-damped sine curve to calculate amplitude. Recordings that deviated significantly from the best fit curve (had a low "goodness of fit") and those that had a period less than 22 or greater than 28 were considered outliers and excluded from analysis. Values were imported to Microsoft Office Excel. Statistical analysis was performed using GraphPad Prism version 5: One-way ANOVA and post-hoc t-tests were used to assess differences in amplitude and period between diagnosis groups. In all tests a p-value < 0.05 was used to determine statistical significance.

2.9 Single Cell Rhythm Analyses

Per2-luc was also used to record *PER2* rhythms of single cells in order to determine the circadian rhythms of individual cells and characterize phase alignment between cells. Neurons were plated on Matrigel-coated 35mm dishes at a density of $90x10^3$ cells per well and matured for 6 – 8 weeks as described above. Neurons were transduced with the surfactant polybrene and a lentiviral Per2-luc construct for 48 hours, and then synchronized with forskolin for 2 hours as described in the previous section. 35mm dishes were sealed with grease and placed on the stage of a bioluminescence microscope (Olympus, Tokyo, Japan, using an Olympus 4x XLFLUOR

objective (NA 0.28)) transmitting to a cooled charge-coupled-device camera (Series 800, Spectral Instruments, Tucson, AZ, USA). Bioluminescence was recorded every 30 minutes with an exposure time of 29.5 minutes for each image. Temperature was maintained at 36°C for the duration of recording. Recording continued for five to seven days and images were compiled into a time-lapse video for analysis with MetaMorph software (Universal Imaging Corp., Buckinghamshire, UK). Single-cell bioluminescence microscopy images were compiled into a time-lapse video using MetaMorph. Fields containing one cell were defined manually and objective brightness over time was logged as a text file using Microsoft Office Excel via MetaMorph, generating a text-converted sine wave file for each cell. Further analysis of each cell's circadian rhythm was conducted using the LumiCycle Analysis program. In the LumiCycle analysis software, rhythms were fit to a damped sine curve and curves were exported and plotted in GraphPad Prism.

2.10 Gene Expression Analysis of Core Clock Components

For quantitative qRT-PCR, NPCs were plated on matrigel-coated 35mm dishes at $400x10^3$ cells/well and differentiated for 6 weeks into neurons. To synchronize rhythms for gene expression analysis, the media was aspirated and replaced with 10 uM forskolin (Tocris) in NDM. Forskolin activates adenylyl cyclase to create cAMP that activates upstream signaling pathways to induce *PER1* and *PER2* and coordinate gene expression. After four hours of forskolin stimulation, the solution was aspirated, the cells were washed in DPBS, and NDM was added and incubated at 35°C for 12 hours.

To measure gene expression over the circadian time course, samples were then collected at regular intervals after synchronization. Samples were collected and frozen starting 12 hours

after synchronization (time 0), and then at 4-hour intervals over a 24 h time course. To freeze the samples, the media was aspirated, cells were washed with 4°C DPBS (Thermo Scientific), and the media completely aspirated again. The dishes were wrapped in plastic wrap and cells were frozen at -80°C until RNA extraction.

RNA was extracted using the Qiagen RNeasy kit (Qiagen #74104) as per manufacturer's instructions. NanoDrop 2000 (ThermoFisher Sci) was used to quantify total RNA. For each sample, 250-500ng total cellular RNA was collected and used to generate cDNA with reverse transcriptase (Tribioscience #TBS4006) and T100 Thermal Cycler (Bio-Rad #1861096). All samples of cDNA were 6.25 ng/ul.

Taqman qRT-PCR was conducted using the C1000 Touch Thermal Cycler and CFX384 Real-Time System (Bio-Rad) with the Taqman Universal PCR Master Mix (Tribioscience #TBS4002). Taqman PCR primers were selected to measure key individual components of the positive and negative limbs of the circadian clock (*PER1/2/3, CRY1/2/3, BMAL1, CLOCK*, *RORα*, and *REV-ERBα*). The mRNA levels for each gene were normalized to the non-rhythmic housekeeping gene *GAPDH* (Kosir et al., 2010). Fold changes for each gene were normalized to the mean expression of controls over 24 hours.

Data analyses were performed with GraphPad Prism version 5. Two-way ANOVA was performed to find the effect of time, diagnosis, and time x diagnosis interactions on gene expression. Tukey's multiple comparisons test was done in individual timepoints to find significant differences between diagnosis groups.

RESULTS

3.1 Immunocytochemistry confirmed stage specific markers in iPSCs and NPCs

Vadodaria et al. previously confirmed that iPSCs that were used for NPC generation had normal karyotype and expressed the pluripotency markers NANOG, cMYC, OCT3/4, SOX2, DMNT3B, TDGF1, and REX1. NPCs were also determined to be positive for Nestin, which is a neural stem cell marker.

3.2 Immunocytochemistry confirmed generation of glutamatergic neurons

We confirmed that we successfully generated glutamatergic neurons from patient-derived iPSCs using immunocytochemistry. All lines were stained for β-III tubulin, a neuron specific marker. For the lines that were stained, we quantified that 90-99% of cells were β-III tubulin positive, with the remaining cells positive for glial cell marker GFAP (data not shown).
The neurons were stained for vesicular glutamate transporter 2 (VGLUT2), which is present preferentially in glutamatergic neurons and commonly used to identify them. We estimate that almost 100% of cells stained with β-III tubulin were positive for VGLUT2, confirming that these were indeed glutamatergic neurons **(Figure 3.1)**.

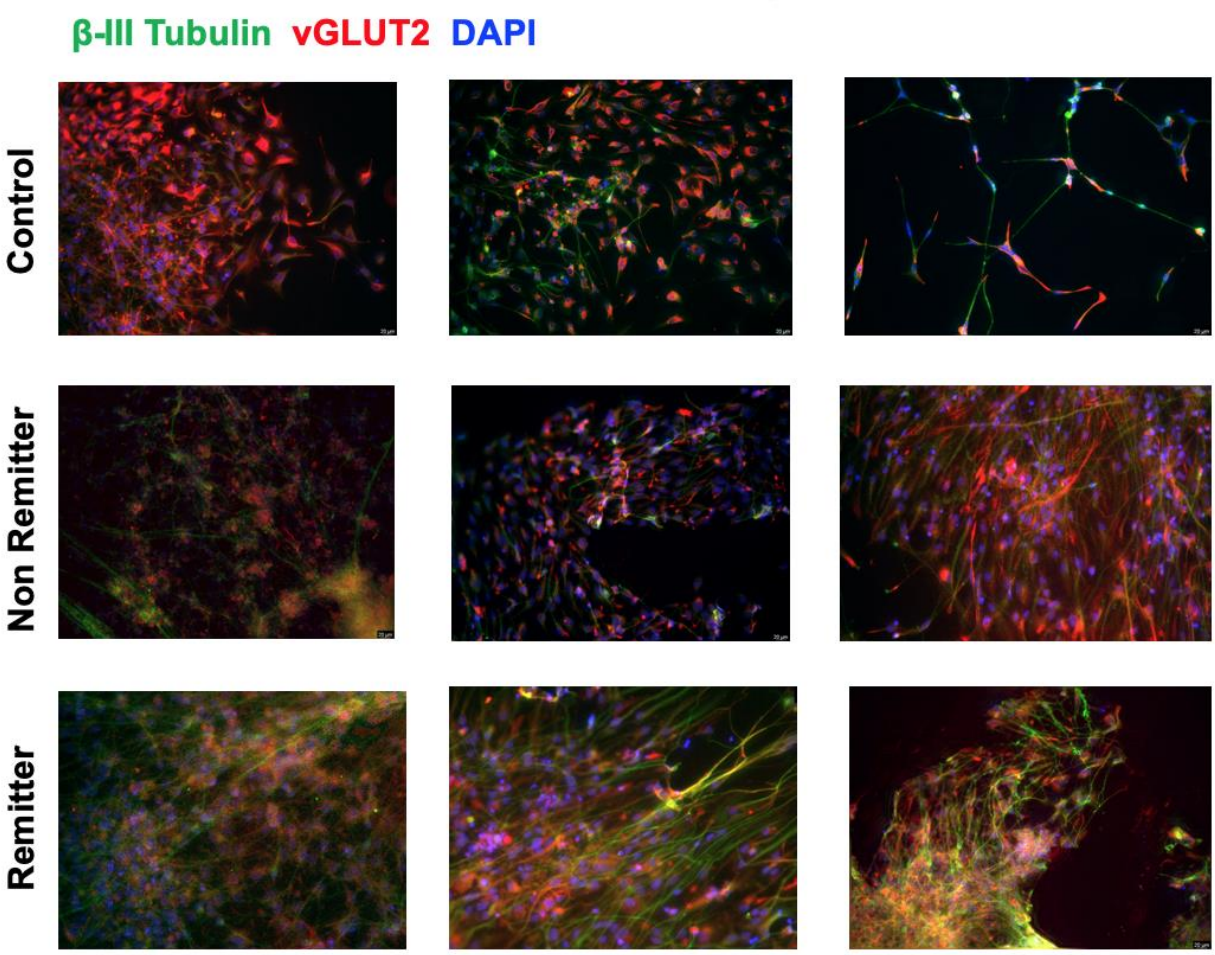


Figure 3.1: Immunocytochemistry confirms generation of glutamatergic neurons for all Control, MDD Non-Remitter, and MDD Remitter Lines.
Each row shows representative images of neurons from each of the three lines in each diagnosis group. Neurons were stained for β-III tubulin and VGLUT2. Approximately 100% of β-III tubulin positive neurons were positive for VGLUT2.

3.3 Amplitude of *PER2* oscillations are decreased in MDD Non-Remitters

In order to measure the circadian rhythms, glutamatergic neurons were transfected with lentiviral bioluminescent reporter *Period2-luciferase (Per2-luc)*. *Per2-luc* contains a firefly luciferase protein that is activated by a mouse *Per2* promoter; thus, luciferase is transcribed rhythmically and emits rhythmic light pulses. Luciferase enzymatically activates the luciferin substrate added to the media, emitting bioluminescence that is measured by the luminometer (Lumicycle, Actimetrics) every 70 secs, therefore allowing us to quantify *PER2* regulation.

The 24-hour temperature cycle succeeded in entraining the cells to a 24-hour rhythm, so as expected, there were no significant differences in period length observed between samples. However, entrained rhythms in MDD non-remitters neurons had a significantly lower amplitude than neurons from controls and remitters **(Figure 3.2 D)**. Neurons from MDD remitters had only a slightly lower amplitude than in controls and were not significantly different. This indicates that under entrained conditions, molecular circadian rhythms are weaker in non-remitters neurons compared to control neurons. Remitter neurons do not exhibit this same lack of robustness in their *PER2* oscillations.

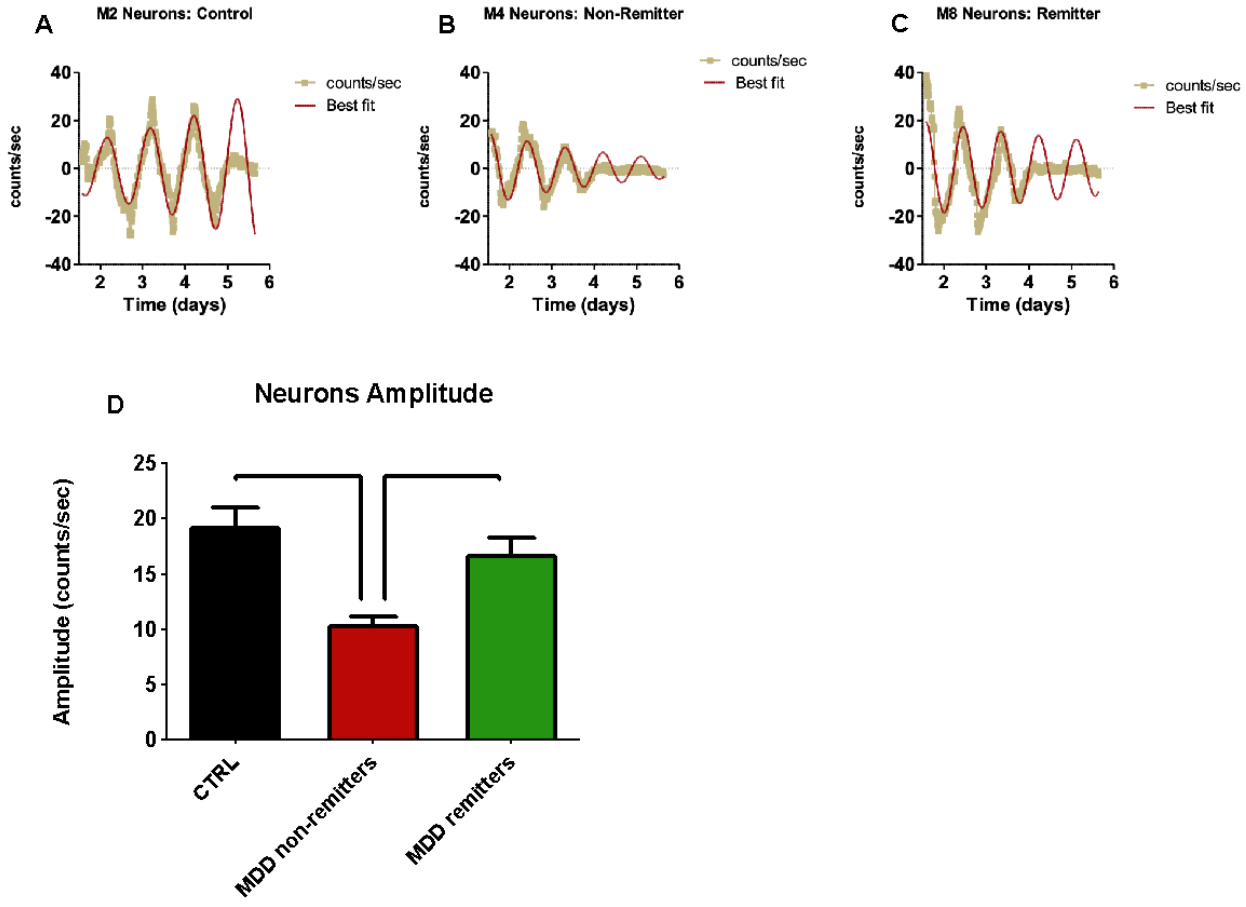


Figure 3.2: Amplitude of *PER2* oscillations are decreased in MDD Non-Remitters. Neurons were transfected with a lentiviral *Per2-luc* construct. Bioluminescence recordings were taken every 70 seconds for 6 days via a luminometer. Luminometer recordings were fit to a damped sine curve. Representative traces are shown for controls **(A)**, MDD non-remitters **(B)**, and MDD remitters **(C)**. Entrained rhythms in MDD non-remitters neurons had a significantly lower amplitude than neurons from controls (p-value = 0.03) and remitters (p-value = 0.04) **(D)**.

3.4 Expression of core circadian clock genes is decreased in MDD Non-Remitters

While *Per2-luc* allowed us to quantify *PER2* expression with high resolution, since recordings were taken of the same sample every 70 seconds, we wanted to learn more about the whole circadian clock. To accomplish this, we quantified gene expression for 9 genes in the core circadian clock using qRT-PCR. For our qRT-PCR time-course, we had a time point every 4 hours for 24 hours. At each time point, we measured a different dish, so we had less resolution to see the change in gene expression in one line over time, but greater specificity with regards to the particular genes involved. Indeed, while there were nominal differences that suggested rhythmic expression across the 24 h collection period, none of the genes showed significant differences over time, as determined by two-way ANOVA analysis.

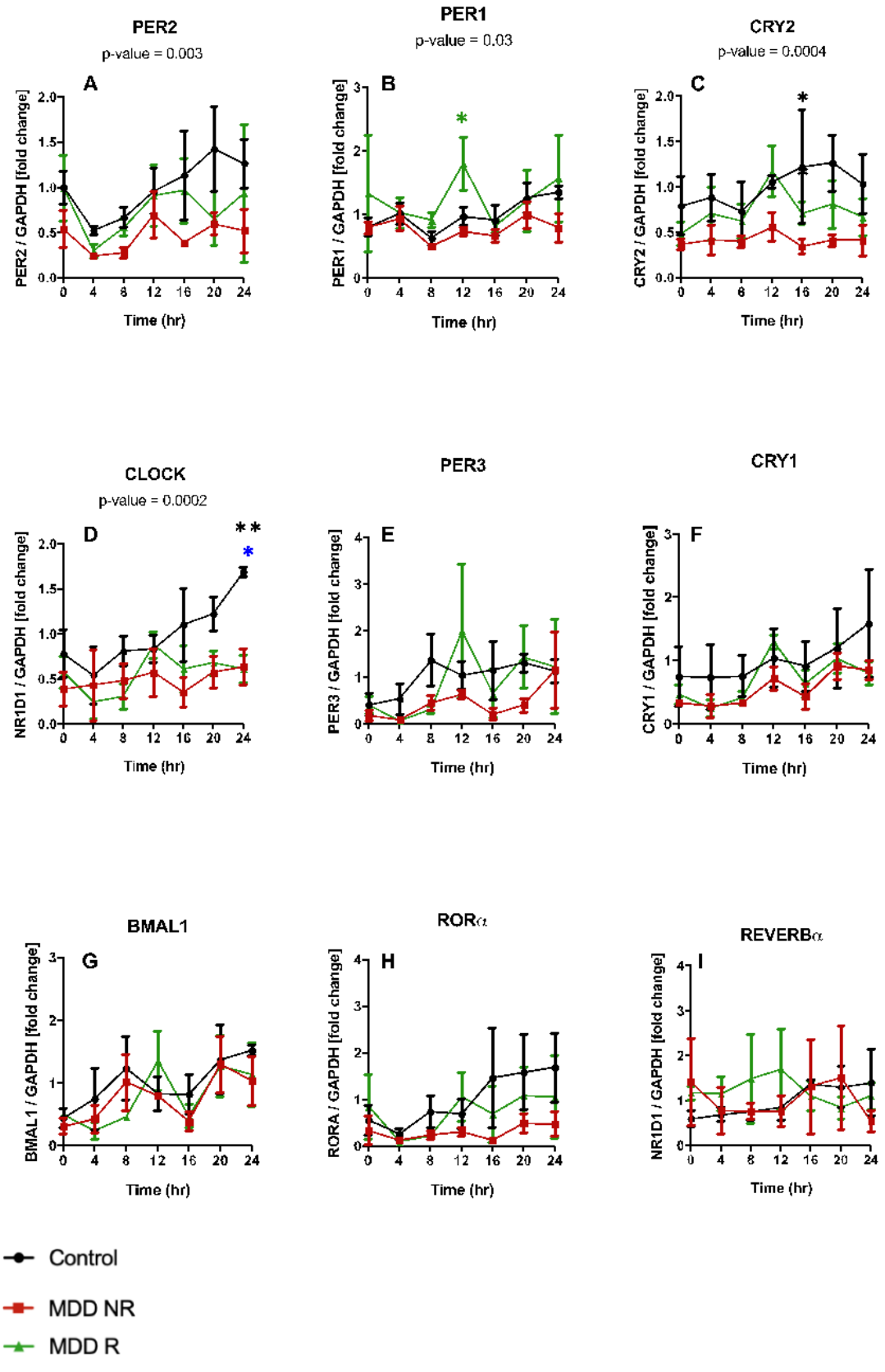
There were, however, significant differences in gene expression between diagnosis groups. Four of the nine core clock genes showed a significant difference in expression depending on diagnoses: *PER2*, *PER1*, *CRY2*, and *CLOCK* **(Figure 3.3 A-D)**. MDD non-remitters had lower expression of all genes at all seven time points. MDD non-remitters also had significantly less expression at certain time points, as determined by Tukey's multiple comparisons test. At the 16-hour time point, *CRY2* expression was significantly less in non-remitters than controls **(Figure 3.3 C)**. At the 24-hour time point, MDD non-remitter expression of *CLOCK* was lower than controls **(Figure 3.3 D)**. Though *PER3*, *CRY1*, *BMAL1*, and *RORα* did not have significantly different expression, they were still expressed less in non-remitters than controls across all timepoints **(Figure 3.3 E-H)**, and differences in expression of *CRY1* and *RORα* based on diagnoses returned p-values less than 0.10.

MDD remitters typically had gene expression levels somewhere between MDD non-remitters and controls that were usually nominally lower than controls. However, its relationship

to the other groups varied between genes and time points. Like non-remitters, MDD remitters had significantly lower *CLOCK* expression compared to controls at the 24 hour time point. Like controls, MDD remitters had higher gene expression than MDD non-remitters at specific time points. MDD remitters had greater expression of *PER1* at the 12-hour time point compared to MDD non-remitter **(Figure 3.3 B)**. At the 24-hour time point, MDD remitters had more expression of *CLOCK* than remitters **(Figure 3.3 D)**.

The results of the qRT-PCR successfully allowed us to investigate all the genes in the core clock and showed us significant differences between MDD non-remitters and controls. Altogether, the results showed there is to be a global reduction in gene expression of the circadian clock in MDD non-remitters. Gene expression is significantly decreased in MDD non-remitters for *PER2*, *PER1*, *CRY2*, and *CLOCK*. Furthermore, while the expression of *PER3*, *CRY1*, *BMAL1*, and *RORα* were not statistically significant, these four other genes had numerically lower amounts of gene expression at all seven timepoints. MDD remitters had gene expression levels between controls and MDD non-remitters. MDD remitters were significantly higher than non-remitters for *PER1* at the 12-hour time point. MDD remitters had significantly lower expression than controls for *CLOCK* at the 24-hour time point. Like in the *Per2-luc* recordings, MDD non-remitters are significantly different from controls. MDD non-remitters have a lower amplitude of gene expression, as shown by *Per2-luc*, and in overall levels of gene expression, as shown by qRT-PCR. *Per2-luc* showed that MDD remitters had a similar amplitude of *PER2* expression as controls, higher than MDD non-remitters. Once again, MDD remitters diverge from MDD non-remitters, with qRT-PCR overall higher levels of gene expression across clock genes. Overall, MDD non-remitters seem to have weaker gene expression of the circadian clock compared to controls and remitters.

Figure 3.3: Expression of *PER2, PER1, CRY2,* and *CLOCK* are decreased in MDD non-remitters.
Quantitative Reverse Transcriptase PCR (qRT-PCR) was performed on glutamatergic neurons. Neuron dishes were collected at t = 0, 4, 8, 12, 16, 20, and 24 hours. Error bars indicate standard error of mean (SEM). Controls are shown in black, MDD non-remitters are shown in red, and MDD remitters are shown in green. Data was analyzed with two-way ANOVA. There were no significant differences in gene expression based on time and time x diagnosis interactions. There were significant differences in gene expression based on diagnosis for four genes: *PER2* **(A)**, *PER1* **(B)**, *CRY2* **(C)**, and *CLOCK* **(D)**. All genes had less expression in MDD non-remitters than in controls over the entire time course. Individual timepoints were assessed for differences between diagnoses groups with Tukey's multiple comparisons test, and significant time points are denoted with *. MDD non-remitters had significantly lower expression than controls for *CRY2* at 16 hours (p-value = 0.047, **C**). MDD non-remitters have significantly lower expression than controls for *CLOCK* at the 24 hour time point (p-value = 0.005, **D**). MDD non-remitters had significantly lower expression than MDD remitters for *PER1* at 16 hours (p-value = 0.043, **B**) and for *CLOCK* at the 24 hours (p-value = 0.011, **D**).


PER2
p-value = 0.003
A
PER2 / GAPDH [fold change]
Time (hr)
PER1
p-value = 0.03
B
PER1 / GAPDH [fold change]
Time (hr)
CRY2
p-value = 0.0004
C
CRY2 / GAPDH [fold change]
Time (hr)
CLOCK
p-value = 0.0002
D
NR1D1 / GAPDH [fold change]
Time (hr)
PER3
E
PER3 / GAPDH [fold change]
Time (hr)
CRY1
F
CRY1 / GAPDH [fold change]
Time (hr)
BMAL1
G
BMAL1 / GAPDH [fold change]
Time (hr)
RORα
H
RORA / GAPDH [fold change]
Time (hr)
REVERBα
I
NR1D1 / GAPDH [fold change]
Time (hr)
Control
MDD NR
MDD R

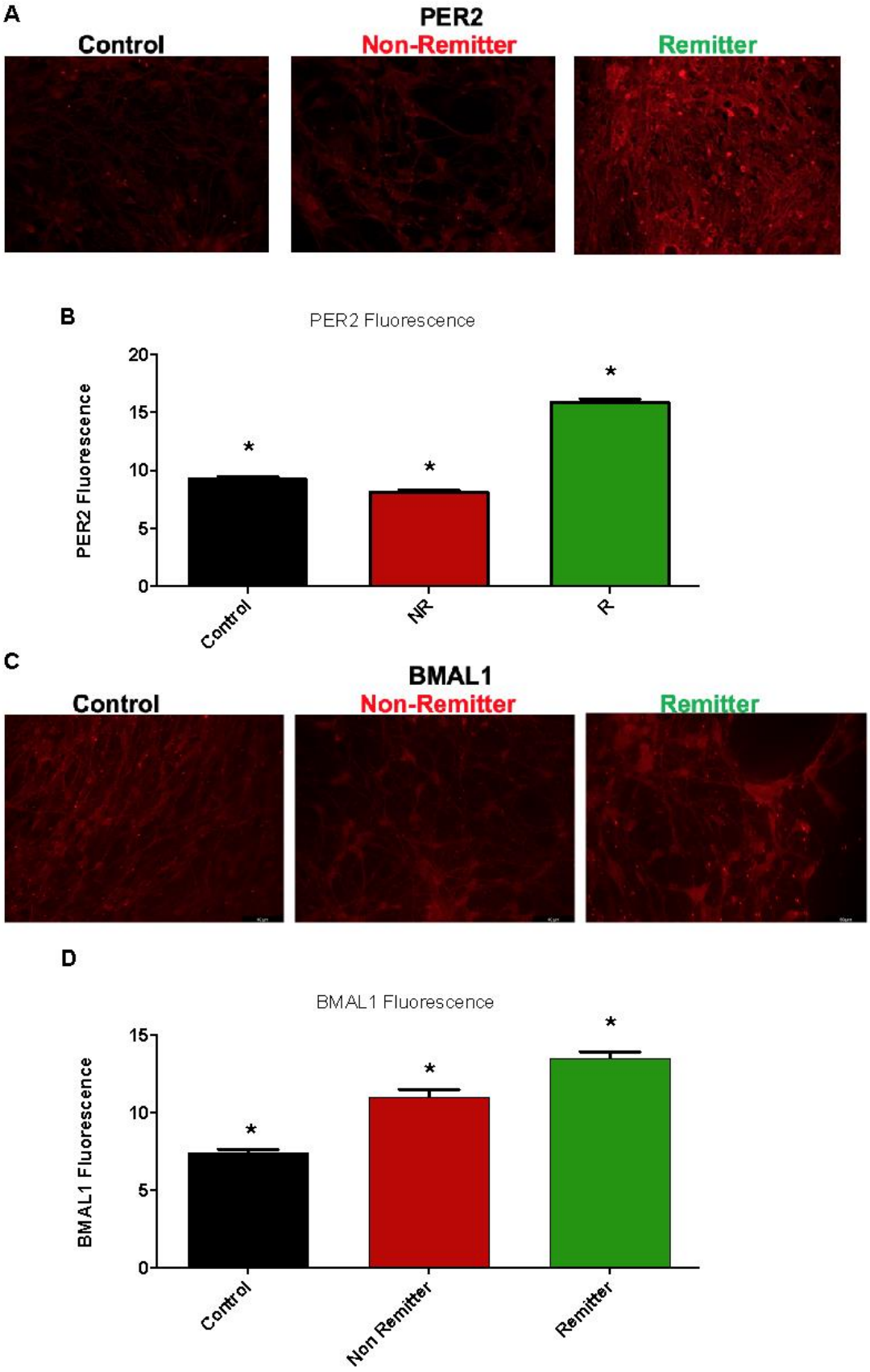
3.5 PER2 and BMAL1 protein signal is elevated in MDD Remitters

We analyzed clock proteins to verify the observed differences in gene expression are present post-translation. Based on qRT-PCR results, we decided to measure one positive regulator of the clock that exists in the nuclei, BMAL1, as well as a negative regulator that exists both in the cytoplasm and nuclei, PER2. To measure protein levels, we used immunocytochemistry. Neurons were stained with antibodies for PER2 (Abcam) and BMAL1 (Invitrogen), widefield fluorescent images were taken (Leica Microsystems #C2343-33722164), and fluorescent intensity in images was measured using ImageJ (**Figure 3.1, A and C).**

Since PER2 exists both in the cytoplasm and nucleus, we quantified the signal in the whole cells **(Figure 3.4, B)**. We found that there were statistically different levels of PER2 between controls, MDD non-remitters, and MDD remitters. One-way ANOVA analysis revealed that there was significantly higher intensity of PER2 signal in MDD remitters compared to controls and MDD non-remitters. There was significantly less PER2 signal in MDD non-remitters compared to controls and MDD remitters. We quantified PER2 levels in DAPI-stained nuclei and found the same relationship between diagnosis groups.

Since BMAL1 exists in nuclei as a transcription factor, BMAL1 intensity was measured only in the nuclei. One-way ANOVA analysis found significantly higher intensity of BMAL1 signal in neuron nuclei in MDD remitters compared to controls and MDD non-remitters, and significantly higher BMAL1 signal in MDD non-remitters compared to controls **(Figure 3.4, D)**.

Figure 3.4: PER2 levels in the nucleus are increased in MDD remitters.
Neurons were stained for clock proteins PER2 **(A)** and BMAL1 **(C)**. Representative images from each diagnosis group are shown. Using Fiji ImageJ, images were quantified for the cellular levels of PER2 signal **(B)**. One-way ANOVA analysis revealed that there was significantly higher intensity of PER2 signal in MDD remitters compared to controls and MDD non-remitters. Additionally, there was significantly less PER2 signal in MDD non-remitters compared to controls and MDD remitters. Nuclear intensity of BMAL1 was also quantified **(D)**. One-way ANOVA analysis found significantly higher intensity of BMAL1 signal in neuron nuclei in MDD remitters compared to controls and MDD non-remitters, and significantly higher BMAL1 signal in MDD non-remitters compared to controls


A
PER2
Control
Non-Remitter
Remitter
B
PER2 Fluorescence
PER2 Fluorescence
20
15
10
5
0
*
*
*
Control
NR
R
C
BMAL1
Control
Non-Remitter
Remitter
D
BMAL1 Fluorescence
BMAL1 Fluorescence
15
10
5
0
*
*
*
Control
Non Remitter
Remitter

DISCUSSION

Previous studies have established a clear link between MDD and circadian rhythms using a variety of models. We have added to this research by modeling the circadian clock in live human neurons, using iPSC-derived neurons from MDD patients. By studying the components of the core circadian TTFL, we found that circadian abnormalities in MDD persist at the molecular level. Interestingly, we found that differences in the circadian clock are based on SSRI response.

Using *Per2-luc* reporter live recordings, we successfully measured circadian rhythms in iPSC-derived neurons under entrained conditions, with *PER2* serving as a model of the circadian clock as a whole. We found that MDD non-remitters had reduced amplitude of *PER2* transcriptional oscillations. On the other hand, MDD remitters had intact, high amplitude circadian rhythms, like controls. This reduction in amplitude and expression could be due to a reduction in the amplitude of individual cells or because the cells in MDD non-remitters are more phase dispersed and experience destructive interference.

Since *Per2-luc* only allowed us to study one part of the clock, we filled in the missing pieces of the clock using the qRT-PCR gene expression assay. We detected significant differences in gene expression based on diagnoses. MDD non-remitters had lower expression of four genes of the core circadian TTFL: *PER2*, *PER1*, *CRY2*, and *CLOCK*. Additionally, *PER3*, *CRY1*, *BMAL1*, and *RORα* expression were nominally decreased in non-remitters at all seven timepoints. This suggests a global reduction in gene expression in the circadian clock in MDD non-remitters. MDD remitter neurons had gene expression lower than controls but higher than MDD non-remitters. Like in the *Per2-luc* recordings, gene expression analyses showed that

MDD non-remitter neurons deviated from controls, while MDD remitters and controls are similar.

In order to see if transcriptional differences resulted in translational differences, we performed immunocytochemistry of one positive regulator and one regulator negative, PER2 and BMAL1. MDD remitters had elevated levels of both PER2 and BMAL1 protein. If this is indeed a permanent feature of MDD remitter neurons, and not a product of phase differences, it could be caused by greater translation or slower breakdown of these proteins. It is unlikely to be caused by phase differences since we do not see rhythms in neurons without temperature entrainment.

The fact that MDD non-remitters and remitters have distinct abnormalities in circadian clock expression, indicates that treatment response history is perhaps a more salient factor than a psychiatric diagnosis of MDD. The diagnosis of MDD may have significant heterogeneity in terms of biological causes, some of which may map onto SSRI response and/or circadian rhythm disruption. The findings of this study could help explain the relationship between circadian rhythms and MDD presentation that we see at the clinical level where MDD patients with circadian disruption respond poorly to SSRI medication (McGlashan et al., 2018; Joyce et al., 2005). Weaker transcription of the clock that is seen in MDD non-remitters, in both amplitude of rhythms and overall gene expression, could contribute to the circadian abnormalities that are observed specifically in SSRI non-responders. Higher levels of PER2 and BMAL1 could be a compensation mechanism for remitters to maintain their oscillatory activity, despite having lower levels of gene expression than controls. Interestingly, the findings of this study have parallels with previous studies on circadian gene expression as delayed to mood disorders. *PER2* is differentially regulated in iPSC-derived neurons from patients with bipolar disorder, with lower

amplitude of *PER2* expression observed in neurons from BD patients (Mishra et al., 2021), similar to what we found in MDD non-remitters neurons. Our gene expression analysis identified CRY2 as a significantly downregulated gene in MDD non-remitters. Similarly, depressed bipolar patients have lower CRY2 gene expression (Lavebratt et al., 2010), and SNPs in CRY2 have been associated with seasonal depression.

Strengths and limitations:

Per2-luc enabled us to track the transcription of *PER2* with high resolution, with recordings taken every 70 seconds, and allowed for longitudinal studies with the same dish over many days. However, *Per2-luc* recordings did not allow us to normalize for cell numbers in dishes. We compensated for this limitation by plating the cells at a standard density, and replication is minimal once the final differentiation process into glutamatergic neurons is started.

During the *Per2-luc* recordings, we did not see rhythms under standard conditions. The neurons expressed fairly constant levels of *Per2-luc* signal. We were only to observe rhythmicity in cells when they were entrained with a 24-hour temperature cycle. Temperature entrainment is a commonly used procedure in circadian research, but it entrains cells to the same phase even if they would be phase dispersed under standard conditions and could potentially cause increases in gene expression in individual cells.

Gene expression analysis with qRT-PCR allowed us to analyze gene expression for all nine positive and negative regulators of the core circadian clock. Though qRT-PCR does not have as high resolution as *Per2-luc* recordings, since samples were only collected every 4 hours, we were able to normalize for differences between samples with the non-rhythmic housekeeping gene GAPDH. This allowed us to normalize for differences in overall gene expression levels, caused by factors such as variation in absolute cell numbers.

For gene expression and protein analysis, our inability to study the same dish across multiple timepoints did not pose a significant problem because gene expression levels under standard conditions remain consistent. The relationship between controls and MDD non-remitters were consistent across all dishes collected across the full 24-hour time course. Two-way ANOVA analysis showed no significant differences in gene expression based on time. Moreover, neurons did not exhibit rhythms without temperature entrainment during *Per2-luc* recordings. Without intact rhythms, it is unlikely that differences in gene or protein expression are caused by differences in phase, and they are more likely caused by differences between diagnosis groups.

Our statistical power was limited by the low number of patient replicates. iPSC-derived neurons are expensive and require a lot of manpower to create and maintain, posing a universal issue in iPSC cell culture-based research.

Future Directions:

McCarthy lab plans to obtain more lines so that we may increase our statistical power and see if the patterns of circadian gene expression that we have observed are generalizable to a greater sample size. In order to evaluate how reduced circadian clock gene expression influences circadian rhythms in MDD patients, we hope to obtain more chronotype information about the subjects of this study and subjects of planned future studies. We also plan to differentiate our existing iPSCs into other cell types that have been implicated in MDD, such as serotonergic neurons (Vadodaria et al., 2019). Studying NPCs could elucidate a developmental component of MDD and circadian rhythms. We could also create neurons that are reprogrammed directly from fibroblasts, so they retain their epigenetic traits (Huh et al., 2016). Further analysis of live NPCs

and a variety of neuron types from patients will provide a more complete picture of the circadian clock's role in MDD.

We plan to perform single cell analysis using the same *Per2-luc* reporter that we used for whole dish recordings. Single cell analysis allows for measurement of rhythms in individual cells, normalizing for differences in cell number between dishes. This method also allows calculation of phase differences between cells in a dish. This will help clarify whether the reduced rhythms we see in MDD non-remitters under entrainment is due to lack of rhythms in the cells or because of phase dispersion. Additionally, it will help us understand the reason dishes do not see rhythms under standard conditions.

To alleviate uncertainties about whether differences in protein levels are caused by differences in phase, we could perform an immunostaining time course, in which we synchronize cells with forskolin and collect samples across multiple timepoints. Since immunostaining is regarded as a semiquantitative rather than a quantitative (Taylor et al., 2006), we plan to optimize this assay by performing more immunostaining controls, such as negative controls, isotype control, and staining with no primary antibody.

We also would like to investigate how other characteristics observed in MDD neurons interact with circadian rhythms. For instance, levels of glutamate have been shown to be elevated in the frontal cortex of people with MDD (Jun et al., 2014; Hashimoto et al., 2007). We could knockdown glutamate levels in MDD neurons and evaluate how that can rescue circadian gene expression. Since serotonin has been shown to cause hyperactivity in serotonergic neurons from the same MDD non-remitters subjects (Vadodaria et al., 2019), we would like to see if the addition of serotonin affects circadian clock expression.

Overall conclusions:

This study demonstrates circadian abnormalities in MDD at the molecular level that could contribute to the relationship between MDD and circadian rhythms that we observe in behavior. It also highlights that circadian rhythms distinguish treatment response from the MDD psychiatric diagnosis. MDD non-remitters had lower amplitude rhythms and generalized weaker transcriptional activity across multiple clock genes. MDD remitters had elevated levels of clock proteins. Findings of behavioral studies could be driven by a certain subset of MDD patients that experience different responses to treatment. Future circadian studies should attempt to separate MDD subjects based on treatment response.

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