Cell-type Specific Regulation of Reward-Related Behavior: The Interface of Metabolic State and the Circadian Molecular Clock

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Substance use disorder (SUD) is a widely prevalent, devastating, and highly complex disease with limited effective treatment options. Given the lack of successful therapeutics, it is crucial to understand better the cellular and molecular level processes that drive reward and how their disruption may contribute to SUDs development and/or perpetuation. The studies outlined here propose that reward may be regulated within the nucleus accumbens (NAc), a key reward region of the brain, through interactions between cellular metabolic state and the circadian molecular clock, a ubiquitous transcription-translation feedback loop that drives endogenous rhythms in physiology and behavior. Moreover, this interaction is mediated at both the molecular level by circadian interfacing redox sensors, namely Neuronal PAS Domain Protein 2 (NPAS2) and Sirtuin 1 (SIRT1), and at the cellular level by astrocytes, highly abundant glial cells integral for circadian, metabolic, and reward function. In our first study, we utilize both molecular and behavioral techniques to demonstrate the circadian redox sensors NPAS2 and SIRT1 interact in the NAc, together regulating reward and metabolic-relevant processes, and this interaction in the NAc is necessary for cocaine reward. Moreover, this interaction introduces a potential mechanism by which cocaine's demonstrated effects on circadian and metabolic gene expression may influence reward. In our second study, we utilize time of day and cell-type-specific RNA-sequencing to demonstrate for the first time that astrocytes exhibit robust rhythmicity in the NAc, with approximately ~43% of their transcriptome showing diurnal variation. Remarkably, this

circadian astrocyte function in the NAc is also important for regulating reward-related behavior, in that mice with genetically induced loss of NAc astrocyte molecular clock function show increased novelty-induced locomotion, exploratory drive, and operant food self-administration and motivation. Finally, this phenotype is also coupled with significant disruptions to NAc metabolic homeostasis in the form of downregulated lactate and glutathione shuttling. Taken together, these two studies not only underscore the multifaceted and interconnected relationship between circadian rhythms, metabolic state, and reward-regulation in the NAc, but also reveal novel mechanisms by which drugs of abuse may influence reward-circuitry to drive the development of SUDs and addiction-like behavior.

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Dedication

I would like to dedicate this dissertation to my parents, Mike and Sandra, and my sister, Bri-Anna, for always believing in me and encouraging me to follow my dreams. Also, to my wife and best friend, Barrett, for being a constant source of unwavering love, support, and inspiration.

1.0 Introduction

1.1 Substance Use Disorders and the Brain's Reward System

With a more than two-fold increase in annual overdose deaths in the past decade and an estimated annual cost of $740 billion, drug addiction or substance use disorder (SUD) remains a devastating public health issue in the United States (Hedegaard et al., 2020; National Institute on Drug Abuse, 2020a). From the Latin verb *addicere* meaning to devote, sacrifice, or consecrate, drug 'addiction' or SUD is a chronically relapsing and debilitating disease characterized by a loss of control in drug intake, compulsion to seek and use a drug, and the persistence or devotion to using despite negative consequences (American Psychiatric Association, 2013). In a 2018 survey, approximately 20.3 million people aged 12 older had a SUD, or roughly 1 in 16 people in the United States (Substance Abuse and Mental Health Services Administration, 2019). While some behavioral and pharmaceutical treatment options have proven to be effective for managing SUDs, drug relapse (i.e., a return to drug use) still occurs in upwards of 60% of people seeking treatment for an SUD (National Institute on Drug Abuse, 2020b). In hopes of developing novel therapeutic options that treat or prevent compulsive drug use, craving, *and* relapse, investigation into the cellular and molecular mechanisms underlying SUD pathophysiology is still of dire need. Notably, to better understand how SUDs emerge, we must continue investigating and building a fundamental understanding of how our brain processes reward and reward-related behavior.

1.1.1 Reward Circuitry and Neurotransmitters

Through both clinical and preclinical studies, it has long been known that SUDs manifest through aberrant functioning of the brain's reward circuitry (Koob and Le Moal, 2001; Koob and Volkow, 2010; Pierce et al., 2020). The primary pathway of the brain's reward system, the mesolimbic pathway, consists of dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NAc) in the ventral striatum (**Figure 1**). When activated, dopamine (DA) is released into the NAc and is thought to be responsible for assigning motivational salience to environmental stimuli, reinforcing rewarding behaviors, and driving goal-directed behaviors – both to natural reward and drugs of abuse (Schultz, 2002; Volkow and Morales, 2015; Morales and Margolis, 2017). The "Dopamine Hypothesis" of SUD posits that substances of abuse, like alcohol, cocaine, nicotine, opiates, and marijuana, act directly on the brain's reward system to increase mesolimbic DA release and promote both reward and subsequent reward-seeking behavior (Koob, 1992; Willuhn et al., 2010; Volkow and Morales, 2015). In fact, psychostimulants like cocaine or amphetamine act directly at NAc synapses to increase the concentration of extracellular DA available either by blocking dopamine transporter (DAT) mediated DA reuptake or promoting greater DA release, respectively (Kalivas, 2007). The increase in DA mediated by substances of abuse directly activates neurons in the NAc through specialized DA receptors, this in turn having significant downstream effects on reward-related behavior given the NAc's position as a nexus of motivated/appetitive behavior and aversion.

Through decades of research, the NAc has been extensively characterized as a key integrator of cognitive, sensorimotor, and affective/limbic information through its DA-ergic and glutamatergic inputs and GABA-ergic (γ-aminobutyric acid) outputs (Floresco, 2015). In addition

to the DA-ergic input from the VTA, the NAc largely receives excitatory glutamatergic input from the prefrontal cortex (PFC), the hippocampus, and the basolateral amygdala (BLA) – essential for relaying information associated with attention / cognitive flexibility, novelty / contextual cues, and aversion / affective salience, respectively. Conversely, the NAc primarily sends inhibitory GABA-ergic projections either directly back to the ventral mesencephalon (including the VTA and substantia nigra; i.e., "direct pathway") or indirectly through the globus pallidus (i.e., "indirect pathway"), both pathways having downstream effects on motor control mediated by the thalamus (Macpherson et al., 2014). The NAc is >90-95% GABAergic medium spiny projection neurons (MSNs) that can be divided into two primary functional subtypes depending on their relative expression of specialized G-protein coupled DA receptors – either dopamine receptor D_1 (encoded by *Drd1*) containing MSNs or dopamine receptor D_2 (encoded by *Drd2*) containing MSNs (Surmeier et al., 2007). While other dopamine receptor subtypes exist ($D_{1\text{-}5}$), the DA receptors are primarily classified as being either D_1-like (D_1 & D_5) or D_2-like (D_2, D_3, & D_4) depending on whether they stimulate levels of secondary messenger cyclic AMP (cAMP) or inhibit it, respectively (Missale et al., 1998; Bhatia et al., 2020). In addition to D_1- vs. D_2-receptor differences in their effects on intrinsic excitability, glutamatergic synaptic plasticity, and signaling cascades (Lu et al., 1998; Surmeier et al., 2007; Baik, 2013), their expression on MSNs has classically served as a functionally defining feature in that D_1-containing MSNs generally makeup the *direct* striatonigral pathway and D_2-containing MSNs generally makeup the *indirect* striatopallidal pathway. While more recent studies suggest this grouping may oversimplify and overlook the nuances of the system (Kupchik et al., 2015), it is largely appreciated that activation of these specific pathways and cell-types differentially affects reward and motivated behavior; e.g., activation of D_1-MSNs and the direct pathway generally promotes reward, while activation of D2-

MSNs and the indirect pathway generally promotes aversion and/or attenuates reward (Hikida et al., 2010, 2013; Lobo and Nestler, 2011; Kravitz et al., 2012; Macpherson et al., 2014).

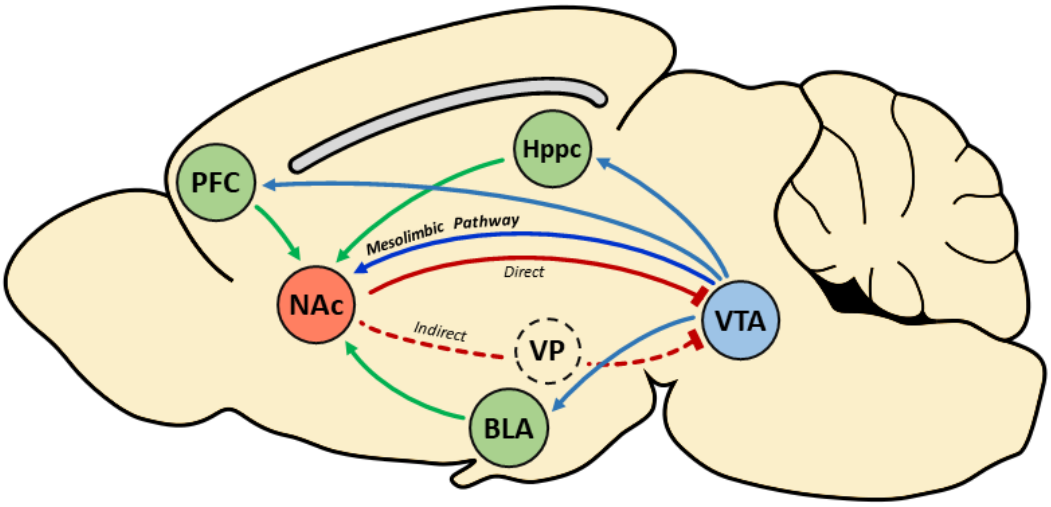

Figure 1. The mesolimbic pathway is the primary reward pathway of the brain. The mesolimbic pathway primarily consists of dopaminergic projections (blue) from the ventral tegmental area (VTA) synapsing on GABAergic medium spiny neurons (MSNs) in the nucleus accumbens (NAc). In response to rewarding stimuli, dopamine (DA) is released into the NAc and activates MSNs through specialized DA receptors (e.g., Drd1, Drd2, & Drd3). The NAc is >90-95% GABAergic and sends GABAergic projections (red) back to the VTA either directly (primarily mediated through reward promoting D1-receptor containing MSNs) or indirectly (dashed) to the VTA through the ventral pallidum (VP; primarily mediated by D2-receptor containing MSNs). The NAc also receives glutamatergic projections (green) from the prefrontal cortex (PFC), hippocampus (Hppc), and basolateral amygdala (BLA), serving as a nexus of reward and motivated behavior through integrating cognitive, sensorimotor, and affective/limbic information. The VTA also sends DA projections to these regions upstream of the NAc (e.g., PFC, Hppc, and BLA), modulating various aspects of reward processing and receiving feedback back through the NAc. This simplified framework is the basis of reward processing, reward learning, motivated behaviors, and aversion in mammals, and disruptions to this system are thought to underly the development of SUDs. Image depicts sagittal mouse brain section.

In addition to subdivisions of the NAc defined by projection and by DA receptor subtype, the NAc can be functionally divided into two primary anatomical subregions – the lateral *core* and the medial *shell*. While both regions play a role in reward processing and reward-related behavior, there are distinct functional differences that separate the two regions - the NAc core predominantly being associated with initiating goal-directed behavior and learning, while the NAc shell being primarily associated with processing hedonic information and assigning value to stimuli (Meredith et al., 2008; West et al., 2018). More specifically, the NAc core is thought to play a role in conditioned and motivational responses to stimuli, spatial and contextual learning/association, and impulsivity, while the NAc shell is believed to be critical for the reinforcing properties of natural reward (e.g., food and sex), drugs of abuse, and novelty (Salgado and Kaplitt, 2015). In the context of drugs of abuse like psychostimulants, the NAc core has been shown to regulate locomotor activity and learned response to drug cues, while the NAc shell is more important for the drugs' rewarding and reinforcing effects (Sellings and Clarke, 2003; Ito et al., 2004). These functional distinctions arise from differential innervation across the NAc from the hippocampus, PFC, amygdala, and other cortico-limbic regions (Meredith et al., 2008; Salgado and Kaplitt, 2015; West et al., 2018). Depending on how drugs of abuse recruit or alter specific subregions, cell-types, or projections of the NAc, there is significant potential to alter reward processing and drive aberrant reward-learning or reward-seeking behavior.

Taken together, the NAc's central role in the processing of reward and motivation positions it to be critical to the development of SUDs through drugs "hijacking" this endogenous reward system; e.g., activating reward promoting D1 / direct neurons while simultaneously inhibiting aversive D2 / indirect neurons, increasing drug reward salience and related cues, and ultimately redirecting behavior toward drug-seeking, despite any aversive consequences (Koob and Volkow,

2010; Volkow and Morales, 2015). While many hypotheses exist as to how drugs of abuse may alter NAc function and "hijack" the endogenous reward system, one notable hypothesis posits that non-neuronal glial cells, specifically astrocytes, are among the most affected by drugs of abuse and contribute to the transition from reward to addiction-like behavior.

1.1.2 Astrocytes in the NAc and Reward Regulation

In addition to the >90-95% MSNs and the remaining neurons being modulatory GABAergic or cholinergic interneurons (Kawaguchi, 1993; Lim et al., 2014; Tepper et al., 2018; Schall et al., 2020), non-neuronal cell types are also incredibly abundant in the NAc and play an integral role in both supporting and regulating overall function (Miguel-Hidalgo, 2009; Linker et al., 2019). One glial cell-type in particular, *astrocytes* or astroglia, is essential to NAc function for regulating glutamatergic transmission, metabolic homeostasis, and synaptic plasticity through their processes occupying synapses, also known as the *tripartite synapse* (Scofield and Kalivas, 2014; Mederos et al., 2018; Khakh, 2019). While once thought to be ten times more abundant than neurons (Hilgetag and Barbas, 2009), current estimates more accurately place astrocyte abundance closer to a 1:1 ratio, depending on species and the specific brain region (Herculano-Houzel, 2014; von Bartheld et al., 2016). Total count aside, astrocytes contribute substantially to synaptic regulation in that a *single* astrocyte is estimated to occupy upwards of 120,000 synapses in rodents and up to 2,000,000 in humans (Bushong et al., 2002; Oberheim et al., 2009). Astrocytes and their projections are not only abundant, but astrocytes also exhibit robust heterogeneity throughout the brain, with functional specificity depending on region-specific demands (Morel et al., 2017; Batiuk et al., 2020; Huang et al., 2020). Interestingly, astrocytes in the striatum are transcriptionally and

functionally distinct, as determined by cell-type-specific analyses (Gokce et al., 2016; Chai et al., 2017; Asanuma et al., 2019). Notably, astrocytes in the NAc are specifically crucial for regulating glutamate homeostasis and neurotransmission at the synapses of the NAc's many glutamatergic inputs (Scofield and Kalivas, 2014; Scofield, 2018).

Astrocytes are critical for maintaining glutamate homeostasis (Danbolt, 2001; Malarkey and Parpura, 2008; Mahmoud et al., 2019). Astrocytes primarily regulate extracellular glutamate levels through astrocyte-specific glutamate transporters: glutamate aspartate transporter (GLAST), glutamate transporter 1 (GLT-1), and the cystine-glutamate antiporter (xCT) (Danbolt, 2001). In the NAc, this function is critical for regulating the many glutamatergic afferents (e.g., prefrontal cortex, hippocampus, amygdala, etc.) that modulate NAc activity (Kalivas, 2009; Scofield et al., 2016a). Significant evidence from the Kalivas lab and colleagues suggests dysregulation of glutamate homeostasis, heavily attributed to astrocyte dysfunction and downregulation of glutamate transporters, may underlie the development of addiction-like behavior – also known as the *glutamate homeostasis hypothesis* (Kalivas, 2009). For example, several studies show rats exposed to cocaine self-administration and extinction have significant NAc-specific reductions in expression and function of GLT-1 and xCT, resulting in decreased glutamate clearance and uptake velocity (Knackstedt et al., 2010; Fischer-Smith et al., 2012; LaCrosse et al., 2017). Pharmacological restoration of GLT-1 and/or xCT expression and function improves glutamate homeostasis and significantly attenuates reinstatement of cocaine-seeking behavior in rats (Baker et al., 2003; Knackstedt et al., 2010; Trantham-Davidson et al., 2012; Fischer et al., 2013; Reissner et al., 2014). In addition to altering glutamate transporters, two recent studies from the Reissner lab demonstrate rats exposed to cocaine self-administration followed by extinction have significantly decreased glial fibrillary acidic protein (GFAP; a widely-used astrocyte marker(Yang

and Wang, 2015)) expression, GFAP-positive astrocytes, smaller astrocyte surface area and volume, and reduced astrocyte synaptic colocalization, all specific to the NAc (Scofield et al., 2016b; Testen et al., 2018). Lastly, altering astrocyte gliotransmission also affects cocaine-seeking behavior in rodents. While astrocytes are not electrically excitable like neurons, astrocytes can modulate synaptic function and plasticity through the calcium-dependent release of "gliotransmitters" (e.g., glutamate, D-serine, and/or ATP) (Harada et al., 2015). Interestingly, transgenic mice unable to release gliotransmitters from astrocytes show a significant attenuation of cue-induced reinstatement to both cocaine CPP and cocaine self-administration (Turner et al., 2013). Furthermore, pharmacologically inducing release of glutamate from NAc astrocytes significantly blocks reinstatement of cocaine-seeking in rats (Scofield et al., 2015). While these studies and others have made great strides in illuminating the role astrocytes may play in the effects of drugs of abuse like cocaine, further investigation is still necessary. How exactly astrocytes contribute to or regulate addiction-related behavior is unclear. Furthermore, there are many astrocyte functions that have yet to be explored in the NAc and/or in the context of reward regulation. One unexplored mechanism may be through astrocyte rhythmicity and their regulation of circadian rhythms.

1.2 Circadian Rhythms and The Endogenous Clock

Roughly every 24 hours, the Earth completes a rotation around its axis with respect to the Sun, resulting in periodic cycling of both available environmental light and temperature. Out of necessity for life on Earth, circadian rhythms convergently evolved to facilitate organisms'

anticipation and adaptation of physiology and behavior to these daily changes in environmental stimuli. Circadian rhythms, derived from Latin *circa diem* or "about a day," are driven by an endogenous autonomous timekeeping biological system called the circadian clock and are highly conserved across nearly all living organisms (e.g., both vertebrate and invertebrate animals, plants, fungi, protists, and bacteria). (Bhadra et al., 2017; Patke et al., 2020) In mammals, both system and cellular level rhythms are generated and maintained within the brain by an anterior hypothalamic region called the suprachiasmatic nucleus (SCN) (Welsh et al., 2010). The SCN consists of a highly coupled network of neuronal and glial oscillators entrained by light information received directly from photosensitive retinal ganglion cells in the retina via the retinohypothalamic tract (Do and Yau, 2010; Hastings et al., 2018). Serving as the central pacemaker, the SCN then relays this temporal information, synchronizing and coordinating the rhythmic activity of a hierarchy of subsidiary peripheral oscillators (e.g., liver, pancreas, gut, etc.) via temperature, hormonal, metabolic, and autonomic signaling (Mohawk et al., 2012; Hastings et al., 2018). While light is the strongest *zeitgeber,* German for "timekeeper," circadian rhythms can also be entrained by non-photic cues – such as temperature, food, exercise, stress, or even substances of abuse (Challet and Pévet, 2003; Mohawk et al., 2012; Gillman et al., 2019). Integrating both photic and non-photic cues from the environment, the SCN and its hierarchy of subsidiary oscillators work dynamically to drive consolidated diurnal rhythms in an organism's sleep/wake activity, behavior, and physiology.

Interestingly, circadian rhythms are also generated and maintained at the molecular level through a complex series of interlacing transcriptional-translational feedback loops (TTFLs). This 'molecular clock' not only drives diurnal rhythms in gene and protein expression through layers of transcriptional, post-transcriptional, translational, and post-translational regulation, but also

controls nearly all aspects of cellular physiology (Partch et al., 2014; Chaix et al., 2016; Takahashi, 2017). In mammals, the molecular clock exists in nearly all somatic cells and across all tissues (Welsh et al., 2004; Yoo et al., 2004; Zhang et al., 2014a; Mure et al., 2018). Through transcriptome-wide sequencing studies, it is estimated that nearly half the genome is rhythmic in rodents (Zhang et al., 2014a) and upwards of 80% in primates (Mure et al., 2018). Across dozens of species, many homologous transcription factors have been identified and characterized as the molecular basis of rhythm generation (Dunlap, 1999), serving similar roles by maintaining an oscillating feedback loop that controls aspects of cellular activity and function.

In mammals, the transcription factors CLOCK (circadian locomotor output cycles kaput) or its paralogue NPAS2 (neuronal PAS domain protein 2) and BMAL1 (brain and muscle ARNT-like protein 1; encoded by *Arntl*) are the key molecular clock proteins that drive the positive arm of a central or main feedback loop (**Figure 2**). First characterized in the late ’90s (Hogenesch et al., 1997; Ikeda and Nomura, 1997; King et al., 1997; Zhou et al., 1997), CLOCK, NPAS2, and BMAL1 are all basic helix-loop-helix (bHLH)-PAS domain-containing transcription factors that heterodimerize and bind to regulatory E-box elements to drive rhythmic expression of many clock-controlled genes (CCGs) – including *Period* (*Per1*, *Per2*, & *Per3*) and *Cryptochrome* (*Cry1* & *Cry2*). Throughout the day, the CLOCK/NPAS2:BMAL1 complex promotes the transcription and expression of the *Per* and *Cry* genes, producing an accumulation of PERs and CRYs in the cytoplasm (Gekakis et al., 1998). Into the night, PERs and CRYs heterodimerize and translocate back into the nucleus to inhibit the activity of CLOCK/NPAS2:BMAL1 and repress their own transcription – completing the negative arm of the main feedback loop (Shearman et al., 2000). This repression is relieved via targeted ubiquitylation-dependent proteasomal degradation of PERs and CRYs (Gallego and Virshup, 2007), thus allowing for the resumption of

CLOCK/NPAS2:BMAL1 activity. This core TTF cycles roughly every 24 hours and is critical for molecular clock function. (Takahashi, 2017)

In addition to the main negative feedback loop produced by CLOCK/NPAS2:BMAL1, there are several auxiliary or supporting TTFLs that help stabilize, increase robustness, and support overall rhythmicity. One notable auxiliary feedback loop consists of the clock-controlled rhythmic expression of RAR-related orphan receptor alpha (RORα) and reverse-ErbA alpha (REV-ERBα) nuclear receptors (encoded by *Nr1f1* and *Nr1d1*, respectively) which bind and compete at retinoic acid-related orphan receptor (ROR) binding elements to regulate the transcription of *Artnl* (i.e., *Bmal1*) and its downstream expression (Preitner et al., 2002; Sato et al., 2004; Akashi and Takumi, 2005). Notably, the REV-ERBα/RORα auxiliary feedback loop runs in antiphase with *Per/Cry* expression and is not only thought to strengthen the main feedback loop (Relógio et al., 2011; Pett et al., 2016), but has also been shown to be important itself for sustaining rhythmicity (Sato et al., 2004; Bugge et al., 2012; Cho et al., 2012). Other clock-driven auxiliary feedback loops include the competing regulation of RORα and PERs by D-box Binding Protein (DBP) and the Nuclear Factor Interleukin3-regulated protein (NFIL3; or E4BP4) (Yamaguchi et al., 2000; Mitsui et al., 2001), and also the more recently discovered rhythmic transcriptional repressor CHRONO (computationally highlighted repressor of the network oscillator; encoded by *Ciart*) regulating CLOCK/NPAS2:BMAL1 activity (Anafi et al., 2014; Goriki et al., 2014). Notably, the existence of multiple interlocking feedback loops creates a system of redundancy and resiliency to protect core clock function, whereby a single loss of any of the molecular clock proteins can be compensated for and does not entirely disrupt circadian molecular clock function and/or behavior rhythms (reviewed in (Partch et al., 2014). The only *single* clock protein shown to be critical to molecular clock function is BMAL1, in that mice lacking BMAL1 (*Bmal1*-/-) show a complete

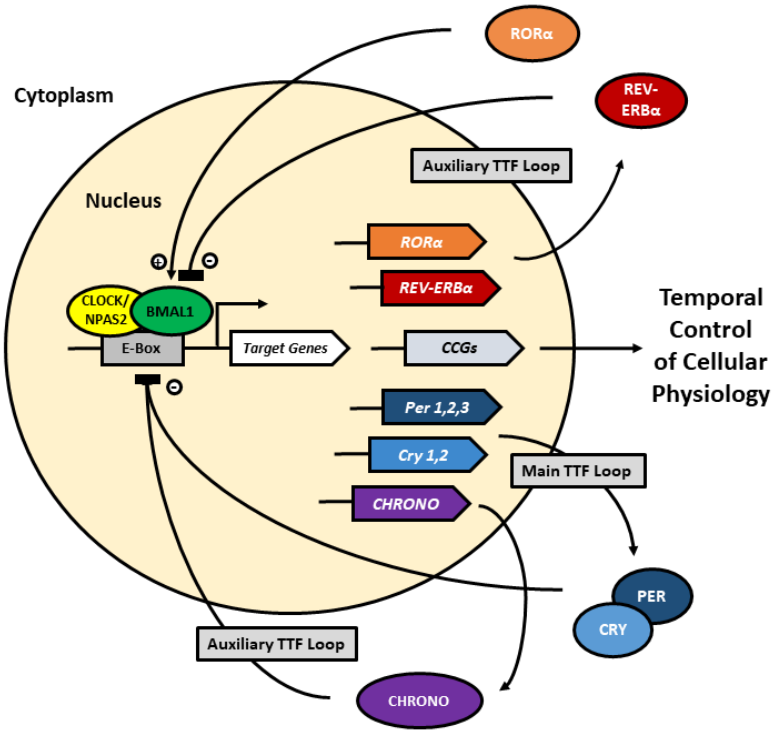

Figure 2. The mammalian circadian molecular clock drives endogenous rhythms. Rhythms are generated by multiple interlocking transcription and translation feedback (TTF) loops. Central to the TTF loops, the transcription factors CLOCK (or NPAS2) and BMAL1 heterodimerize and bind to the enhancer box (E-Box) sequence to promote transcription of many target and clock-controlled genes (CCGs). The main TTF loop is achieved when PERIOD (PER1,2,3) and CRYPTOCHROME (CRY1,2) proteins accumulate, dimerize, undergo phosphorylation, and shuttle back into the nucleus to inhibit both CLOCK/NPAS2:BMAL1 activity and, as a result, their own transcription. This negative feedback loop cycles roughly every ~24 hours and is crucial for the regulation of circadian rhythm. Among its target genes, CLOCK/NPAS2 : BMAL1 also regulates the expression of the nuclear receptors RORα and REV-ERBα, both of which can regulate BMAL1 activity via binding at a response element in its promoter. A recently discovered circadian protein, CHRONO, is clock-regulated and can also inhibit CLOCK/BMAL1 activity via interactions at the E-Box. Together, these proteins make up auxiliary TTF loops that work to both stabilize and reinforce both the main TTF and rhythmic output as a whole. Arrows and (+) indicate promote/activate; Bars and (-) indicate repress/inhibit. Adapted from (Becker-Krail and McClung, 2016).

lack of both molecular and behavioral rhythms (McDearmon et al., 2006). However, mice globally lacking *Bmal1* also show significantly disrupted metabolism, decreased body weight, reduced activity, and significantly shortened lifespan (Kondratov et al., 2006; McDearmon et al., 2006; Sun et al., 2006) – indicating the relative importance of the circadian molecular clock and overall circadian rhythms for physiology and behavior.

Taken together, the molecular clock is a dynamic and highly coordinated system of interlacing TTFLs that work to maintain temporal control of cellular physiology. This orchestration of rhythmicity is granted both by the abundance of cis-regulatory elements (CREs; e.g., E-boxes, D-boxes, and ROR-elements) throughout the genome (Ueda et al., 2005; Koike et al., 2012) and CLOCK's interaction with and own post-translational modification activity. In addition to interacting with histone acetyltransferases (HATs) like CREB-binding Protein (CBP) or p300 (Etchegaray et al., 2003), CLOCK itself has been shown to have intrinsic HAT activity itself to promote acetylation of proteins and histone H3 (Hirayama et al., 2007; Masri et al., 2013). The acetylation of histones unfurls tightly wound chromatin, providing access for recruitment of transcriptional machinery (including RNA polymerase II) and activity of the CLOCK/NPAS2:BMAL1 complex to drive transcription of CCGs (Etchegaray et al., 2003; Le Martelot et al., 2012). Additionally, the CLOCK complex has been shown to directly interact with the histone-deacetylase (HDAC) Sirtuin 1 (SIRT1) and the mixed-lineage leukemia (MLL1 & MLL3) histone methyltransferases (Nakahata et al., 2008; Katada and Sassone-Corsi, 2010; Valekunja et al., 2013) – adding another layer of post-translational control of chromatin state and thus circadian regulated transcription of CCGs. Ultimately, the circadian coordinated epigenetic regulation of chromatin state and subsequent circadian transcription regulation are what drive the great degree of rhythmicity seen across the genome and afford temporal control of physiology.

While the core mechanism in which the molecular clock regulates transcription of CCGs may be consistent across the organism, the CCGs themselves and their rhythmic expression vary in a cell-type and tissue-specific manner (Zhang et al., 2014a). This is especially true within the central nervous system (CNS), as CCGs can vary in a region-specific manner depending on the inherent cell-types and functional characteristics of the region – a phenomenon best demonstrated recently in the SCN (Wen et al., 2020). Notably, within the SCN, several recent studies have revealed an integral role for non-neuronal cell-types, namely astrocytes, in regulating circadian rhythms and downstream circadian regulated behaviors (Ruben and Hogenesch, 2017).

1.2.1 Astrocytes Regulate Circadian Rhythms

While astrocytes have been extensively shown to play integral roles in both supporting and modulating neuronal function in regions like the hippocampus and the striatum (Ota et al., 2013; Khakh, 2019), the role of astrocytes in the regulation of circadian rhythms has been largely underappreciated and understudied until recently. Like neurons, astrocytes also contain a circadian molecular clock (Prolo et al., 2005; Yagita et al., 2010; Chi-Castañeda and Ortega, 2016), and many essential astrocyte functions are regulated by core clock genes (Gwak et al., 2007; Beaulé et al., 2009; Marpegan et al., 2011). Interestingly, several recent studies have demonstrated astrocyte rhythmicity is also important for regulating overall circadian rhythmicity. First, a study from the Hastings lab demonstrated SCN astrocytes show anti-phasic rhythmicity relative to SCN neurons, whereby they suppress neuronal activity during the night via regulation of extracellular glutamate (Brancaccio et al., 2017). This anti-phasic relationship was found to be crucial for not only regulating SCN circadian timekeeping, but also circadian patterns of locomotor activity in mice

(Brancaccio et al., 2017). These findings were further supported by a study from the Herzog lab, in which they demonstrated loss of *Bmal1* specifically in SCN astrocytes altered circadian rhythmicity of both SCN clock gene expression (*in vitro* and *in vivo*) and wheel-running activity in mice (Tso et al., 2017). Most notably, circadian astrocyte function in the SCN may also regulate both rhythmicity and function of regions *outside* of the SCN. In addition to showing altered SCN rhythmicity and wheel running, another study demonstrated loss of *Bmal1* in SCN astrocytes both alters GABA signaling and significantly dampens molecular clock rhythmicity in cortical and hippocampal regions (Barca-Mayo et al., 2017). Strikingly, these mice also displayed significant memory impairments across short-term, long-term, and spatial memory tasks (Barca-Mayo et al., 2017). While the exact mechanism by which these changes occur still remains to be elucidated, more recent findings suggest a loss of *Bmal1* in astrocytes significantly alters the morphology of hippocampal astrocyte processes, potentially disrupting localization and function at synapses (Ali et al., 2020). Moreover, BMAL1 itself may regulate astrocyte activation as measured by GFAP (Lananna et al., 2018; Ali et al., 2020), and loss of *Bmal1* in astrocytes suppresses astrocyte metabolic-related processes like glutathione-S-transferase signaling and glucose homeostasis (Lananna et al., 2018; Barca-Mayo et al., 2020). However, while these findings begin to shed light on the importance of astrocyte rhythmicity, future investigation is still necessary to understand how exactly circadian astrocyte function is necessary both for the astrocyte itself and within a specific regional context.

Taken together, these studies highlight a novel role for astrocytes in the regulation of circadian rhythms through their dynamic interaction with neurons in the SCN. However, the importance of astrocyte circadian functions outside of the SCN has yet to be investigated. More specifically, whether astrocytes exhibit a similar anti-phasic relationship with neurons in other

regions of the brain is unknown. Of particular interest, no studies to date have investigated NAc astrocyte rhythmicity and/or its potential role in the known association between circadian rhythm disruptions and the pathophysiology of SUDs (McClung, 2007; Logan et al., 2014).

1.3 Circadian Rhythms and SUDs

In the past couple of decades, a growing body of evidence suggests a link between disruptions in circadian rhythms and the development or perpetuation of SUDs. For example, individuals with a SUD often present with significantly impaired sleep/wake patterns, e.g., disruptions to sleep onset, duration of sleep, and/or sleep quality (Angarita et al., 2016; Gates et al., 2016; Koob and Colrain, 2020). Moreover, substance abuse has been shown to disrupt other circadian rhythms, including the diurnal variation of core body temperature, melatonin, cortisol, adrenocorticotropic hormone (ACTH), β-endorphins, testosterone, and other hormones (Bolelli et al., 1979; Vescovi et al., 1992; Danel et al., 2001; Rupp et al., 2007; Conroy et al., 2012). These disruptions have been associated with acute substance use, chronic substance use, and even withdrawal from substance use (Hasler et al., 2012a; Angarita et al., 2016). Interestingly, this connection between circadian rhythm disruption and SUDs is also thought to be bidirectional. For example, those with sleep problems often have an increased propensity to abuse substances (likely as a form of self-medication) and develop a SUD (Breslau et al., 1996; Brower et al., 2001; Johnson and Breslau, 2001; Dolsen and Harvey, 2017; Goodhines et al., 2019). This is hypothesized to be due to desynchronization of circadian rhythms through "social jet lag" or the discrepancy/misalignment of biological rhythms with one's daily "social clock" determined by

work, school, and/or other social obligations (Roenneberg et al., 2003; Wittmann et al., 2006). This often takes form as a weekly alternating between sleep cycles due to school or shift work disrupting sleep during the week, followed by sleeping in or "catching up" on sleep during the weekend. This weekly disturbance is thought to be particularly detrimental during adolescence, as the discrepancy between school start times and preferred sleep schedule is associated with increased drug and alcohol use (Hasler et al., 2012b, 2015; Hasler and Clark, 2013; Touitou, 2013; Haynie et al., 2018; Logan et al., 2018). Interestingly, the natural shift toward an evening preference/chronotype, or "night owls," during adolescence also coincides with the vulnerability period in which people are more likely to develop SUDs and other psychiatric disorders (Hasler and Clark, 2013; Gulick and Gamsby, 2018; Logan et al., 2018). Other studies have also shown more generally that "night owls" show greater substance use and a higher likelihood of developing a SUD and/or other psychiatric disorders (Adan, 1994; Broms et al., 2011; Kivelä et al., 2018; Hug et al., 2019). Finally, polymorphisms or variants in core circadian molecular clock genes have also been shown to correlate with increased substance use in humans. Several studies have identified single nucleotide polymorphisms (SNPs) and variants in the *ARNTL*, *PER,* and *CLOCK* genes to be associated with increased cocaine use, alcohol consumption, and reward dependence (Spanagel et al., 2005; Comasco et al., 2010; Kovanen et al., 2010; Sjöholm et al., 2010; Dong et al., 2011; Shumay et al., 2012; Tsuchimine et al., 2013; Bi et al., 2014). While these studies offer a glimpse into how core molecular clock genes may play a role in SUD relevant behavior in humans, much of our understanding of how reward is regulated in the brain and how molecular clock function directly impacts this regulation has come from pre-clinical translational research in mice.

1.3.1 Circadian Regulation of Reward and the Effects of Drugs of Abuse

Through animal model studies, we have long appreciated the circadian regulation of reward processing in the NAc and other reward regions of the brain (Parekh and McClung, 2015; DePoy et al., 2017). At the behavioral level, decades of work has characterized diurnal variations in reward sensitivity, locomotor sensitization, conditioned place preference, and operant self-administration of drugs of abuse, with peaks being in the animal's active phase (Webb et al., 2015). This is also true for natural reward, in that daily patterns of food intake are under circadian regulation and mostly overlap with the animal's active phase (Challet, 2019). While this is partly explained by the fact that both food and drugs of abuse are strong zeitgebers that entrain rhythms (Gillman et al., 2019), the reward circuity itself possesses functioning molecular clocks, and its activity is tightly regulated by the circadian system. For example, our lab has previously demonstrated whole NAc rhythmicity through both luminescence rhythms using *Per2:Luc* mice (Logan et al., 2015) and diurnal variation in the expression of core molecular clock genes *Clock*, *Npas2*, and *Per1,2,3* in mouse NAc tissue (Falcon et al., 2013). Further supporting these studies, a time of death analysis of gene expression in human post-mortem tissue revealed many of the canonical molecular clock genes (e.g., *Bmal1*, *Per1,2,3*, *Npas2*, etc.) were highly rhythmic in the NAc of healthy controls (Li et al., 2013). In addition to the molecular clock being rhythmically expressed in the NAc, nearly all aspects of mesolimbic function at the cellular and molecular levels are under circadian influence and display circadian variation (**Figure 3**) (reviewed in (Webb et al., 2015).

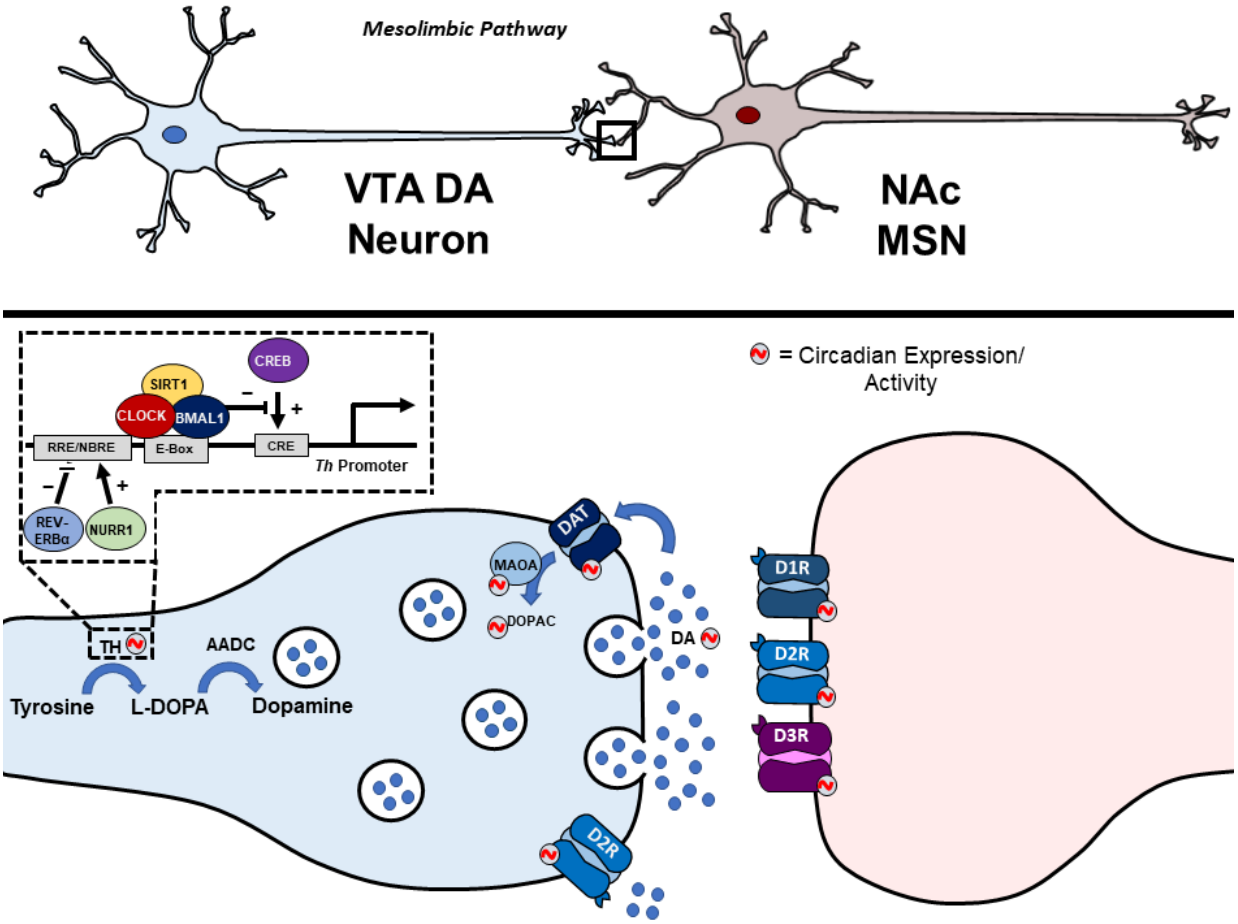

Figure 3. The circadian molecular clock regulates dopamine neurotransmission. Clock genes regulate components of dopaminergic transmission within the primary reward pathway, the mesolimbic pathway. The mesolimbic pathway consists of DA neurons in the VTA projecting to and synapsing on MSNs in the NAc. Components of the DA synapse involved in the synthesis, uptake, and degradation of DA show circadian rhythms in expression and/or activity, including TH, monoamine oxidase A (MAOA), DAT, and dopamine receptors type 1 (D1R), type 2 (D2R), and type 3 (D3R). *Tyrosine hydroxylase* transcription is activated by cAMP response element-binding protein (CREB)-mediated binding to CRE sites in the *Th* promoter. The CLOCK/BMAL1 complex interacts with the histone and protein deacetylase, Sirtuin 1 (SIRT1), to repress CREB-induced *Th* transcription in a time-of-day dependent manner. There are also circadian rhythms in DA levels itself and the DA metabolite DOPAC; Arrows and (+) indicate promote/activate, Bars and (-) indicate repress/inhibit; Modified and adapted from (Ketchesin et al., 2020).

In rodents, levels of DA, glutamate, GABA, and their metabolites all show diurnal rhythms in the NAc (Castañeda et al., 2004; Ferris et al., 2014; Alonso et al., 2020). The neuropeptide cholecystokinin (CCK), known to modulate DA release and activity, also shows diurnal variation and is directly regulated by the molecular clock (Schade et al., 1995; Rotzinger and Vaccarino, 2003; Arey et al., 2014). Moreover, work from our lab and others have demonstrated diurnal variations in the expression of DA receptors and DAT, as well as levels/activity of tyrosine hydroxylase (TH) and monoamine oxidase A (MAOA), the enzymes respectively involved in the synthesis and degradation of DA (**Figure 3**) (Sleipness et al., 2007; Hampp et al., 2008; Chung et al., 2014; Ozburn et al., 2015; Logan et al., 2019). Interestingly, the genes *Th*, *Dat*, *Maoa*, and *Drd1,2,3* have all been shown in rodents to be directly regulated by the circadian molecular clock through knockdown studies and/or the identification of circadian CREs (e.g., E-boxes or ROR-elements) in the genes' promoter regions (Ueda et al., 2005; Hampp et al., 2008; Ikeda et al., 2013; Chung et al., 2014; Ozburn et al., 2015). This is particularly relevant given that our lab and others have shown substances of abuse, like psychostimulants and alcohol, lead to upregulation or disrupted diurnal variation of core clock gene expression (e.g., *Clock, Npas2, Bmal1, Cry1*, *Per2*, etc.) in the striatum and mesocortical areas (Lynch et al., 2008; Perreau-Lenz and Spanagel, 2008, 2015; Falcon et al., 2013).

In addition to the molecular clock regulating reward neurophysiology, studies investigating genetic knockouts, functional mutations, and/or reward-region-specific knockdowns of core circadian clock genes have illuminated the functional importance of the molecular clock in reward-related behaviors. Extensive work from our lab and others have shown the core clock genes, including *Clock* and *Npas2*, play a significant role in regulating reward through their expression

in reward-regions of the brain. Interestingly, mice carrying a functional mutation in the transactivational domain of *Clock*, the *Clock*Δ19 mouse model (King et al., 1997), exhibit a hyper-hedonic behavioral phenotype. Relative to WT mice, *Clock*Δ19 mice show increased cocaine preference, increased cocaine locomotor sensitization, and both increased TH expression and DA neuron excitability in the VTA (McClung et al., 2005; Roybal et al., 2007). Further corroborating this finding, *Clock*Δ19 mice trained in a translational operant intravenous self-administration (IVSA) task also show increased cocaine self-administration, increased motivation to self-administer, increased propensity to initiate use, and increased sensitivity to reinforcing properties of cocaine, relative to WT mice (Ozburn et al., 2012). In addition to increased cocaine reward, *Clock*Δ19 mice show increased locomotor response to novelty, increased exploratory drive, increased preference for sucrose, increased ethanol intake in a two-bottle choice task, and increased propensity (Roybal et al., 2007; Ozburn et al., 2013). Notably, many of these findings were recapitulated through a VTA-specific knock-down of *Clock* (Mukherjee et al., 2010; Ozburn et al., 2013) – highlighting the integral role CLOCK plays in regulating reward through its expression and role in mesolimbic circuitry, particularly in the VTA.

1.3.2 NPAS2 is Highly Expressed in the NAc and Regulates Reward

Of particular interest for reward-regulation, our lab has been studying the differential regulation of reward between CLOCK and its paralog NPAS2. Structurally and functionally similar to CLOCK, NPAS2 also binds to BMAL1 to drive the expression of clock genes *Per* and *Cry*, and can functionally compensate in conditions where CLOCK is nonfunctional or lower in abundance (Reick et al., 2001; DeBruyne et al., 2007; Bertolucci et al., 2008; Landgraf et al.,

2016). This functional compensation is further evidenced by the fact that knockout mice of *Clock* and *Npas2* separately still maintain consolidated rhythmic activity, but double knockouts of both *Clock* and *Npas2* completely abolishes activity rhythms (DeBruyne et al., 2007), similarly seen with *Bmal1* knockouts (McDearmon et al., 2006). Notably, though CLOCK and NPAS2 are functionally similar in the context of core circadian function, their expression patterns set them apart. While CLOCK is ubiquitously expressed, NPAS2 is thought to primarily be expressed in nervous tissue with enriched expression in the forebrain (Zhou et al., 1997; Garcia et al., 2000; Reick et al., 2001). More specifically, NPAS2 is highly expressed in the NAC and particularly enriched in the reward-promoting D_1-receptor containing MSNs (**Figure 4**) (Garcia et al., 2000; Ozburn et al., 2015). This unique expression pattern seemed to position NPAS2 to play a role in reward-related behavior, and thus motivated its functional characterization both by our lab and others.

The first functional knockout studies of *Npas2* found that *Npas2* mutant mice demonstrated impaired cue and contextual memory and an impaired ability to acquire food anticipatory behavior in response to food restriction (Garcia et al., 2000; Dudley et al., 2003; Wu et al., 2010). Following these studies, work from our lab looked to characterize the role of NPAS2 in reward processing and reward-related behavior. Interestingly, *Npas2* null mutant mice show decreased cocaine preference in the conditioned place preference (CPP) task, a measure of reward and a drug's reinforcing effects (Ozburn et al., 2015). Notably, this decrease in cocaine preference was recapitulated through NAc specific viral-mediated knockdown of *NPAS2*, but not *Clock*, and is thought to be attributed to *Npas2*'s restricted expression in D_1-receptor containing MSNs and regulation of the DA receptor expression (Ozburn et al., 2015). Recent follow-up studies further confirmed these findings in that cell-type-specific viral-mediated knockdown of *Npas2* in D_1-

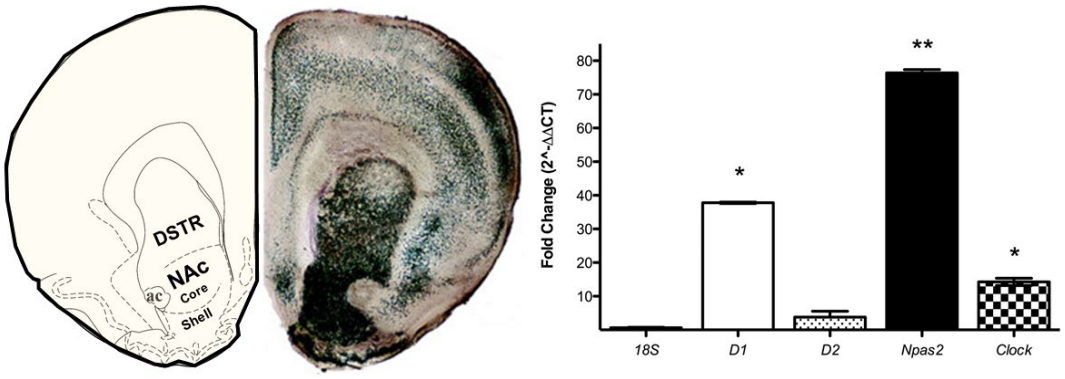

Figure 4. NPAS2 is highly enriched in D_1-receptor containing MSNs in the NAc. Original studies from the McKnight lab determined NPAS2 to be highly enriched in the forebrain, specifically in the NAc, through light microscope imaging of β-Gal expression in NPAS2-lacZ mice. Work from our lab expanded on these findings utilizing tissue from GENSET *Drd1-Td-Tomato* mice and fluorescence-activated cell sorting (FACS) to isolate D1-receptor positive and negative MSNs in the NAc. Reverse transcriptase quantitative PCR (RT-qPCR) for *Drd1*, *Drd2*, *Npas2*, and *Clock* revealed *Npas2* was highly enriched in D_1-containing MSNs relative to *Clock* and *Drd2*. Figure adapted and modified from (Garcia et al., 2000; Ozburn et al., 2015).

receptor containing MSNs in the NAc recapitulated the decreased cocaine preference phenotype, but not D_2-specific knockdown (Parekh et al., 2019). NAc specific knockdown of *Npas2* in mice also increased excitatory drive onto D1-receptor containing MSNs and increased excitatory synaptic strength, as measured by mini excitatory postsynaptic current (mEPSC) amplitude and frequency, and the AMPA /NMDA glutamate receptor ratio, respectively (Parekh et al., 2019). Conversely, in a recent unpublished study from our lab, *Npas2* mutant mice trained in a cocaine IVSA task surprisingly demonstrated a significantly greater propensity to self-administer, greater cocaine intake, greater reinforcement, and greater motivation to self-administer cocaine, relative to WT mice (DePoy et al., 2020). Moreover, these differences were observed in a sex- and circadian-dependent manner, with both males and females showing these increases during the

light/inactive phase and females exhibiting a greater phenotype in the dark/active phase. Further supporting these self-administration findings, *Npas2* null mutant mice also show increased locomotor response to novelty and increased exploratory drive (Ozburn et al., 2017). While it might seem counterintuitive, it is not unusual to see these divergent behavioral effects. Studies investigating the transcription factor cAMP response element-binding protein (CREB) in the NAc have also found opposite cocaine CPP and cocaine IVSA behaviors in rodents (Carlezon et al., 1998; Larson et al., 2011). This is because CPP and self-administration are measuring and modeling fundamentally discrete features of addiction-like behavior. While cocaine CPP measures the interoceptive, subjective, and pleasurable effects of passive cocaine exposure, the cocaine IVSA model measures active, volitional, chronic drug intake, as well as both the reinforcing and motivational properties of cocaine. Considering the limitations of cocaine CPP as it translates to SUDs (Bardo and Bevins, 2000), it is worth highlighting many studies have found a significant correlation between increased locomotor response to novelty / exploratory drive and substance abuse vulnerability (Piazza et al., 1989; Hooks et al., 1991; Stead et al., 2006; Flagel et al., 2010, 2014; Zhou et al., 2019b) – which is further supported by the *Npas2* mutant mouse phenotype.

Taken together, both NPAS2's enriched expression in the NAc and complex role in regulating reward-related behavior underscores its functional relevance and warrants further investigation into mechanisms underlying NPAS2's role in the NAc. One potential mechanism of interest is through interactions between the circadian molecular clock, cellular metabolic state, and the reward system.

1.4 Metabolic State at the Interface of Molecular Clock Function and Reward Regulation

As the primary rewarding and reinforcing mechanism, drugs of abuse acutely lead to increased DA signaling of the mesolimbic pathway and increased extracellular DA in the NAc (Feltenstein et al., 2020). With increased and sustained overactivation of this circuit, the metabolic needs of the cells can become too demanding and lead to oxidative stress. There is significant evidence to suggest substances of abuse, especially cocaine and amphetamine, significantly alter the regulation of cellular metabolic state and lead to increased oxidative stress and damage (Cunha-Oliveira et al., 2013; Uys et al., 2014; Jang et al., 2015; Pavlek et al., 2020). This altered cellular metabolic state may play a direct role in the development and/or perpetuation of addiction-like behavior, in that astrocytes and the circadian molecular clock both respond to and directly regulate cellular metabolic state (Bailey et al., 2014; Freyberg and Logan, 2018; Deitmer et al., 2019), while also playing significant roles in the processing of reward and reward-related behavior.

1.4.1 Astrocytic Regulation of Cellular Metabolic State and its Implications for Reward

At the cellular level, astrocytes play an integral role in regulating cellular metabolic state through "neurometabolic coupling" – a process by which astrocytes detect the activity of neurons through levels of glutamate and in response support their activity through shuttling of lactate (a source of energy) and/or glutathione (GSH; an antioxidant) for neuronal uptake (Bélanger et al., 2011; Bolaños, 2016; Gonçalves et al., 2018). In mammals, glucose serves as the primary energy source for the brain; however, it must be converted into pyruvate via glycolysis before it can be utilized in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) to

produce adenosine triphosphate (ATP), the primary energy substrate in biochemistry. In the brain, astrocytes play an integral role in utilizing glycolysis to produce lactate, a metabolic substrate that can be readily interconverted to pyruvate by the enzyme lactate dehydrogenase A (LDHA). Notably, lactate serves as the primary feeder of the TCA cycle, in that neurons convert lactate to pyruvate for the subsequent TCA cycle and OXPHOS mediated generation of ATP. (Magistretti and Allaman, 2018; Rabinowitz and Enerbäck, 2020). The astrocyte-to-neuron lactate shuttle (ANLS) hypothesis proposes that increased neuronal activity and subsequent glutamate uptake by astrocytes triggers astrocytic intake of glucose from blood capillaries, increased production of lactate from glycolysis, and then increased shuttling of lactate via monocarboxylate transporters (MCTs) to neurons (**Figure 5**) (Pellerin and Magistretti, 1994, 2012; Magistretti and Pellerin, 1996; Magistretti and Allaman, 2018). While some debate exists as to whether astrocytes are the sole contributors of glycolysis-derived lactate in the brain (Dienel, 2012; Díaz-García et al., 2017), there is extensive evidence to demonstrate astrocytes play a critical role in this process nonetheless (Magistretti and Allaman, 2018).

In addition to providing neurons with lactate to meet their energetic demands, astrocytes also shuttle glutathione (GSH) to help neurons combat reactive oxygen species (ROS) accumulation and oxidative stress (Bolaños, 2016). Much like the ANLS hypothesis, it is thought that activation of nuclear factor erythroid-related factor 2 (NRF2) in astrocytes, the master antioxidant transcription factor, is coupled with glutamatergic neurotransmission of neurons whereby glutamate triggers activation of the NRF2 antioxidant signaling pathway (**Figure 5**) (Habas et al., 2013; Jimenez-Blasco et al., 2015; Bolaños, 2016; McGann and Mandel, 2018). NRF2 regulates the expression of enzymes crucial for GSH biogenesis (GCLC, GCLM, GSH synthetase, etc.) and other antioxidants like HO-1, NAD(P)H Quinone Dehydrogenase 1 (N

QO1), and superoxide dismutase (SOD) (Ma, 2013; Baxter and Hardingham, 2016; Tu et al., 2019). GSH primarily acts to remove ROS (e.g., hydrogen peroxide, superoxide, etc.) that would otherwise damage DNA, RNA, proteins, and lipids, also known as oxidative stress (Schieber and Chandel, 2014); this is achieved by GSH reducing ROS to water and/or oxygen through its oxidation into glutathione disulfide (GSSG), which is later converted back to GSH (O'Donovan and Fernandes, 2000). Interestingly, a recent study found the NRF2-antioxidant signaling pathway may also be coupled with the ANLS process, in that lactate itself may be able to activate the NRF2 pathway and other pro-survival pathways to help maintain metabolic homeostasis (Tauffenberger et al., 2019). Taken together, the regulation of lactate and GSH by astrocytes underscores their functional significance in the maintenance of both metabolic functions and overall neuronal function.

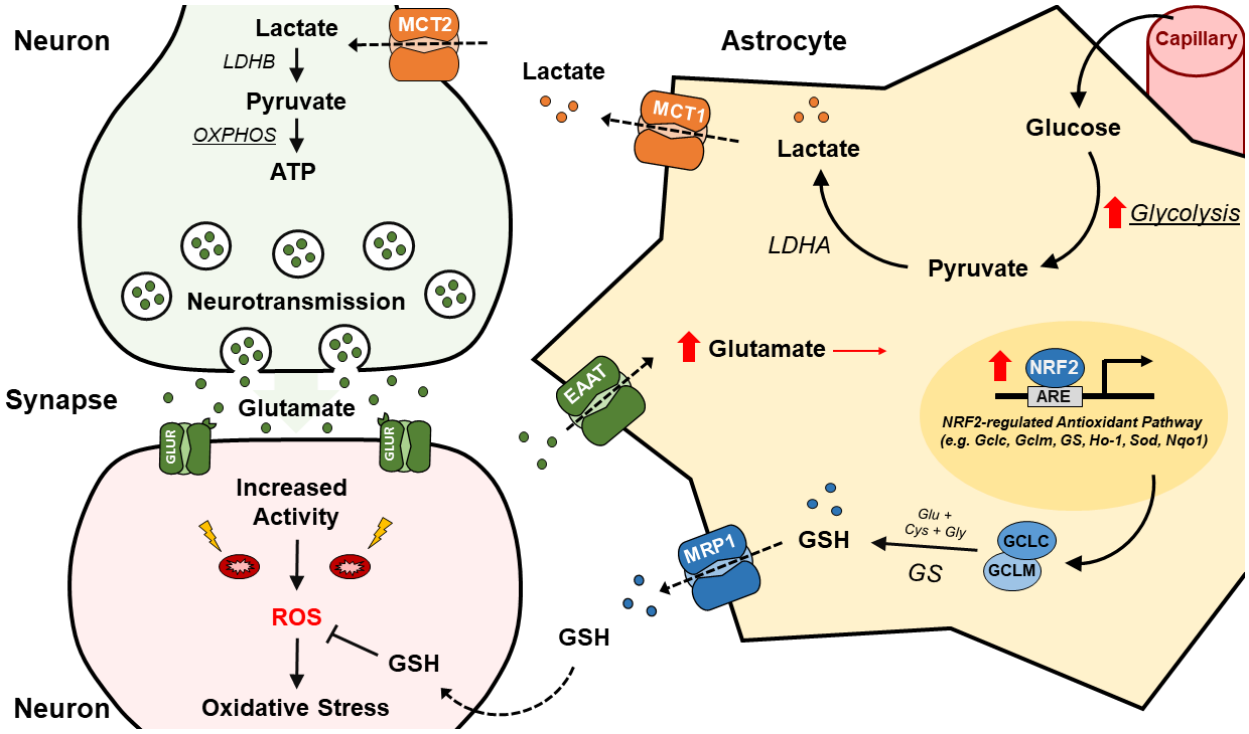

Figure 5. Astrocytes support neuronal energetic demands and mitigate oxidative stress. Activation of presynaptic neurotransmission leads to the release of glutamate from neurons into the synapse, thus activating post-synaptic neurons through the binding of glutamate receptors (GLUR). The uptake of glutamate by astrocytes through excitatory amino acid transporters (EAATs; e.g., Glutamate Transporter 1, GLT-1) stimulates increased glucose intake from blood capillaries and subsequent upregulation of glycolysis. Pyruvate generated from glycolysis is converted to lactate via lactate dehydrogenase A (LDHA) before being shuttled out into the synapse through monocarboxylate transporter 1 (MCT1) or MCT4 (not pictured). Lactate is then taken up by neurons through the neuron-specific MCT2 to be used to generate its own energy. Lactate in neurons is converted to pyruvate via LDHB to feed the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS) that in turn generate adenosine triphosphate (ATP), the primary energy molecule of the cell. This ATP is used to fuel continued synaptic transmission, intracellular signaling, membrane transport, enzymatic activity, DNA/RNA synthesis, etc. In addition to lactate shuttling, the uptake of glutamate by astrocytes also activates the nuclear factor erythroid 2-related factor 2 (NRF2) antioxidant pathway, crucial for the biogenesis and shuttling of glutathione (GSH), one of the primary antioxidants of the cell. Activation drives NRF2-mediated transcription through binding of antioxidant response elements (ARE) of antioxidant genes like heme oxygenase 1 (*Ho-1*), superoxide dismutase (*Sod*), NAD(P)H dehydrogenase quinone 1 (*Nqo1*), as well as

several enzymes integral for GSH synthesis. Glutamate-cysteine ligase (GCLC and GCLM) and glutathione synthetase (GS) convert the amino acids glutamate (Glu), cysteine (Cys), and glycine (Gly) into GSH before being shuttled out into the synapse by multidrug resistance protein 1 (MRP1). This GSH is used by neurons to quench increased reactive oxygen species (ROS) generated from neuronal activation and increased mitochondrial activity. If untreated, ROS would otherwise damage proteins, DNA/RNA, and lipids, also known as oxidative stress. Together, neuron activity coupled shuttling of astrocyte-derived lactate and GSH are integral to the maintenance of neurometabolic homeostasis and normal neuron functioning.

Notably, both lactate production and GSH biogenesis are under direct circadian regulation, in that the molecular clock has been shown to regulate the expression of LDHA, MCTs, NRF2, GCLC, GCLM, and other factors (Rutter et al., 2001; Musiek et al., 2013; Pekovic-Vaughan et al., 2014; Henriksson et al., 2017). Given that production of lactate and GSH are known to be coordinated with glutamate uptake, their diurnal variation may be further regulated by astrocytes' circadian-timed uptake of glutamate for timekeeping seen in the SCN (Brancaccio et al., 2017). Most notably, this circadian regulation results in diurnal variation in both lactate and GSH levels in the brain (Kinoshita et al., 2014; Wallace et al., 2020). While studies have shown either blocking lactate shuttling or treatment with ROS scavengers can attenuate cocaine CPP and self-administration in rodents(Numa et al., 2008; Jang et al., 2015; Boury-Jamot et al., 2016a, 2016b; Zhang et al., 2016), no studies to date have investigated how circadian regulation of astrocytes and their metabolic functions in the NAc may play a role in reward regulation.

1.4.2 Interactions Between the Circadian Molecular Clock and Metabolic State: Implications for Reward

At the molecular level, the circadian molecular clock both regulates and receives feedback from the redox state of the cell. The ratio of NAD+/NADH serves as a key measure of cellular metabolic homeostasis in that many metabolic processes in the cell (e.g., glycolysis, the TCA cycle, oxidative phosphorylation, etc.) utilize NAD+ and NADH to transfer hydrogen/electrons (Ying, 2008; Houtkooper et al., 2010; Cantó et al., 2015) - specifically relevant for the aforementioned interconversion of lactate and pyruvate that depend on this ratio (Rabinowitz and Enerbäck, 2020). Nicotinamide adenine dinucleotide (NAD) is a cofactor that is central to cellular metabolism and exists in both an oxidized (NAD+) and reduced (NADH) state. In mammals, NAD+ is either synthesized *de novo* from the amino acid tryptophan or salvaged/recycled from nicotinamide (NAM) by nicotinamide phosphoribosyltransferase (NAMPT), the rate-limiting enzyme in the conversion of nicotinamide mononucleotide (NMN) to NAD+ (Houtkooper et al., 2010; Cantó et al., 2015). The gene that encodes NAMPT (*Nampt*) has been shown to be under direct circadian regulation by the core circadian heterodimer CLOCK:BMAL1 and exhibit robust diurnal variation, and by consequence levels of NAD+ also exhibit robust rhythms (Nakahata et al., 2009; Ramsey et al., 2009; Peek et al., 2013; Logan et al., 2019). This regulation of metabolic state by the molecular clock is further evidenced by *Bmal1*-/- mice exhibiting significant oxidate stress, induction of redox response pathways, and increased astrocyte reactivity (Musiek et al., 2013). Interestingly, levels of NAD+ and NADH can feedback and directly regulate the DNA binding activity of NPAS2, whereby low NAD+/NADH levels or high NADH enhance DNA binding of NPAS2:BMAL1(Rutter et al., 2001; Dioum et al., 2002; Yoshii et al., 2015). NPAS2

and CLOCK can also bind heme as a prosthetic cofactor, directly tying its transcriptional activity to the levels of carbon monoxide (CO) – a byproduct created when the antioxidant enzyme heme oxygenase 1 (HO-1) utilizes NADH to breakdown heme (Dioum et al., 2002; Wu and Wang, 2005; Araujo et al., 2012; Klemz et al., 2017; Minegishi et al., 2018). In addition to the core clock genes acting directly as redox sensors themselves, the NAD+ dependent histone and protein deacetylase SIRT1 may also serve to integrate metabolic state with molecular clock function.

In mammals, SIRT1 has been extensively characterized to be a master regulator of cellular metabolic homeostasis and oxidative resilience (Cantó and Auwerx, 2012; Boutant and Cantó, 2014). Interestingly, SIRT1's activity is directly coupled with the redox state of the cell through its dependence on NAD+ for activity as a deacetylase and can shuttle between the nucleus and the cytoplasm to regulate both transcription and protein function. In response to changes in NAD+ driven by oxidative stress or other cellular stressors, SIRT1 activation can regulate mitochondrial function, antioxidant production, and/or inflammation response via interacting with and deacetylating key transcription factors like PGC1-α, FoxOs, NF-κ, etc. (Li, 2013; Salminen et al., 2013; Xu et al., 2018). Studies from Sassone-Corsi, Bass, and Schibler have provided compelling evidence demonstrating SIRT1 to connect cellular metabolism to the molecular clock through directly regulating the activity and amplitude of circadian clock-controlled gene expression. In the liver, this is achieved by SIRT1 interacting with the CLOCK:BMAL1 complex to regulate its activity through deacetylation of BMAL1 or deacetylation of PER2, promoting its degradation (Asher et al., 2008; Nakahata et al., 2008; Ramsey et al., 2009). Moreover, this function was found to be necessary for proper circadian transcription (Hirayama et al., 2007; Asher et al., 2008; Nakahata et al., 2009). This interaction is particularly interesting, given SIRT1's role in regulating reward and reward-related behavior.

While much of SIRT1's role in regulating metabolic processes has been characterized in the liver, SIRT1 in the brain has been shown to play an integral role in the hippocampus, hypothalamus, and in the NAc (Herskovits and Guarente, 2014; Fujita and Yamashita, 2018). Work from the Nestler lab and colleagues first identified SIRT1 in the NAc to be upregulated following chronic cocaine exposure through binding of ΔFosB at its promoter (Renthal et al., 2009), a transcriptional regulator extensively implicated in the development of addiction-like behavior (Nestler et al., 2001; Nestler, 2008; Ruffle, 2014). In addition to identifying increased acetylation at histone H3 on *Sirt1* promoters, mice exposed to chronic cocaine also showed significant upregulation of *Sirt1* mRNA and SIRT1 enzymatic activity in the NAc. In acute NAc slice preps, administration of SIRT1 modulators, resveratrol (agonist) or sirtinol (antagonist), directly increased or decreased electrical excitability of NAc MSNS, respectively. Most notably, administration of resveratrol significantly increased cocaine CPP in mice, while sirtinol significantly decreased cocaine CPP and cocaine IVSA. (Renthal et al., 2009) Follow-up studies by Ferguson and colleagues further confirmed SIRT1 mRNA and protein induction following chronic cocaine exposure, and NAc specific viral-mediated overexpression of *Sirt1* significantly increased cocaine CPP, cocaine locomotor sensitization, and induction of both synaptic plasticity genes and dendritic spine density (Ferguson et al., 2013). Interestingly, work from our lab recently demonstrated these effects might partly be driven by an interaction between SIRT1 and the molecular clock, in that CLOCK and SIRT1 together regulate mesolimbic function through a bidirectional regulation of *Th* transcription in the VTA. Viral-mediated overexpression of SIRT1 in the VTA or activation of SIRT1 by the administration of either resveratrol or the NAD+ precursor NMN significantly increases *Th* expression in the VTA and increases cocaine CPP in wild-type mice; however, lack of a functional CLOCK (e.g., *Clock*Δ19 mutation) abolished these

effects. (Logan et al., 2019) This is further supported by findings demonstrating NAMPT, SIRT1, NAD+, and NMN are all significantly upregulated in the VTA following cocaine exposure, and VTA-specific NAMPT inhibition significantly attenuates cocaine CPP in WT mice that is dampened in SIRT1 VTA knockout mice (Kong et al., 2018). While these latter findings highlight an intriguing mechanism by which SIRT1 regulates reward through an interaction with the molecular clock in the VTA, such an interaction between SIRT1 and NPAS2 remains to be investigated in the NAc.

2.0 Dissertation Aims

The studies contained within this dissertation investigate the interaction between the circadian molecular clock and cellular metabolic, with a specific interest in the role of NPAS2 and SIRT1 in the NAc together may play in reward regulation. Moreover, this dissertation seeks to uncover the role of circadian astrocyte function in the NAc in regulating both reward and relevant metabolic processes. The central hypothesis of this dissertation is that reward is regulated in the NAc through interactions between the circadian molecular clock and cellular metabolic state, which is mediated at both the cellular level by astrocytes and at the molecular level by circadian interfacing redox sensors. This hypothesis is tested through the following experimental aims:

Aim 1: Determine cocaine's effects on molecular clock function and redox balance in the NAc, and whether NPAS2 and SIRT1 interact in the NAc to regulate reward-related behavior.

Aim 2: Determine the circadian regulation of astrocytes in the NAc.

Aim 3: Determine if circadian rhythmicity of astrocytes is important for overall NAc function and/or regulation of cellular metabolic state.

3.0 Circadian Transcription Factor NPAS2 and NAD+-Dependent Deacetylase SIRT1 Interact in the Mouse Nucleus Accumbens to Regulate Cocaine Preference

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(Submitted: *Frontiers in Molecular Neuroscience*)

3.1 Overview

Accumulating evidence from both clinical and pre-clinical studies suggests substance use disorders (SUDs) are associated with disruptions to both circadian rhythms and cellular metabolic state. At the molecular level, the circadian molecular clock and cellular metabolic state may be interconnected through interactions with the nicotinamide adenine dinucleotide (NAD+) dependent deacetylase, Sirtuin 1 (SIRT1). In the nucleus accumbens (NAc), a region important for reward and motivation, both SIRT1 and the core circadian transcription factor neuronal PAS domain protein 2 (NPAS2) are highly enriched, and both have been shown to be directly regulated by the concentration of the metabolic cofactor NAD+. Substances of abuse, like cocaine, can significantly disrupt cellular metabolism and promote oxidative stress; however, their effects on NAD+ in the brain remain unclear. Interestingly, cocaine can also increase the expression of both NPAS2 and SIRT1 in the NAc, and both have independently been shown to regulate cocaine reward in mice. However, whether NPAS2 and SIRT1 interact in the NAc and/or whether together

they regulate cocaine reward is unknown. In the present study, we demonstrate that expression of *Npas2*, *Sirt1*, and NAD+ exhibit diurnal variation in the NAc, which is disrupted by cocaine exposure, primarily driven by an upregulation across time of day. Additionally, co-immunoprecipitation studies reveal NPAS2 and SIRT1 interact in the NAc, and a cross-analysis of NPAS2 and SIRT1 chromatin immunoprecipitation sequencing (ChIP-seq) studies reveal several reward-relevant and metabolic-related pathways enriched among their shared gene targets. Most notably, viral-mediated knock-down of *Npas2* in the NAc abolishes a SIRT1 agonist-mediated increase in cocaine preference, further supporting a shared role in the regulation of reward. Taken together, our results demonstrate that cocaine disrupts diurnal expression of core circadian and metabolic factors in the NAc relevant for reward regulation and that NPAS2 and SIRT1 interact in the NAc to integrate these changes and together regulate reward-related behavior.

3.2 Introduction

SUDs are widely prevalent and debilitating psychiatric disorders with devastating social and economic burdens. While the exact mechanisms contributing to the development of SUDs are still unknown, emerging evidence suggests disruptions in both circadian rhythms and redox metabolism as key contributors (Logan et al., 2014; Uys et al., 2014). For example, drugs of abuse are known to disrupt circadian rhythms and sleep (Hasler et al., 2012a), as well as redox homeostasis (Cunha-Oliveira et al., 2013). Conversely, disruption to circadian genes directly alters reward regulation in rodent models (Parekh et al., 2015), and pharmacologically targeting redox

regulation has shown promise as a treatment option for SUDs in both clinical and pre-clinical animal studies (Womersley et al., 2019). Interestingly, few studies have looked at how crosstalk between circadian rhythms and redox homeostasis may contribute to SUD vulnerability, despite the growing understanding of how the circadian and metabolic systems are interconnected at the molecular level (Eckel-Mahan and Sassone-Corsi, 2013).

Nearly all cells in mammalian organisms express a circadian molecular clock, consisting of several transcription and translation feedback loops (Partch et al., 2014). At its core, Circadian Locomotor Output Cycles Kaput (CLOCK) or its homolog, Neuronal PAS Domain 2 (NPAS2), hetero-dimerize with Brain and Muscle ARNT-like 1 (BMAL1) to drive transcription of many output genes, including *Period* and *Cryptochrome* genes. Throughout the day, translated Period (PER) and Cryptochrome (CRY) proteins accumulate in the cytoplasm and eventually shuttle back into the nucleus as a heterodimer within a complex to inhibit their own transcription. An additional auxiliary feedback loop involves the transcriptional regulation of *Bmal1* and *Npas2* by the clock-controlled nuclear receptors RAR Related Orphan Receptor A (RORα) and REV-ERBα (or NR1D1), with RORα promoting their transcription and REV-ERBα repressing (Preitner et al., 2002; Sato et al., 2004; Crumbley et al., 2010). Together, these negative feedback loops cycle roughly every 24 hours and control cellular physiology across the day, with nearly half of all protein-coding genes being regulated by the molecular clock (Partch et al., 2014; Zhang et al., 2014a). Notably, NPAS2 is structurally and functionally similar to CLOCK (DeBruyne et al., 2007; Landgraf et al., 2016), but the two proteins differ in their expression across the brain; while CLOCK is ubiquitously expressed, NPAS2 is enriched in specific regions, including the forebrain (Reick et al., 2001). Unlike CLOCK, NPAS2 is highly enriched in the striatum and nucleus accumbens (NAc) (Garcia et al., 2000), regions integral to processing reward and motivated

behaviors (Volkow and Morales, 2015). Previous work from our lab has demonstrated that mutations in *Clock* increase cocaine and alcohol intake (McClung et al., 2005; Ozburn et al., 2012, 2013); however, mutations or NAc knock-down of *Npas2* reduce cocaine preference (Ozburn et al., 2015; Parekh et al., 2019). This differential regulation of reward may arise through NAc-specific interactions between NPAS2 and cellular metabolic state.

In addition to NPAS2's direct regulation of key metabolic pathways (O'Neil et al., 2013), NPAS2 can integrate metabolic/redox information itself. Work from the McKnight lab and others have demonstrated NPAS2's ability to bind the heme cofactor and act as a gas-responsive redox sensor (Dioum et al., 2002; Uchida et al., 2005). Moreover, NPAS2's DNA binding activity is also regulated by changes in pH and the mitochondrial co-factor nicotinamide adenine dinucleotide (NAD) (Rutter et al., 2001; Yoshii et al., 2015). NAD exists in both an oxidized (NAD+) and reduced form (NADH) that both respond to changes in redox state and help maintain cellular metabolic homeostasis (Ying, 2008; Houtkooper et al., 2010). While levels of NAD have been shown to affect NPAS2 function under ultra-physiological conditions *in vitro* (Rutter et al., 2001), a more likely mechanism *in vivo* may be through interactions with the NAD+-dependent deacetylase, sirtuin 1 (SIRT1).

SIRT1 is a histone and protein deacetylase whose activity is directly coupled to redox balance via NAD+ levels and it regulates transcriptional control of cellular metabolic state (Cantó and Auwerx, 2012). SIRT1 has also been shown to play an integral role in regulating NAc function and reward in mice. Work from the Nestler lab and colleagues has found that SIRT1 regulates drug-associated synaptic plasticity, including NAc dendritic spine density and plasticity-related gene expression (Ferguson et al., 2013). Furthermore, both SIRT1 overexpression in the NAc and activation via the SIRT1 agonist *resveratrol* increase cocaine preference in mice, while antagonists

decrease preference (Renthal et al., 2009; Ferguson et al., 2013). One mechanism that may underlie these findings is the known integration of SIRT1 metabolic signaling with the circadian molecular clock. Extensive work from Sassone-Corsi, Schibler, and others illustrate how SIRT1 directly regulates the molecular clock through both NAD+ dependent PER2 deacetylation and/or repression of CLOCK/BMAL1 transcription (Asher et al., 2008; Nakahata et al., 2008). This crosstalk is thought to be governed by a transcriptional-enzymatic feedback loop through circadian control of the nicotinamide phosphoribosyltransferase (NAMPT) NAD+ salvage pathway by CLOCK and SIRT1 (Nakahata et al., 2009; Ramsey et al., 2009). However, to date, the characterization of this relationship has primarily been in the liver. Interestingly, recent work from our lab has demonstrated in the ventral tegmental area (VTA) that CLOCK and SIRT1 regulate dopaminergic activity and cocaine reward, mediated by NAD+ cellular redox (Logan et al., 2019). This work is further supported by recent findings in the VTA showing NAMPT-mediated NAD biosynthesis directly regulates cocaine reward in a SIRT1 dependent manner (Kong et al., 2018). However, it remains unclear whether similar mechanisms hold true in the NAc and/or with CLOCK's homolog NPAS2. No studies to date have investigated whether SIRT1 and NPAS2 interact in the NAc or whether together they may be necessary for regulating reward. In the present study, we find diurnal variation in expression of *Npas2* and *Sirt1* in the mouse NAc that is disrupted by cocaine exposure, as well as a disrupted diurnal variation of *Nampt* expression and NAD+ levels. Most notably, we demonstrate NPAS2 and SIRT1 interact in the NAc and share several reward-relevant and metabolic-related gene targets, and this interaction is necessary for the SIRT1 agonist-mediated increase in cocaine preference in mice.

3.3 Materials and Methods

3.3.1 Animals

Experiments utilized either male C57BL/6J mice (The Jackson Laboratory; Bar Harbor, ME; IMSR Cat# JAX:000664, RRID:IMSR_JAX:000664) or *Npas2* mutant and wild-type (WT) littermates (maintained on a C57BL/6J background) from the Weaver lab (Dallmann et al., 2011), ages 8-12 weeks. *Npas2* mutant mice carry a mutation resulting in NPAS2 lacking its functional domain and rendering it incapable of binding BMAL1 (Garcia et al., 2000). All mice were maintained on a 12:12 light-dark cycle (lights on at 0700, zeitgeber time (ZT) 0, and lights off at 1900, ZT12) with food and water provided *ad libitum*. Behavioral testing occurred between ZT2-5, with at least 30 minutes of habituation to the room. Separate cohorts of mice were used for each of the experiments described below, unless otherwise indicated. Animal use was conducted in accordance with the National Institute of Health guidelines, and all procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.3.2 Drug Administration

Cocaine hydrochloride was provided by the National Institute on Drug Abuse (NIDA) and dissolved with 0.9% saline (Fisher Scientific). Mice were injected intraperitoneally (i.p.) at 10 ml/kg with cocaine (5 mg/kg for CPP or 20 mg/kg for molecular experiments), resveratrol (20 mg/kg; SelleckChem, Houston, TX, USA), or 0.9% saline. Systemic administration of resveratrol has been shown to cross the blood-brain barrier readily (Baur and Sinclair, 2006). For molecular

experiments, mice were administered saline (10 ml/kg i.p.), chronic cocaine (20 m/kg i.p. for 14 days), or acute cocaine (saline for 13 days and cocaine for 1 day at 20 m/kg i.p.). Drugs were administered between ZT4-8.

3.3.3 Viral-Placement Surgery and Verification

Npas2 knock-down stereotaxic surgery was performed as previously described and validated in (Ozburn et al., 2015). Briefly, male C57BL/6J mice were given bilateral stereotaxic 1 µl injections into the NAc (Bregma: angle 10˚, AP +1.5, ML ± 1.5, DV -4.4 mm) of purified high titer adeno-associated virus (AAV2) containing shRNA for *Npas2* or a scrambled sequence, both expressing a GFP reporter (UNC Vector Core; Chapel Hill, NC, USA; RRID:SCR_002448). To minimize pain and discomfort, surgeries were quickly performed under isoflurane anesthesia, and mice were given pain treatment up to 2 days following surgery. Mice recovered for at least 2 weeks to allow for sufficient viral expression before behavioral testing. Following testing, viral placement and spread were verified using immunofluorescence (IF). Mice were perfused with ice-cold phosphate-buffered saline (1x PBS) followed by paraformaldehyde in PBS (4% PFA at pH 7.4). Brains were post-fixed for 24 hours and then transferred to 30% sucrose for another 24 hours. Brains were cryo-sectioned at 40-um sections before processing for IF. Green Fluorescent Protein (GFP) signal was enhanced using a primary anti-GFP antibody (Abcam, Cambridge, UK; Cat# ab13970; RRID:AB_300798) before sections were mounted on slides with VECTASHIELD mounting medium plus DAPI (Vector Laboratories; Burlingame, CA, USA; Cat# H-1500, RRID:AB_2336788). Sections were imaged at 4x magnification using an Olympus epifluorescence microscope (Olympus Corporation; Shinjuku, Tokyo, Japan).

3.3.4 Behavioral Testing

Cocaine conditioned place preference (CPP) was performed as previously published (Parekh et al., 2019). A biased conditioning protocol was utilized whereby cocaine pairing is performed on the mouse's least preferred side, as determined during the pre-test. Briefly, mice were first allowed to freely explore a 3-chambered apparatus (Med Associates; Fairfax, VT) for 20 minutes to test for inherent bias. Mice exhibiting greater than 10 minutes in any chamber during the pre-test were excluded. On days 2 and 4 of CPP, mice were injected with saline (i.p) and placed in their initially preferred chamber of the apparatus for 20 minutes. On days 3 and 5 of CPP, mice were injected with cocaine (5 g/kg, i.p.) and placed in their initially non-preferred chamber for 20 minutes. On the final day (Day 6), mice were placed into the 3-chambered apparatus for 20 minutes and allowed to explore freely. Time spent in each chamber was recorded. CPP score was calculated by subtracting the pre-conditioning time spent in the cocaine-paired side from the test-day time on that side.

3.3.5 Gene Expression: RNA isolation and RT-qPCR

Mice were sacrificed across 4 times of day (ZT 5, 10, 17, 22), brains were rapidly extracted, and the NAc was quickly micro-dissected before being snap-frozen on dry ice. Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen; Hilden, Germany) and converted to complementary DNA (cDNA) using the SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific; Waltham, MA, USA). Gene expression was measured using quantitative polymerase chain reaction (qPCR) with primers for circadian and metabolic genes of interest, performed in a

CFX96 Touch Real-Time PCR detection system (Bio-Rad; Hercules, CA, USA). The following primers were utilized (**Table 1**):

Table 1. Circadian and Metabolic Gene Expression Mouse Primers

Primer	Forward (5' – 3')	Reverse (5' – 3')
18s	ACCGCAGCTAGGAATAATGGA	GCCTCAGTTCCGAAAACCA
Arntl	CTTGCAAGCACCTTCCTTCC	GGGTCATCTTTGTCTGTGTC
Npas2	GACACTGGAGTCCAGACGCAA	AATGTATACAGGGTGCGCCAAA
Nr1d1	GCAAATCAGATCTCAGGTGC	AGGTGATAACACCACCTGTG
Nr1f1	ACGCCCACCTACAACATCTC	ACATATGGGTTCGGGTTTGA
Sirt1	TCTGTCTCCTGTGGGATTCC	GATGCTGTTGCAAAGGAACC
Nampt	TACAGTGGCCACAAATTCCA	AATGAGCAGATGCCCCTATG

The comparative cycle threshold (ct) method (i.e., $2^{-\Delta\Delta CT}$ method) was used to calculate relative gene expression (Schmittgen and Livak, 2008), normalized to the 18s reference gene. Relative gene expression values were reported as the mean normalized Ct value ± SEM.

3.3.6 Co-Immunoprecipitation (Co-IP) Assay

Mice were sacrificed at 2 times of day (ZT 5 and 17), brains were rapidly extracted, and the NAc was quickly micro-dissected before being snap-frozen on dry ice. NAc punches were homogenized mechanically and with sonication in pierce immunoprecipitation (IP) lysis buffer (Thermo Fisher Scientific) plus protein phosphatase and protease inhibitor cocktails. A detergent

compatible (DC) protein assay (Bio-Rad; Hercules, CA, USA) was performed in an Epoch Microplate Spectrophotometer (BioTek; Winooski, VT, USA) to determine total protein concentrations. Interaction between NPAS2 and SIRT1 was assessed using the Dynabead Co-Immunoprecipitation Kit (Invitrogen; Carlsbad, CA, USA), as per manufacturer instructions. Briefly, samples were incubated with anti-NPAS2 (Santa Cruz; Dallas, TX, USA; Cat# sc-28708, RRID:AB_2282741) coupled M-270 Epoxy Dynabeads and the NPAS2 containing protein complex was eluted. Detection of SIRT1 in the protein complex was assessed using SDS-PAGE followed by immunoblotting for SIRT1. Briefly, eluted NPAS2-IP samples were run in a 7.5% mini-gel at 100V (Bio-Rad) and transferred overnight. Blotting for SIRT1 was achieved using the LI-COR Odyssey Imaging System (LI-COR Biosciences; Lincoln, NE, USA) with antibodies for SIRT1 (Santa Cruz; Cat# sc-15404, RRID:AB_2188346) or GAPDH (Millipore; Burlington, MA; Cat# MAB374, RRID:AB_2107445).

3.3.7 NAD+ Measurement

Mice were sacrificed across 4 times of day (ZT 5, 10, 17, 22), brains were rapidly extracted, and the NAc was quickly micro-dissected before being snap frozen using liquid nitrogen. High-Performance Liquid Chromatography (HPLC) quantification of NAD+ was performed as previously published (Yoshino and Imai, 2013; Stromsdorfer et al., 2016). Briefly, NAc punches were rapidly homogenized in perchloric acid and then neutralized in potassium carbonate. NAD+ concentrations were determined using an HPLC system (Prominence; Shimadzu Scientific Instruments; Columbia, MD, USA) and a SUPELCOSIL LC-18-T column (Sigma Aldrich; St. Louis, MO, USA). NAD+ concentrations were normalized to weights of the frozen tissue samples.

3.3.8 Chromatin Immunoprecipitation sequencing (ChIP-seq) Pathway Analyses

Previously, we performed ChIP-seq for NPAS2 in the NAc to identify novel DNA binding targets (Ozburn et al., 2015). NAc SIRT1 ChIP-seq data from Ferguson et al. (2015) was utilized for comparative analyses investigating shared binding targets between NPAS2 and SIRT1 in the NAc. Here, both Ingenuity Pathway Analysis (IPA) software (QIAGEN; Hilden, Germany; RRID:SCR_008653) (Krämer et al., 2014) and the online bioinformatics database Metascape (https://metascape.org/; RRID:SCR_016620) (Zhou et al., 2019a) were used to identify enriched pathways and processes among the shared binding targets gene list between NPAS2 and SIRT1. For both analyses, shared genes were analyzed as follows: (1) The 3189 genes bound by SIRT1 (e.g., gene body and/or promoter; Saline; ZT 2) and the 2347 genes bound by NPAS2 at ZT2 (e.g., gene body and/or promoter) were first input through the Bioinformatics Evolutionary Genomics Venn Diagram Tool to identify 390 shared gene binding targets (http://bioinformatics.psb.ugent.be/webtools/Venn/); (2) the 390 shared genes bound were then used as an input list for both IPA and Metascape analyses, with 11,005 total identified genes from both datasets used as a reference gene set; and (3) for identification of significant pathways and processes, a significance threshold of $p<0.05$ (or a -log10(p-value) of 1.3 in figures) was utilized. IPA software was used to identify enriched canonical molecular pathways and *top disease and biological functions*, while Metascape was used to identify enriched biological processes using only *Gene Ontology (GO) Biological Processes* as the ontology source. Within Metascape, all statistically enriched terms, accumulative hypergeometric p-values, and enrichment factors were automatically calculated and used for filtering. The remaining significant terms were then hierarchically clustered into a tree based on Kappa-statistical similarities among their gene

memberships, with a 0.3 kappa score applied as the threshold to cast the tree into term clusters. A subset of representative terms from this cluster were then converted into an enrichment network using Cytoscape (v3.1.2; RRID:SCR_003032) (Shannon et al., 2003), with each term represented by a circle node colored by cluster identity and terms with a similarity score > 0.3 linked together (the thickness representing the similarity score). (Zhou et al., 2019a) In figures, enriched processes and pathways of relevance to reward regulation are indicated in bold.

3.3.9 Statistical Analysis

For all statistical analyses, GraphPad Prism 9 software was used (GraphPad Software; San Diego, CA, USA; RRID:SCR_002798). Statistical analysis of more than two groups was performed using either a one-way or two-way analysis of variance (ANOVA) with significant interactions followed by Bonferroni *post-hoc* tests corrected for multiple comparisons. Otherwise, statistical analysis of just two groups was performed using a Student's t-test. Across all data sets, outliers were tested for using the Grubbs' test. Data throughout are expressed as mean ± SEM with $\alpha = 0.05$ considered statistically significant.

3.4 Results

3.4.1 The molecular clock and regulators of cellular metabolic state show diurnal variation in the NAc

Accumulating evidence suggests circadian molecular clock function may be coupled with the cellular metabolic state through a transcriptional-enzymatic feedback loop via SIRT1 and the NAD+ salvage pathway (Eckel-Mahan and Sassone-Corsi, 2013). However, this phenomenon has largely been characterized in the liver and primarily in the context of SIRT1 and CLOCK/BMAL1. To investigate whether this relationship exists in the NAc and/or with NPAS2/BMAL1, we first sought to confirm and expand our previous findings that core molecular clock genes are rhythmic in the NAc (Falcon et al., 2013). We performed reverse transcriptase quantitative PCR (RT-qPCR) with whole NAc tissue that was collected across 4 times of day (ZT 5, 10, 17, and 22) from saline-injected C57BL/6J mice (10 ml/kg, i.p., 14 days), utilizing primers for core circadian genes *Npas2*, *Bmal1*, *Rev-erbα*, and *RORα* (normalized to *18s*). Much like our previous findings, *Npas2* showed diurnal variation in expression (**Figure 6A**), with a highly significant main effect of time ($F_{(3, 20)} = 18.34$; $p<0.0001$). Expanding on our previous findings, *Bmal1* and the auxiliary feedback loop members *Rev-erbα* and *RORα* also showed diurnal variation in the NAc, all with significant main effects of time ($F_{(3, 20)} = 15.72$, $p<0.0001$; $F_{(3, 20)} = 7.94$, $p=0.001$; $F_{(3, 20)} = 10.94$, $p=0.0002$, respectively).

While SIRT1 is known to be expressed in the NAc (Renthal et al., 2009; Ferguson et al., 2015), no studies have investigated its expression across time of day. Here we find *Sirt1* expression shows a diurnal variation in the NAc (**Figure 6B**), with a highly significant main effect of time

($F_{(3, 19)} = 14.32$; $p<0.0001$). Moreover, we find *Nampt,* a clock-controlled enzyme in the NAD+ salvage pathway and proposed transcriptional-enzymatic feedback loop (Ramsey et al., 2009), also display diurnal variation in the NAc (**Figure 6B**), with a significant main effect of time ($F_{(3, 18)} = 7.5$; $p=0.001$). Most notably, we also investigated the diurnal variation of NAD+ in the NAc. While work from the Bass lab has characterized rhythmicity of NAD+ in the liver (Ramsey et al., 2009), characterization in the brain has yet to be done outside of our previous study investigating the VTA (Logan et al., 2019). Importantly, using HPLC, we find NAD+ shows a diurnal variation in the NAc (**Figure 6C**), with a significant main effect of time ($F_{(3, 20)} = 3.26$, $p<0.05$). Together, these results indicate *Npas2*, *Sirt1,* and the NAD+ salvage pathway are circadian regulated in the NAc, with peaks of expression, or acrophase, generally around ZT22 during the light phase (**Figure 6D**).

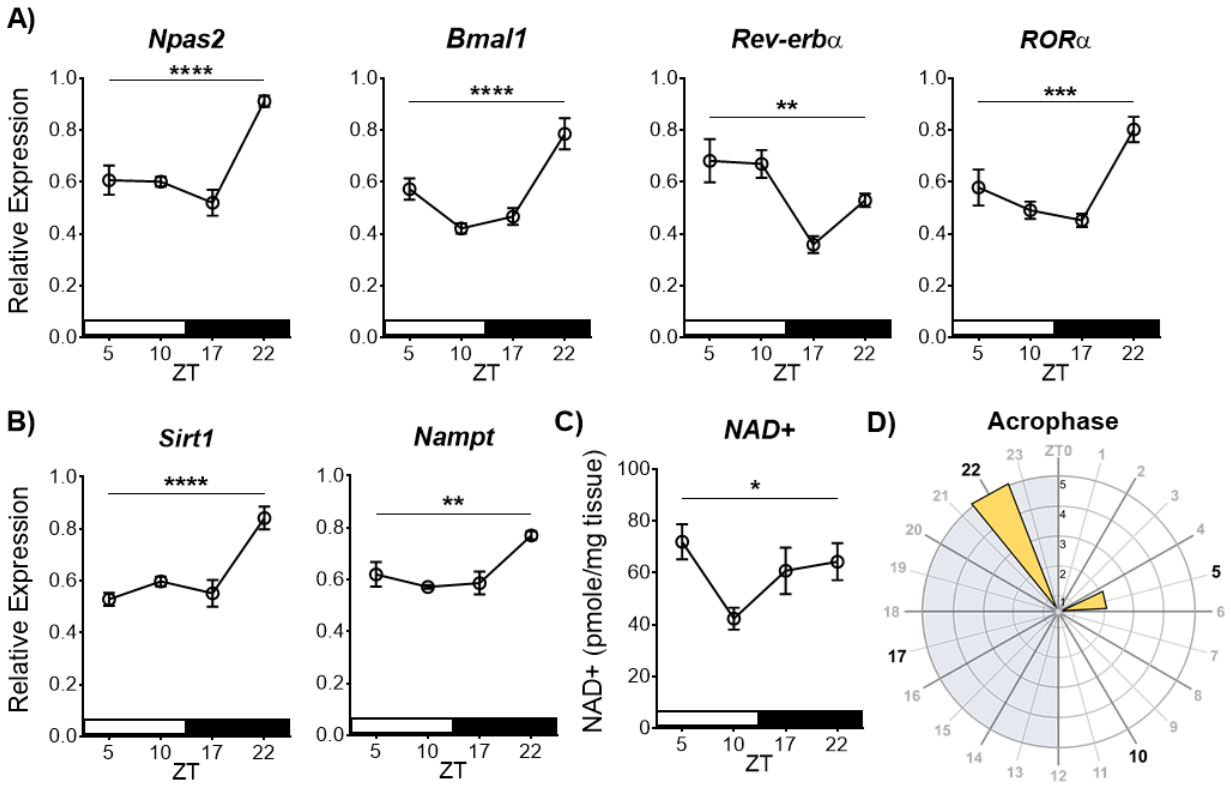

Figure 6. The molecular clock and regulators of cellular metabolic state show diurnal variation in the NAc. (**A**) Core molecular clock gene expression of *Npas2*, *Bmal1* (i.e., *Arntl*), Rev-erbα (i.e., *Nr1d1*), and RORα (i.e., *Nr1f1*) show diurnal variation in the NAc, as well as the (**B**) metabolic genes Sirt1 and Nampt – measured using RT-qPCR. Relative expression normalized to the reference gene 18s. (**C**) HPLC analysis of NAD+ levels also show diurnal variation in the NAc. (**D**) Rose plot of the peaks of expression, or acrophase, illustrates peaks are primarily at ZT22 during the dark phase. Asterisks and bar indicate a significant main effect of time measured in a one-way ANOVA (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$). Data represented as Mean ± SEM (n=5-6 / ZT). White stripe indicates lights on; black stripe indicates lights off. Grey shading on the rose plot indicates lights off, and the inset axis shows the number of genes. ZT 0 = 7 am.

3.4.2 Diurnal Variations of the molecular clock, SIRT1, and NAD+ are Affected by Cocaine

Previous studies have found that NPAS2 and SIRT1 expression are induced by cocaine when measured at a single time of day (Renthal et al., 2009; Falcon et al., 2013). We next looked

to extend these findings by investigating how expression is affected by cocaine across time of day in the NAc. Mice were injected with either saline (10 ml/kg, i.p., 14 days) or cocaine (acute: 20 mg/kg, i.p., 1 day; chronic: 14 days) and sacrificed across 4 times of day (ZT 5, 10, 17, 22), for NAc tissue to be used in gene expression analyses (**Figure 7A**). Notably, diurnal expression of *Npas2* is upregulated by both acute and chronic cocaine exposure (**Figure 7B**), with a significant interaction and main effects of time and treatment (Interaction: $F_{(6, 59)} = 5.134$, p=0.0003; Time: $F_{(3, 59)} = 5.331$, p=0.002; Treatment: $F_{(2, 59)} = 10.47$, p=0.0001). Acute cocaine significantly increases *Npas2* expression across ZT5, 10, and 17 ($p<0.05$), leading to a phase shift in peak expression, or acrophase, to the light phase. Chronic cocaine significantly increases *Npas2* expression only at ZT 17 ($p<0.001$), relative to saline. Diurnal expression is also altered by cocaine exposure for both *Bmal1* (Interaction: $F_{(6, 59)} = 2.293$, $p<0.05$; Time: $F_{(3, 59)} = 29.46$, $p<0.0001$; Treatment: $F_{(2, 59)} = 6.768$, p=0.002) and *RORα* (Interaction: $F_{(6, 60)} = 5.297$, p=0.0002, p=0.0003; Time: $F_{(3, 60)} = 7.912$, p=0.0002; Treatment: $F_{(2, 60)} = 6.148$, p=0.003), but not *Rev-erbα,* which only shows a main effect of time ($F_{(3, 60)} = 21.85$, $p<0.0001$).While previous studies suggest NAc *Sirt1* expression is induced by cocaine exposure (Renthal et al., 2009), no studies have investigated how cocaine affects *Sirt1* expression across time of day. Here we find that diurnal variation of *Sirt1* expression is disrupted by both acute and chronic cocaine exposure (**Figure 7C**), with a significant interaction and main effects of time and treatment (Interaction: $F_{(6, 59)} = 2.808$, p=0.01; Time: $F_{(3, 59)} = 3.179$, $p<0.05$; Treatment: $F_{(2, 59)} = 3.628$, $p<0.05$). Much like *Npas2*, acute cocaine upregulates Sirt1 expression at ZT10 ($p<0.05$), leading to a phase shift in peak expression to the light phase, while chronic cocaine significantly increases *Sirt1* only at ZT 17 (p=0.05). Moreover, the gene encoding the NAD+ salvage pathway enzyme, *Nampt,* also shows significant upregulation of expression (**Figure 7C**), with a significant interaction and a main effect of time

(Interaction: $F_{(6, 57)} = 4.084$, $p=0.001$; Time: $F_{(3, 57)} = 7.639$, $p=0.0002$). Chronic cocaine's upregulation of *Nampt* expression at ZT5 and ZT10 ($p<0.05$) also leads to a phase-shifting of acrophase to the light phase.

Extending our finding that NAD+ shows diurnal variation in the NAc (**Figure 7C**), we next wanted to investigate how cocaine exposure may affect NAD+ levels across time of day (**Figure 7D**). Interestingly, diurnal variation of NAD+ levels in the NAc are significantly altered by cocaine exposure, with significant main effects of time and treatment (Time: $F_{(3, 54)} = 6.157$, $p=0.001$; Treatment: $F_{(2, 54)} = 3.895$, $p<0.05$), no significant interaction ($F_{(6, 54)} = 1.884$, $p=0.1$), and a shift in acrophase with cocaine exposure to the dark phase. Together, these data indicate cocaine significantly alters diurnal variation of *Npas2*, *Sirt1*, and related metabolic regulators in the NAc through upregulation of expression, and a phase shifting of acrophase, resulting in more peaks in expression during the light phase (**Figure 7E**).

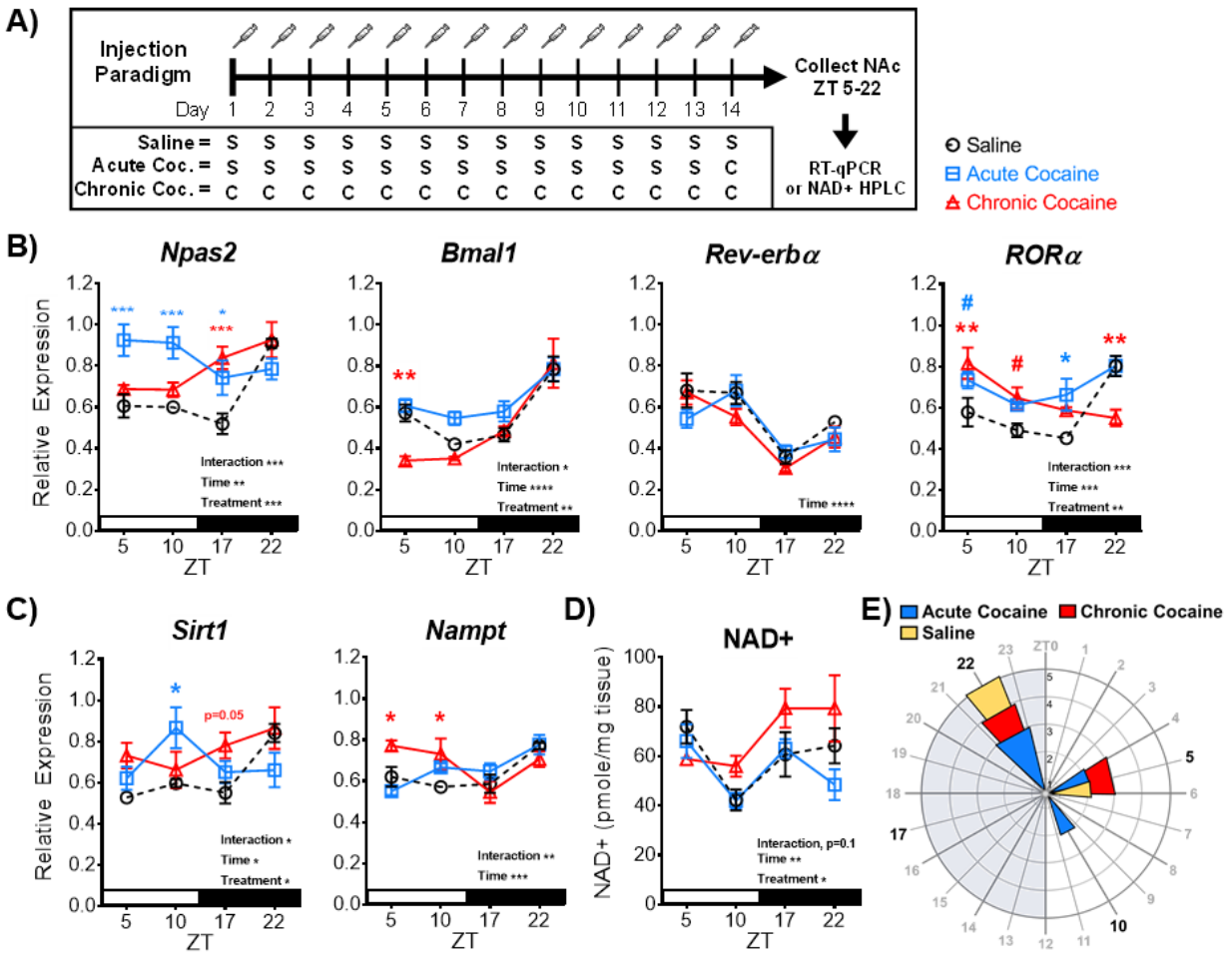

Figure 7. Cocaine exposure alters diurnal variation of the circadian molecular clock and cellular metabolic state in the NAc. (**A**) Schematic outlining the two-week injection paradigm. Mice were given either saline (S; 10 ml/kg, i.p.), chronic cocaine (C; 20 mg/kg, i.p.), or acute cocaine (13 x saline, 1 x cocaine). NAc tissue was collected from mice across the 4 times of day (ZT5, 10, 17, & 22) following the last injection. (**B**) In mice exposed to cocaine, diurnal variation of core molecular clock gene expression of *Npas2*, *Bmal1* (i.e., *Arntl*), and *Rorα* (i.e., *Nr1f1*) , as well as (**C**) the metabolic genes *Sirt1* and *Nampt*, were significantly disrupted in the NAc – as indicated by significant interactions and main effects of time and/or treatment by 2-way ANOVA. (**D**) HPLC analysis revealed cocaine exposure also significantly disrupts diurnal variation of NAD+ levels in the NAc, with significant main effects of time and treatment. (**E**) An acrophase rose plot illustrates peaks are primarily at ZT22 during the dark phase, but cocaine exposure shifts more peaks of expression to the light phase. Asterisks above the plotted lines indicate a significant difference relative to saline, revealed in a Bonferroni post-hoc analysis. (# p≤0.07, * p<0.05, ** p<0.01, *** p<0.001).

Data represented as Mean ± SEM (n=5-6 / ZT). White stripe indicates lights on, and black stripe indicates lights off. Grey shading on the rose plot indicates lights off and the inset axis shows the number of genes. ZT 0 = 7 am.

3.4.3 NPAS2 and SIRT1 are important for cocaine conditioned place preference

Previous work from our lab has demonstrated NPAS2 is important for cocaine preference, in that mice carrying a functional mutation in *Npas2* (*Npas2* mutant mice) show decreased cocaine CPP (Ozburn et al., 2015). Additionally, work from our lab and others have demonstrated that SIRT1 is important for cocaine preference, in that activation of SIRT1 via resveratrol increases cocaine CPP (Renthal et al., 2009; Logan et al., 2019). Before determining whether *together* NPAS2 and SIRT1 are necessary for cocaine preference, we first wanted to re-confirm the findings from previous studies. To accomplish this, *Npas2* mutant mice were run through the cocaine CPP paradigm (**Figure 8A**) and conditioned to both saline and 5 mg/kg cocaine (i.p.) paired chambers. Consistent with our previous findings, *Npas2* mutant mice show a decrease in cocaine CPP, as compared to WT littermates ($t_{(20)=}2.082$, p=0.05; **Figure 8B**). To test the effects of resveratrol, C57BL/6J mice were run through the cocaine CPP paradigm and conditioned to both saline and 5 mg/kg cocaine paired chambers; however, during conditioning days, mice were injected with either saline or 20 mg/kg resveratrol (i.p) 30 minutes prior to conditioning. As previously published, mice injected with the SIRT1 agonist resveratrol show a significant increase in cocaine CPP (**Figure 8C**), relative to saline controls ($t_{(14)=}2.243$, $p<0.05$). Together, these findings further indicate NPAS2 and SIRT1 are important for cocaine preference.

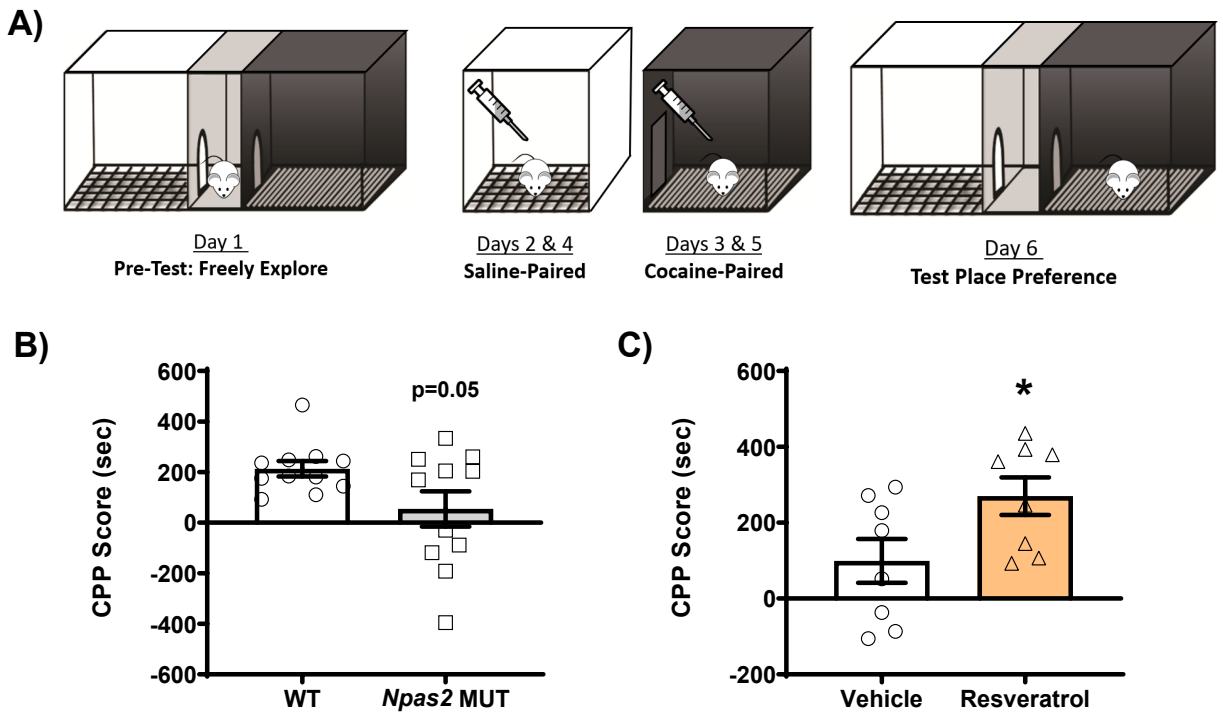

Figure 8. NPAS2 and SIRT1 are important for cocaine preference. (**A**) Schematic showing the cocaine conditioned place preference (CPP) paradigm. Mice were conditioned to saline (10 ml/kg, i.p.) or cocaine (5 mg/kg, i.p.) on conditioning days. (**B**) *Npas2* mutant mice show decreased cocaine preference relative to wild-type (WT) littermates. (**C**) C57BL/6J mice were administered vehicle or resveratrol (20 mg/kg; i.p.) 30 minutes prior to conditioning. Mice administered resveratrol showed a significant increase in cocaine preference relative to vehicle controls. Data represented as Mean ± SEM (n= 8-11). CPP score is calculated by subtracting the initial pre-test time in the cocaine paired chamber minus test-day time. Asterisk indicates significance relative to controls (* $p<0.05$).

3.4.4 NPAS2 and SIRT1 interact in the NAc and Share Reward-Relevant and Metabolic-Related Gene Targets

While studies done in the liver suggest SIRT1 interacts with CLOCK/BMAL1 to integrate metabolic information and regulate molecular clock function (Nakahata et al., 2008, 2009), no

studies have investigated whether this interaction holds true with CLOCK's homolog NPAS2 and/or within the NAc. Given the previous findings indicating both NPAS2 and SIRT1 are induced in the NAc by cocaine exposure (Renthal et al., 2009; Falcon et al., 2013) and they play a role in regulating cocaine reward (Renthal et al., 2009; Ferguson et al., 2013; Ozburn et al., 2015), we wanted to investigate whether NPAS2 and SIRT1 interact in the NAc. To assess a potential interaction, co-immunoprecipitation of NPAS2 followed by western blot analysis for SIRT1 was performed in NAc tissue from C57BL/6J mice. Across two times of day, SIRT1 was detected in NPAS2 pulldown (**Figure 9A**), indicating NPAS2 and SIRT1 interact in the same protein complex in the NAc. Previous work from our lab and Ferguson et al. utilizing chromatin immunoprecipitation sequencing (ChIP-seq) in NAc tissue demonstrated NPAS2 and SIRT1 regulate transcription of reward-relevant genes (Ferguson et al., 2015; Ozburn et al., 2015); however, investigation into potential shared gene targets has yet to be studied. Cross analysis of the NPAS2 and SIRT1 ChIP-seq datasets revealed 2,347 and 3,189 genes bound (e.g., promoter or gene body) at ZT2 by NPAS2 and SIRT1, respectively, with 390 bound genes shared between the two (**Figure 9B**). To investigate the functional importance of these 390 shared gene targets, we performed Ingenuity Pathway Analysis (IPA) and Metascape analysis (Zhou et al., 2019a) to uncover molecular pathways and biological processes enriched among the shared gene set. Interestingly, IPA revealed NRF2-mediated oxidative stress response and NF-kB activation to be among the top shared canonical pathways (**Figure 9C**), both pathways are known to mitigate oxidative stress and promote metabolic homeostasis (Mauro et al., 2011; Ma, 2013; Vomund et al., 2017; Lingappan, 2018). Moreover, top biological functions enriched among the shared genes include behavioral conditioning, synaptic transmission, and protection of dopamine neurons (**Figure 9C**) – all relevant to the regulation of reward and reward-related behavior. These results

are further supported by Metascape (GO) Biological Processes analysis revealing enriched catecholamine and dopamine-related processes, as well as regulation of synaptic transmission (**Figure 9D**); notably, when plotted as an enrichment network visualization, these synaptic transmission, catecholamine, and dopamine-related processes are highly interconnected (**Figure 9E**). These findings not only expand upon previous data implicating NPAS2 and SIRT1 in the regulation of reward, but also suggest an interaction in the NAc that facilitates co-regulation of reward-relevant genes/processes.

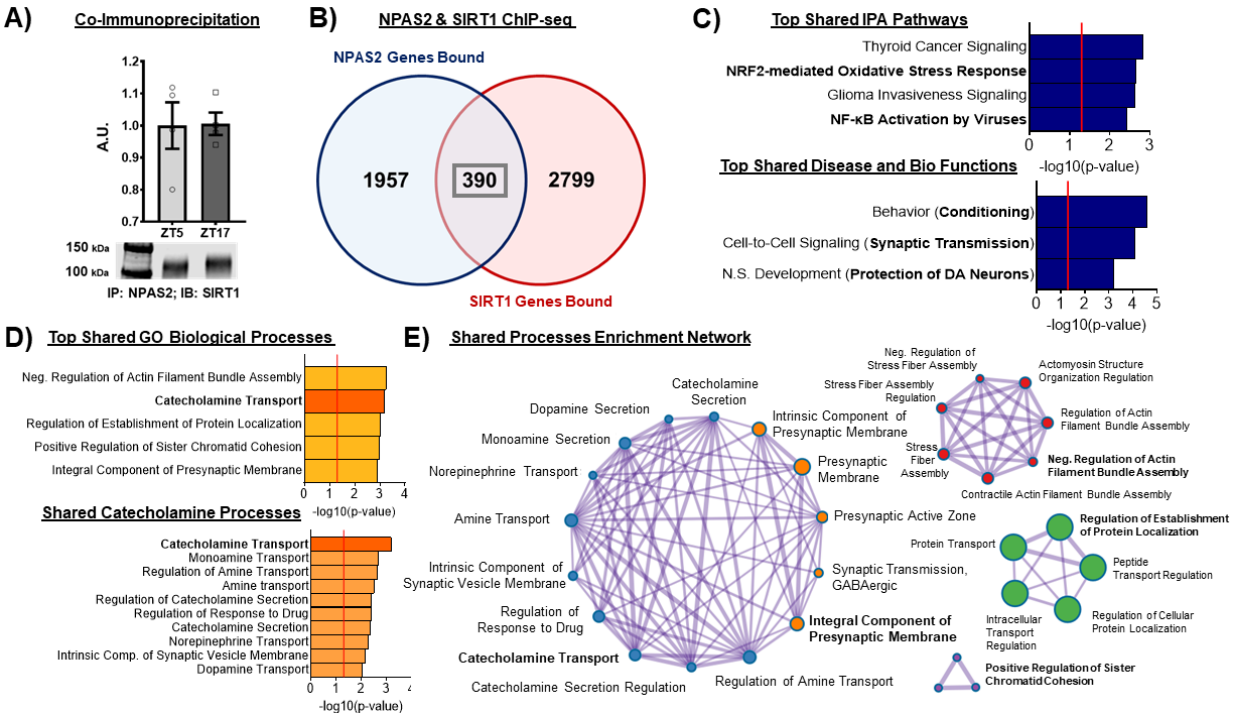

Figure 9. NPAS2 and SIRT1 interact in the NAc and share reward-relevant gene targets. (**A**) Co-immunoprecipitation (Co-IP) of NPAS2 from whole NAc tissue and immunoblotting for SIRT1 revealed a band for SIRT1 at ~120kD across time of day. GAPDH was used as a negative control and was not detected. (**B**) Cross analysis of chromatin immunoprecipitation sequencing (ChIP-seq) for NPAS2 and SIRT1 revealed 2,347 and 3,189 genes bound (e.g., promoter or gene body) by NPAS2 and SIRT1, respectively, with 390 genes bound shared between the two. (**C**) The top pathways enriched among these 390 shared genes include metabolic and reward-relevant biological functions. (**D**) Synaptic transmission, catecholamine transport and other dopamine relevant mechanisms are among the top biological processes enriched among NPAS2 and SIRT1's shared gene targets. (**E**) Among the enriched shared biological processes, the catecholamine nodes and synaptic transmission nodes show a high degree of interconnectivity. Red line in bar graphs indicates significance threshold of $p<0.05$ or -log10(p-value) > 1.3.

3.4.5 NPAS2 is necessary for the SIRT1 agonist-mediated increase in cocaine CPP

Given that expression of *Npas2* and *Sirt1* are both induced in the NAc following cocaine exposure (**Figure 7B, C**) and NPAS2 and SIRT1 interact in the NAc to regulate reward-relevant genes (**Figure 9**), we next wanted to investigate whether *together* they regulate reward-related behavior in the NAc. To determine if NPAS2 in the NAc is necessary for the increase in CPP seen with the SIRT1 agonist resveratrol (**Figure 8C**), BL6 mice were first injected bilaterally into the NAc with an AAV2-*Npas2*-shRNA or scramble control (**Figure 10A**). Mice given the shRNA have a NAc specific knockdown of NPAS2, previously shown in our lab to result in a 4-fold decrease in expression of *Npas2* (Ozburn et al., 2015). Mice were then run through the cocaine CPP paradigm and conditioned to both saline and 5 mg/kg cocaine paired chambers, with the administration of either saline or 20 mg/kg resveratrol 30 minutes prior to conditioning. Strikingly, knock-down of *Npas2* in the NAc abolishes the resveratrol mediated increase in cocaine CPP seen in scramble controls (**Figure 10B**), with significant main effects of both virus and resveratrol treatment (Virus: $F_{(1, 51)} = 4.513$, p=0.0385; Treatment: $F_{(1, 51)} = 9.003$, p=0.004). Taken together, these data indicate that NPAS2 in the NAc is necessary for the increase in cocaine reward seen with SIRT1 activation by resveratrol.

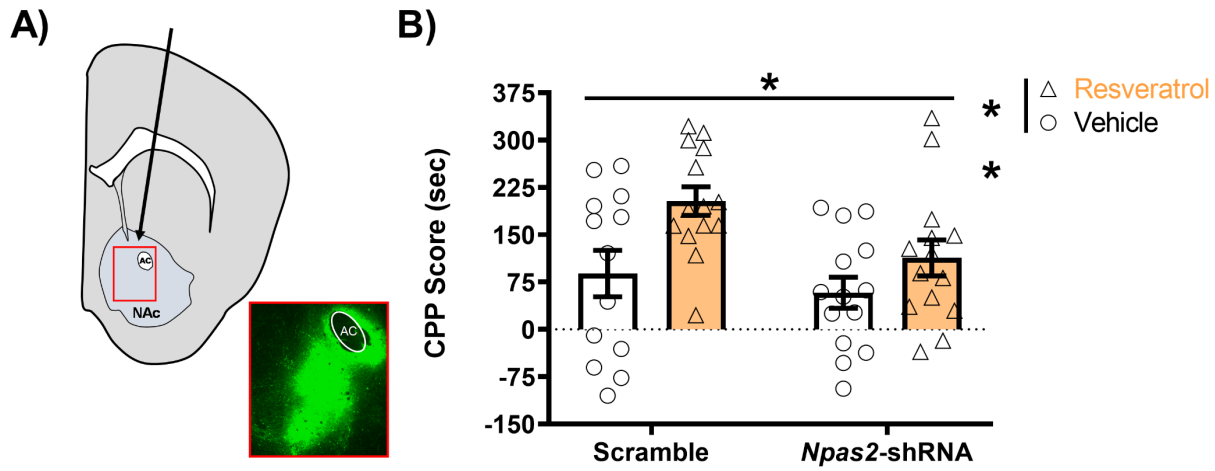

Figure 10. NPAS2 in the NAc is necessary for resveratrol mediated increase in cocaine preference. (**A**) C57BL/6J mice were injected bilaterally into the NAc with either AAV2-*Npas2*-shRNA-GFP or scramble control. Inset box depicts a representative image of virus placement and spread, as measured by GFP immunofluorescence. AC indicates anterior commissure, while arrow indicates virus injection path. (**B**) Following 2 weeks of recovery, mice injected with either Npas2-shRNA or scramble were conditioned to saline or cocaine (5 mg/kg) and injected with vehicle or resveratrol (20 mg/kg, i.p.) 30 minutes prior to conditioning. Viral-mediated knock-down of *Npas2* in the NAc significantly attenuated the increase in cocaine CPP seen with resveratrol treatment in scramble controls – with both main effects of virus and treatment measured by a 2-way ANOVA. Asterisk and horizontal bar indicate a significant main effect of the virus, while the asterisks and vertical bar indicate a significant main effect of treatment. (* $p<0.05$, ** $p<0.01$) Data represented as Mean ± SEM (n= 13-15).

3.5 Discussion

Emerging evidence suggests disruptions in both circadian rhythms and redox metabolism as key contributors underlying SUDs (Logan et al., 2014; Uys et al., 2014). Interestingly, circadian molecular clock function has been shown to be integrated with the cellular metabolic state through

a transcriptional-enzymatic feedback loop involving SIRT1 and the clock-controlled NAD+ salvage pathway (Nakahata et al., 2009; Ramsey et al., 2009). However, this interaction has yet to be explored in the NAc and/or in the context of the circadian transcription factor NPAS2. Results from this study illustrate how cocaine exposure affects the circadian expression of core circadian and metabolic genes, namely *Npas2* and *Sirt1*, and how together these two may interact in the NAc to control reward regulation. Core circadian genes *Npas2*, *Bmal1*, and their regulators *Rev-erbα* and *RORα* all show diurnal variation in the NAc. Expression of metabolic regulators *Sirt1*, *Nampt*, and levels of the mitochondrial coenzyme NAD+ also showed diurnal variation in the NAc. Interestingly, the diurnal variation in expression of the molecular clock and regulators of metabolic state were all shown to be altered in the NAc following cocaine exposure. Specifically, *Npas2*, its transcriptional activator *RORα*, and the metabolic regulators *Sirt1*, and *Nampt* were all phase-shifted in response to cocaine exposure, primarily driven by upregulation and shifting of acrophase to the light phase; NAD+ levels; NAD+ was also upregulated by cocaine exposure, but showed a shifting of acrophase to the dark phase. Most notably, in addition to reconfirming past findings showing NPAS2 and SIRT1 are important for cocaine CPP, we found NPAS2 and SIRT1 interact in the mouse NAc and *together* these proteins are essential in the regulation of cocaine reward. Through immunoprecipitation of NPAS2 in the NAc followed by western blot analysis, we found that SIRT1 interacts in a protein complex with NPAS2 across time of day. Furthermore, cross-analysis of NPAS2 and SIRT1 ChIP-seq datasets revealed several metabolic and reward-relevant pathways to be enriched among their shared gene targets; these pathways and processes included NRF2-mediated oxidative stress response and NF-kB activation, as well as many processes important for transport of dopamine, synaptic transmission, regulation of response to drug, and behavioral conditioning. Further confirming the findings that NPAS2 and SIRT1 interact in the

NAc and regulate many reward-relevant gene targets, knock-down of *Npas2* in the NAc abolishes the increase in cocaine CPP seen with SIRT1 activation via resveratrol, suggesting a shared role in this reward-relevant behavior. Taken together, these findings illustrate for the first time an interaction between NPAS2 and SIRT1 in the NAc and that this interaction is important in the regulation of cocaine reward.

Our findings that molecular clock genes show diurnal variation in the NAc are consistent with the previous literature from our lab and others illustrating that the NAc is under circadian control. Previous work from our lab has shown in mice that core clock genes *Npas2*, *Clock*, and *Period* all show diurnal variation in the NAc (Falcon et al., 2013), as well as rhythmicity measured by *Per2:*luciferase luminescence assays (Logan et al., 2015). This is also seen in time of death analyses of gene expression in human post-mortem tissue, where the canonical clock genes *Bmal1*, *Npas2*, and *Period* were all highly rhythmic in the NAc of healthy controls (Li et al., 2013). Here we further support these findings and expand upon them by showing *Npas2*, *Bmal1*, and their transcriptional regulators *Rev-erbα* and *RORα* all show significant diurnal variation in the NAc. This circadian regulation also extends to the regulation of *Sirt1*, *Nampt*, and levels of the mitochondrial coenzyme NAD+, which all show diurnal variation in the NAc. While these metabolic genes and cofactors have previously been shown to be diurnally regulated in the liver (Nakahata et al., 2008, 2009; Ramsey et al., 2009), our findings are the first to suggest this relationship also exists in the NAc. Future investigation with more time points may be necessary to fully capture the rhythmicity across time of day, especially considering the potential for bimodal circadian oscillations as seen with NAD+ in the liver (Ramsey et al., 2009). Furthermore, future investigation may also explore these changes in a cell-type-specific manner to fully appreciate the regulation of circadian rhythms in the NAc.

Through decades of research, aberrant functioning of the brain's reward circuitry, including dopamine signaling in the NAc, has been linked to the manifestation of SUDs (Koob and Le Moal, 2001; Koob and Volkow, 2010; Pierce et al., 2020). The dopamine hypothesis posits that substances of abuse act directly on the brain's reward system to increase mesolimbic DA release and promote both reward and subsequent reward-seeking behavior (Koob, 1992; Willuhn et al., 2010; Volkow and Morales, 2015). In fact, psychostimulants like cocaine or amphetamine act directly at NAc synapses to increase the concentration of extracellular DA available either by blocking dopamine transporter (DAT) mediated DA reuptake or promoting greater DA release, respectively (Kalivas, 2007). Interestingly, through our NPAS2 and SIRT1 ChIP-seq cross-analysis, we found catecholamine/dopamine transport, catecholamine secretion, regulation of response to drugs, and regulation of presynaptic membrane synaptic transmission to all be significantly enriched biological processes among shared gene targets between NPAS2 and SIRT1 in the NAc. This is further evidenced by IPA pathway analyses revealing behavioral conditioning, synaptic transmission, and protection of dopamine neurons to all be significantly enriched biological functions among shared gene targets. One gene target in particular, glial cell-derived neurotrophic factor (GDNF), is shared among all the enriched processes and pathways and has been extensively shown to be important for dopamine neuron survival and function (Airaksinen and Saarma, 2002). GDNF and its signaling pathways have also been associated with reward physiology relevant for the development of SUDs - specifically shown to regulate dopamine neuronal excitability, synaptic transmission, synaptic plasticity, and sensitivity to psychostimulants (Ron and Janak, 2005; Carnicella and Ron, 2009; Ghitza et al., 2010). While particularly interesting in the context of our current findings, the relationship between NPAS2, SIRT1, and GDNF, and how this shared gene target may contribute to reward regulation remains

to be studied. Taken together, the many dopamine-related processes enriched among shared gene targets between NPAS2 and SIRT1 in the NAc further highlights their functional relevance for reward processing and warrants future mechanistic investigation.

An abundance of evidence suggests substances of abuse disrupt both circadian rhythms and metabolic redox state of the cell (Hasler et al., 2012a; Cunha-Oliveira et al., 2013; Uys et al., 2014). Here, our findings further support these observations showing a diurnal variation of both core clock genes (e.g., *Npas2*, *Bmal1*, and *RORα*) and regulators of metabolic state (e.g., *Sirt1*, *Nampt*, and NAD+ levels) are significantly disrupted by exposure to cocaine. The disruption in diurnal variation in expression of *Npas2*, *Sirt1,* and the NAD+ salvage pathway may point toward a bi-directional mechanism in which cocaine alters both circadian and metabolic regulation of the NAc. In both human and animal studies, cocaine has been shown to produce increased oxidative stress/damage and subsequent compensatory upregulation of glutathione antioxidant signaling (Cunha-Oliveira et al., 2013; Uys et al., 2014). This is further supported by the human and rodent literature suggesting therapeutic targeting of redox signaling through antioxidants (e.g., n-acetylcysteine) may prove beneficial in treating SUDs (Womersley et al., 2019). Interestingly, NRF2-mediated oxidative stress response and NF-kB activation are highly enriched pathways among NPAS2 and SIRT1's gene targets. Both pathways are known to mitigate oxidative stress and promote redox homeostasis through antioxidant expression (Mauro et al., 2011; Ma, 2013; Vomund et al., 2017; Lingappan, 2018). Levels of NAD+ are directly correlated with the redox state of the cell (Ying, 2008) and can directly impact circadian molecular clock function, either through direct actions on CLOCK or NPAS2/BMAL1 (Rutter et al., 2001) or through SIRT1 (Asher et al., 2008; Nakahata et al., 2008). Our findings that cocaine exposure significantly upregulates and disrupts diurnal variation of NAD+ levels and members of the SIRT1/NAMPT

transcriptional-enzymatic feedback loop might suggest a mechanism by which cocaine directly modulates molecular clock function through NPAS2. However, while our findings suggest NPAS2 and SIRT1 do interact in the same protein complex in the NAc, future investigation would be needed to determine whether this upregulation of NAc NAD+ levels leads to altered molecular clock function through direct impacts on the clock, through SIRT1, or both. Nevertheless, our findings underscore the complexity of the bidirectional relationship between cellular metabolic state and the circadian molecular clock, while also revealing this interaction may be necessary in the NAc.

While work from our lab and others in the VTA suggest SIRT1 interacts with CLOCK to regulate dopaminergic activity via NAMPT driven NAD+ biosynthesis (Kong et al., 2018; Logan et al., 2019), it was unclear if this would hold true with CLOCK's functional homolog NPAS2 and/or in the NAc, a region downstream of the VTA. The findings presented in this study are the first to show that NPAS2 and SIRT1 interact in the same protein complex and together they are important for regulating cocaine reward. However, further mechanistic investigation into how this interaction is modulating reward is still necessary. Interestingly, work from Nestler and colleagues, in addition to a recent study published from our lab, might suggest that regulation of synaptic plasticity is at the root of this interaction. Work from our lab has recently demonstrated the importance of NPAS2 for regulating drug-related synaptic plasticity in the NAc through cell-type-specific modulation of Drd1-medium spiny neurons at the glutamatergic inputs onto the NAc (Parekh et al., 2019). This is particularly relevant, considering SIRT1 is a key mediator of drug-related molecular and cellular plasticity through the regulation of numerous synaptic proteins and dendritic spines in the NAc (Ferguson et al., 2013). Supporting this idea, in addition to regulation of dopamine transport and synaptic transmission, regulation of actin filament bundle assembly was

the top enriched biological process among the shared gene targets between NPAS2 and SIRT1 revealed by ChIP-seq analysis. Essential to synaptic plasticity, the post-synaptic elements of excitatory synapses consist of actin-rich protrusions from dendrites called dendritic spines that adapt in shape and size in response to synaptic activity; the dynamics of dendritic spine formation and modulation are driven by regulation of actin filament bundles and actin-binding proteins (Lin and Webb, 2009; Gordon-Weeks and Fournier, 2014; Konietzny et al., 2017; Borovac et al., 2018). It has long been appreciated that drug-induced long-term changes in synaptic plasticity and spine density/morphology in the NAc are associated with addiction pathophysiology and behavior (Kauer and Malenka, 2007; Russo et al., 2010). Studies have shown actin and its cycling between filamentous and depolarized forms is immediately relevant for psychostimulant reward-related behavior (Toda et al., 2006; Shibasaki et al., 2011; Young et al., 2015; Areal et al., 2019). Perhaps together the interaction between NPAS2 and SIRT1 shown in this study may be important for regulating drug-related synaptic transmission and plasticity in the NAc, and thus drug reward; however, future investigation into this idea is still needed.

While this study focused on NPAS2 and SIRT1 in the NAc, a role for CLOCK in the NAc is still possible. Though NPAS2 is highly enriched in the mouse forebrain and specifically within the striatum and NAc (Garcia et al., 2000), CLOCK is still expressed in these regions (Falcon et al., 2013; Ozburn et al., 2015). Outside of the NAc, complete loss of CLOCK can be compensated by NPAS2 and vice versa due to independent interactions with BMAL1 to regulate the molecular clock (DeBruyne et al., 2007; Bertolucci et al., 2008; Landgraf et al., 2016). While CLOCK and NPAS2 may be regulating molecular clock function similarly, their differential contribution to cocaine reward highlights their divergence in functions outside of the transcription-translation feedback loop. While *Clock* mutant mice show increased cocaine reward (McClung et al., 2005;

Ozburn et al., 2012), *Npas2* mutant mice show decreased cocaine reward that is recapitulated through knock-down of *Npas2* in the NAc (Ozburn et al., 2015); knock-down of *Clock* in the NAc fails to produce an effect on cocaine reward (Ozburn et al., 2015) – further supporting the notion that NPAS2 and CLOCK serve different roles in regulating reward, especially within the NAc. However, despite these differences in cocaine preference, a recent study from our lab found similarities in operant cocaine self-administration. Much like how *Clock* mutant mice show increased cocaine self-administration and motivation (Ozburn et al., 2012), *Npas2* mutant mice also show increased intravenous cocaine self-administration, but in both a sex- and time-specific manner (DePoy et al., 2020). Considering these new findings and the limitations associated with our current study utilizing only male mice, future studies will benefit from including both sexes in the continued investigation of NPAS2 in the NAc. Finally, while an interaction between CLOCK and SIRT1 in the VTA is important for regulating dopaminergic activity and reward (Logan et al., 2019), whether CLOCK and SIRT1 interact in the NAc and whether this interaction may be important remains to be investigated. Future studies may continue to tease apart the role of NPAS2 versus CLOCK in the NAc, and how their interaction with SIRT1 may differ.

Ultimately, the results of this study demonstrate that NPAS2 and SIRT1 together play an important role in regulating both cocaine's actions and cocaine reward-related behavior. NPAS2 and SIRT1 interact in the NAc and introduce a potential mechanism by which cocaine's effects on the cellular metabolic state may affect circadian regulation of the NAc and downstream reward function. These proteins together may have important implications for the development of substance use disorders and future investigation into the therapeutic potential of targeting NPAS2 and SIRT1 for novel treatments is necessary.

3.6 Acknowledgments

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4.0 Astrocyte Molecular Clock Function in the Nucleus Accumbens is Important for Reward-Related Behavior in Mice

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4.1 Overview

Substance Use Disorder (SUD) is a widely prevalent and devastating disease associated with disruptions in both sleep and circadian rhythms. Work from our lab and others have extensively demonstrated the functional importance of circadian molecular clock function for reward regulation in mice. However, the investigation into this phenomenon has primarily been focused within whole reward regions of the brain, like the nucleus accumbens (NAc), or within neurons exclusively. No studies to date have explored the role of circadian *astrocyte* function specifically in the NAc and/or its role in reward. Astrocytes are a highly abundant CNS glial cell type essential for numerous modulatory and support functions, including regulation of circadian rhythms. Notably, loss of astrocyte function in the NAc has also been implicated in the development of addiction-like behavior; however, an investigation into specific mechanisms is still necessary. Here, we investigate astrocyte circadian rhythmicity in the NAc and how a loss of astrocyte molecular clock function affects NAc function and NAc-regulated behaviors in mice. Through NAc astrocyte-specific RNA-sequencing across time of day, we determined ~43% of the

astrocyte transcriptome is rhythmic and key metabolic pathways were enriched among the top rhythmic genes. To test the functional relevance of this rhythmicity, mice with a viral-mediated loss of molecular clock function specifically in NAc astrocytes were assessed across a panel of behaviors testing locomotor response to novelty, exploratory drive, natural reward, and motivation. Strikingly, mice with a loss NAc astrocyte circadian function showed a significant increase in locomotor response to novelty and exploratory drive across five separate assays, and a significant increase in operant food self-administration levels and motivation. Most notably, a preliminary investigation into molecular mechanisms underlying this phenotype revealed whole NAc expression changes in genes important for metabolic homeostasis, along with significant downregulation of both lactate and glutathione levels in the NAc. Taken together, these findings highlight a novel role for circadian astrocyte function in the regulation of the NAc and reward-related behaviors.

4.2 Introduction

Addiction, or substance use disorder (SUD), remains a devastating public health issue in the United States (Hedegaard et al., 2020; National Institute on Drug Abuse, 2020a). With a lack of successful therapeutics (National Institute on Drug Abuse, 2020b), the investigation into underlying mechanisms is critical for the development of novel treatment options. One potential mechanism may be through disruption of circadian rhythms. While it has long been known that individuals with SUDs have disrupted circadian and sleep/wake rhythms (Hasler et al., 2012a; Angarita et al., 2016), there is a growing body of evidence suggesting a bidirectional relationship

between circadian rhythm disruption and substance abuse. Both human and animal studies indicate disruptions to circadian rhythms and sleep/wake may increase SUD vulnerability (Logan et al., 2014). Specifically, changes at the molecular level of the circadian system may play an essential role in the establishment and/or reinforcement of drug addiction.

In mammals, circadian rhythms are controlled by a transcriptional/translational feedback loop (TTFL) that cycles every 24 hours (Reppert and Weaver, 2002). This 'molecular clock' consists of the core circadian transcription factors CLOCK or NPAS2, which form a complex with BMAL1 and drive the expression of the proteins Period (PER) and Cryptochrome (CRY). PER and CRY heterodimerize in the cytosol, translocate into the nucleus, and interact with CLOCK/NPAS2:BMAL1 to inhibit their own transcription. This molecular clock is in nearly every cell throughout the body and is organized into a synchronous, hierarchical system by a "central clock" in the suprachiasmatic nucleus (SCN) of the hypothalamus. This SCN-regulated circadian system ultimately works to control physiology and behavior in a time-dependent manner (Partch et al., 2014; Takahashi, 2017). Notably, disrupted rhythmicity through mutations in the circadian molecular clock genes has been associated with altered drug reward. In humans, variants in the *BMAL1*, *PER,* and *CLOCK* are associated with increased cocaine use, alcohol consumption, and reward dependence (Spanagel et al., 2005; Comasco et al., 2010; Kovanen et al., 2010; Sjöholm et al., 2010; Dong et al., 2011; Shumay et al., 2012; Tsuchimine et al., 2013; Bi et al., 2014). In mice, mutations in the *Per* genes alter both cocaine locomotor sensitization (C-LMS) and cocaine conditioned place preference (CPP)(Abarca et al., 2002). Additionally, our lab has extensively shown the importance of CLOCK and NPAS2 for reward regulation in mice. Mice carrying a functional mutation in *Clock,* the *Clock*Δ19 mouse model, show more significant cocaine CPP and self-administration, increased C-LMS, and increased reward value for cocaine (McClung et al.,

2005; Roybal et al., 2007; Ozburn et al., 2012). While mice carrying a global null mutation in *Npas2*, a functional homolog to *Clock,* show decreased cocaine CPP, but increased exploratory drive and increased cocaine self-administration and motivation to self-administer (Ozburn et al., 2015, 2017; DePoy et al., 2020). This is likely due to NPAS2's enriched expression in D1 medium spiny neurons (MSNs) of the nucleus accumbens (NAc) (Garcia et al., 2000; Ozburn et al., 2015), a region highly involved in regulating reward and motivation (Mannella et al., 2013; Scofield et al., 2016a). Taken together, these studies highlight the role a functional molecular clock and its core genes may play in regulating cocaine's effects and related behavior. However, most research to date has focused mainly on molecular clock function in neurons specifically or in whole reward regions. Interestingly, a growing body of work suggests *astrocytes* play a critical role in both regulating circadian rhythms and reward processing (Scofield and Kalivas, 2014; Kim et al., 2018).

Astrocytes are a highly abundant glial cell type essential for many modulatory and support functions across the central nervous system (Sofroniew and Vinters, 2010), including regulation of glutamate levels and neurometabolic homeostasis (Danbolt, 2001; Malarkey and Parpura, 2008; Bolaños, 2016; Gonçalves et al., 2018). Like neurons, astrocytes also contain a circadian molecular clock (Prolo et al., 2005; Yagita et al., 2010; Chi-Castañeda and Ortega, 2016), and core astrocyte functions have been shown to be regulated by clock genes (Gwak et al., 2007; Beaulé et al., 2009; Marpegan et al., 2011). Interestingly, several recent studies have demonstrated astrocyte rhythmicity is also important for regulating overall circadian rhythmicity. First, a study from the Hastings lab demonstrated SCN astrocytes show anti-phasic rhythmicity relative to SCN neurons, whereby they suppress neuronal activity during the night via regulation of extracellular glutamate (Brancaccio et al., 2017). This anti-phasic relationship was found to be important for not only regulating SCN circadian timekeeping, but also circadian patterns of locomotor activity in mice

(Brancaccio et al., 2017, 2019). These findings were further supported by a study from the Herzog lab, in which they demonstrated loss of *Bmal1* specifically in SCN astrocytes altered circadian rhythmicity of both SCN clock gene expression (*in vitro* and *in vivo*) and wheel-running activity in mice (Tso et al., 2017). Most notably, SCN astrocyte rhythmicity may also regulate the rhythmicity and function of regions outside of the SCN. In addition to showing altered SCN rhythmicity and wheel running, another study demonstrated loss of *Bmal1* in SCN astrocytes both alters GABA signaling and significantly dampens molecular clock rhythmicity in cortical and hippocampal regions (Barca-Mayo et al., 2017). Strikingly, these mice also displayed significant memory impairments across short-term, long-term, and spatial memory tasks (Barca-Mayo et al., 2017). Finally, *Bmal1* deletion in cortical astrocytes was also shown to regulate astrocyte activation via a glutathionylation-dependent mechanism; however, its functional relevance for behavior was not investigated (Lananna et al., 2018). Taken together, these studies highlight a novel role for astrocytes in the regulation of circadian rhythms through their dynamic interaction with neurons in the SCN. However, the importance of astrocyte circadian functions outside of the SCN still requires further investigation. More specifically, no studies to date have investigated NAc astrocyte rhythmicity and/or its potential role in NAc function and reward-related behavior.

Here, we demonstrate for the first time that the astrocyte transcriptome in the NAc is highly regulated in a circadian manner, with ~43% of detected genes showing rhythmicity. Notably, pathway analyses of NAc astrocyte-specific RNA-seq across time of day revealed circadian rhythm signaling, circadian regulation of gene expression, NRF2-mediated oxidative stress, and ATPase Activity pathways and processes were enriched among the most significantly rhythmic genes. Interestingly, in testing the functional significance of this circadian regulation, mice with a loss of circadian astrocyte function in the NAc show significant alterations in reward-related

behavior. More specifically, through a viral-mediated astrocyte-specific functional ablation of *Bmal1* in the NAc*,* mice show increased locomotor response to novelty, increased exploratory drive, and increased food self-administration motivation in an operant task. Notably, a preliminary investigation into molecular mechanisms driving this phenotype revealed significant alterations in metabolic gene expression and disruptions in both lactate and glutathione levels in the NAc.

4.3 Materials and Methods

4.3.1 Animals

Behavioral experiments utilized male and female *Bmal1* floxed (BMFL) mutant mice, ages 10-14 weeks old (*Bmal1*^{lox}, B6.129S4-Arntltm1Weit/J; Stock No: 007668, The Jackson Laboratory; Bar Harbor, ME). When Cre recombinase is expressed in BMFL mice, the exon encoding BMAL1's basic helix-loop-helix (bHLH) functional domain is deleted, resulting in loss of circadian clock regulated rhythmicity (Storch et al., 2007). Rhythmicity characterization experiments utilized male and female Aldh1l1-eGFP mice (JAX; RRID:IMSR_JAX:030247) expressing an eGFP fluorophore under the *Aldh1l1* promotor, an established pan-astrocyte marker (Cahoy et al., 2008; Yang et al., 2011; Zhang et al., 2014b). All mice were maintained on a 12:12 light-dark cycle (lights on: 0700 zeitgeber time (ZT) 0; lights off: 1900, ZT12) and provided *ad libitum* food and water access unless otherwise indicated. Animal use was conducted in accordance with guidelines set by the National Institutes of Health (NIH), and all procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC).

4.3.2 Viral-Placement Surgery and Verification

BMFL mice were injected at 8 weeks of age with either AAV8-GFAP-eGFP or AAV8-GFAP-Cre-GFP viral vectors (UNC Viral Vector Core; Chapel Hill, NC; RRID:SCR_002448) into the NAc following protocols previously published (Ozburn et al., 2015). Briefly, male and female BMFL mice under isoflurane anesthesia were given bilateral stereotaxic 1 µl injections into the NAc (Bregma: AP +1.5, ML ±1.5, DV -4.4 mm; angle 10°) of respective purified high titer adeno-associated virus. To allow for sufficient viral expression, mice were allowed at least 2 weeks to recover. Expression of Cre recombinase in the NAc under the *Gfap* promoter induces a functional ablation of BMAL1 specifically in astrocytes and results in loss of astrocyte rhythmicity, as previously demonstrated and validated by Lananna et al. (2018) and Tso et al. (2017). Viral placement, spread, and specificity were verified qualitatively using immunofluorescence (IF). In short, mice were perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) at pH 7.4, and brains were isolated for post-fixation plus 30% sucrose saturation. Brains were then cryo-sectioned at 40 microns before processing for IF. Green Fluorescent Protein (GFP) signal was enhanced using a primary anti-GFP antibody (ab13970; Abcam, Cambridge, UK; RRID:AB_300798), and specificity was verified against anti-BMAL1 (NB100-2288; Novus Biologicals; Centennial, CO; RRID:AB_10000794) or anti-GFAP (NBP1-05197; Novus; RRID:AB_1555288), as previously validated (Lananna et al., 2018). Sections were mounted on slides with VECTASHIELD mounting medium plus DAPI (Vector Laboratories; Burlingame, CA, USA; RRID:AB_2336788). Sections were imaged at 10x and 20x magnification using an Olympus Fluoview FV1200 confocal microscope (Olympus Corporation; Shinjuku, Tokyo, Japan).

4.3.3 Behavioral Testing

A battery of behavioral tests assessing reward-related behaviors was conducted with both BMFL-NAc: GFAP-Cre mice and eGFP control mice. Behavioral testing was conducted between ZT2-6, with at least 30 minutes of habituation to the room prior to testing.

Locomotor Response to Novelty (LRN). Mice were placed individually in 25 x 45 cm plexiglass boxes equipped with photo beams (Kinder Scientific Smart Cage Rack System; Poway, CA). Distance traveled (cm) was recorded for 2 hours in 5-minute bins, as well as overall total distance.

Open Field (OF). Under 20 lux lighting, mice were placed individually in a large black plexiglass arena (52 x 52 x 25 cm) and allowed to explore for 10 min freely. Behavior was recorded and analyzed using Ethovision XT 13 (Noldus; Leesburg, VA), calibrated by blind hand scoring. Time spent in the center of the arena (24 x 24 cm square in the center), center entries, and distance traveled were recorded.

Light / Dark Box (L/D). Mice were placed individually in a 25 x 45 cm plexiglass arena equipped with photo beams, divided into two equally sized chambers (Kinder Scientific Smart Cage Rack System; Poway, CA) – one side brightly lit (~880 lux) and the other containing a black opaque box. Mice were placed on the dark side for 2 min before a door opened, allowing mice to explore both sides for 20 minutes freely. Distance traveled, light entries, and time spent in the light side were all recorded.

Elevated Plus Maze (EPM). Under 20 lux lighting, mice were placed in the center of an elevated plus maze (81 cm off the ground), consisting of 2 open arms and 2 enclosed arms oriented perpendicularly (each 30 x 5 cm). Mice started facing an open arm and were allowed to explore

the maze for 10 minutes freely. Behavior was recorded and analyzed using Ethovision XT 13, calibrated by blind hand scoring. Arm entries (both open and closed), time spent in the open arm, and distance traveled were recorded.

Novelty Suppressed Feeding (NSF). Mice were food deprived exactly 24 hours prior to the start of the NSF task. On test day, were placed individually in the corner of a brightly lit (700-800 lux) large black plexiglass arena (52 x 52 x 25 cm) filled with fresh bedding and a pellet of fresh food placed in the center of the arena. The mouse was allowed to explore the arena for 12 minutes *or* until the mouse takes a bite of the food. Following test completion, the mouse was returned individually to its home cage and allowed free access to 1 pellet of food for 8 minutes. Latency to eat was recorded during the test by a blind-trained scorer. Post-test food consumption (grams) and total weight loss were recorded as controls for any potential feeding differences.

Operant Food Self-Administration (Food SA). Mice were initially food-restricted overnight and then maintained at 85-90% free-feeding weight throughout the behavior. For all operant testing, mice were placed individually in an operant conditioning chamber equipped with a pellet dispenser, a food trough with 2 levers on either side, a cue light above each lever, and an overhead chamber light (Med Associates Inc; Fairfax, VT). To test food self-administration, mice were first trained on a fixed ratio (FR) 1 schedule (1 press = 1 pellet) in daily 1 hour sessions or until mice consumed 30 pellets (chocolate-flavored 20 mg, grain-based precision pellets; Bio-Serv; Flemington, NJ). During the session, the overhead chamber light and active lever cue light were continuously lit until the end of the session. Mice were considered to have achieved acquisition criteria if they reached ≥ 25 pellets for 3 sessions in a row. After 7 sessions of food self-administration testing, motivation was then tested by increasing mice to FR3 for 3 sessions, FR5

for 2 sessions, and then FR10 for 2 sessions. Mice were returned to free-feeding weight following operant training.

4.3.4 RNA isolation and RT-qPCR for Gene Expression

BMFL-NAc: GFAP-Cre and eGFP control mice were sacrificed and the NAc was quickly micro-dissected before being snap-frozen on dry ice. Total RNA was isolated using the RNeasy Plus Mini Kit (Qiagen; Hilden, Germany), and 100ng was converted to complementary DNA (cDNA) using the iScript cDNA synthesis kit (Bio-Rad; Hercules, CA). Quantitative polymerase chain reaction (qPCR) was then used to measure gene expression of metabolic and astrocyte genes of interest. Briefly, 1 ng of cDNA was mixed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad; Hercules, CA, USA) and both forward and reverse primers. Samples were run in triplicate in a 96-well plate, performed using a CFX96 Touch Real-Time PCR detection system (Bio-Rad). The following primers were utilized (**Table 2**):

Table 2. Astrocyte and Metabolic Gene Expression Mouse Primers

Primer	Forward (5' – 3')	Reverse (5' – 3')
18s	ACCGCAGCTAGGAATAATGGA	GCCTCAGTTCCGAAAACCA
Aldh1l1	CCAGCCTCCCAGTTCTTCAA	GGACATTGGGCAGAATTCGC
Gfap	GAAACCAACCTGAGGCTGGA	CCACATCCATCTCCACGTGG
Nrf2	TTCTTTCAGCAGCATCCTCTCCAC	ACAGCCTTCAATAGTCCCGTCCAG
Pgc1a	TCACACCAAACCCACAGAAA	TCTGGGGTCAGAGGAAGAGA
Gclc	ACATCTACCACGCAGTCAAGGACC	CTCAAGAACATCGCCTCCATTCAG
Ldha	AGGTTACACATCCTGGGCCATT	TCAGGAGTCAGTGTCACCTTCACA
Mct1	CATTGGTGTTATTGGAGGTC	GAAAGCCTGATTAAGTGGAG
Mct2	CACCACCTCCAGTCAGATCG	CTCCCACTATCACCACAGGC;

The comparative cycle threshold (ct) method (i.e., $2^{-\Delta\Delta CT}$ method) was used to calculate relative gene expression (Schmittgen and Livak, 2008), normalized to the 18s reference gene. Relative gene expression values were reported as the mean normalized Ct value ± SEM.

4.3.5 Immunoprecipitation (IP) and RNA extraction from NAc Astrocytes

NAc tissue was collected from both male and female Aldh1l1-eGFP/Rpl10a (B6;FVB-Tg(Aldh1l1-EGFP/Rpl10a)JD133Htz/J; JAX Stock No: 007668; RRID:IMSR_JAX:030247) mice across 6 times of days (ZT2,6,10,14,18, & 22; 10M/8F per ZT). Immunoprecipitation of polyribosomes from Aldh1l1-eGFP/Rpl10a was processed using a modified protocol similarly described previously (Sanz et al., 2009; Chandra et al., 2015). For each sample, the NAc from 2

mice were pooled and homogenized in a Dounce homogenizer with a buffer containing 50 mM Tris-HCl, 100 mM KCl, 12 mM MgCl2, and 1% NP-40, supplemented with 1 mM DTT, 100 μg/ml cycloheximide (Sigma-Aldrich; St. Louis, MO), RNasin® Plus ribonuclease inhibitor (Promega; Madison, WI), and EDTA-free protein inhibitor cocktail (Roche; Basel, CH). Following a 10 minute 10,000g centrifugation step, the supernatant was transferred to low-protein binding tubes and mixed with 20 μg of anti-GFP antibody (ab290; Abcam; Cambridge, UK), rotating overnight at 4°C. The following day, 12 mg of Dynabeads Protein G (Invitrogen) were washed once in non-supplemented homogenization buffer and then added to antibody-bound lysate samples for an additional overnight incubation step at 4°C. Finally, beads were washed 3 times in a high salt buffer containing 50 mM Tris-HCl, 300 mM KCl, 12 mM MgCl2, 1% NP-40, 1 mM DTT, and 100 μg/ml cycloheximide. Elution of polyribosomes from beads was performed in TRK lysis buffer + β-ME provided in the Omega Micro Elute Total RNA kit (Omega Bio-Tek; Norcross, GA). The supernatant was removed from beads using the DynaMag-2 magnetic rack (Invitrogen) and then processed for RNA following the kit provided manufacturer instructions. Final eluted NAc astrocyte-specific RNA was used for RNA-sequencing.

4.3.6 Library preparation and next-generation RNA sequencing

Prior to library preparation, isolated RNA samples were quantified by fluorometry using the Qubit RNA High Sensitivity assay (Qubit 4 Fluorometer; Invitrogen; Carlsbad, CA; RRID:SCR_018095) and assessed for quality/integrity with chromatography using the Agilent RNA 6000 Pico Kit (2100 Bioanalyzer; Agilent, Santa Clara, CA; RRID:SCR_018043). Across the 54 pooled samples, the average RNA integrity number (RIN) was ~7, with an average

concentration of ~18 ng/µl. Library preparation was performed with >100 ng of RNA from each sample using the TruSeq Stranded Total RNA Kit (Illumina; San Diego, CA), as per the manufacturer's instructions. Briefly, Total RNA input was depleted for rRNA and fragmented. Random primers initiated first and second-strand cDNA synthesis. Adenylation of 3' ends was followed by adapter ligation and library amplification with indexing. One sample from ZT 14 did not generate a library and was therefore excluded. Single-read 75bp sequencing at 40 million reads per sample was performed using the Illumina NextSeq 500 platform through the University of Pittsburgh Health Sciences Sequencing Core at UPMC Children's Hospital of Pittsburgh.

4.3.7 RNA-sequencing Analysis

Pre-Processing. FastQC v0.11.7 (https://bioinformatics.babraham.ac.uk/projects/fastqc/) was performed to assess the quality of the data. Per base sequence quality was high (Quality score generally > 30), indicating good data quality. HISAT2 (HISAT2 v2.1) was then used to align reads to the reference genome (Mus musculus Ensembl GRCm38 – mm10) using default parameters (Kim et al., 2019). The resulting bam files were converted to expression count data using HTSeq (HTSeq v0.10) with default union mode (Anders et al., 2015). One sample from ZT 18 was removed due to its consistently low expression. RNA-seq count data were transformed to log2 continuous counts per million (cpm) data using the cpm function of the Bioconductor edgeR package (Robinson et al., 2010; McCarthy et al., 2012). Genes were retained for analysis if log2(cpm) was >1 in 50% or more of samples. All Y-chromosome genes were also removed from the analysis. After filtering, 12,739 genes remained for further analysis.

Rhythmicity Detection. Across the 54 pooled samples (9 samples per 6 ZT), the JTK_CYCLE package for R was used to detect circadian rhythmicity of NAc astrocyte-specific genes, with parameters set to 22-26hr to fit time-series data to periodic waveforms (RRID:SCR_017962) (Hughes et al., 2010; Wu et al., 2014). The JTK_Cycle statistical algorithm utilizes a non-parametric harmonic regression / sinusoidal fit test to output both a Bonferonni-Adjusted p-value (Adj.P) and a Benjami-Hochberg q-value (BH.Q), distinguishing between rhythmic and non-rhythmic transcripts. This algorithm also assesses other circadian parameters, including period, phase, and amplitude. From the 12,739 genes, 5527 transcripts were determined to rhythmic at the $p<0.05$ Adj.P cutoff, and 1,131 transcripts at the more statistically rigorous BH.Q cutoff. A heatmap of the top 200 circadian transcripts was generated using the gplots R package (v.3.1), where the expression levels for each top gene were Z-transformed, ordered on the y-axis descending by significance, and each column along the x-axis representing a sample ordered by phase (i.e., ZT). A radar plot of phase distribution across all rhythmic transcripts ($p<0.05$; 5,527) was created using DisplayR online data analysis and visualization software (https://www.displayr.com/).

4.3.8 Enriched Pathways and Biological Processes Analysis

Both Ingenuity Pathway Analysis (IPA) software (QIAGEN; Hilden, Germany; RRID:SCR_008653) (Krämer et al., 2014) the online bioinformatics database Metascape (https://metascape.org/; RRID:SCR_016620) (Zhou et al., 2019a) were used to identify enriched molecular pathways and processes in both the top enriched (200 genes with highest Log2CPM) and top rhythmic (BH $q<0.0001$) gene lists. For both analyses, gene lists were analyzed as follows:

(1) after filtering, the remaining 12,739 annotated genes were used as the background/reference gene list; (2) the lists of top 200 enriched genes (highest Log2CPM) and 1,141 top rhythmic genes ($q<0.0001$) were used as input lists for both IPA and Metascape analyses; and (3) for identification of significant pathways and processes, a significance threshold of $p<0.05$ (or a -log10(p-value) of 1.3 in figures) was utilized. IPA software was used to identify enriched canonical molecular pathways, while Metascape was used to identify enriched biological processes using only *Gene Ontology (GO) Biological Processes* as the ontology source. Within Metascape, all statistically enriched terms, accumulative hypergeometric p-values, and enrichment factors were automatically calculated and used for filtering. The remaining significant terms were then hierarchically clustered into a tree based on Kappa-statistical similarities among their gene memberships, with a 0.3 kappa score applied as the threshold to cast the tree into term clusters. The most significant term in each cluster served as the cluster title. (Zhou et al., 2019a)

4.3.9 RNAscope® in situ hybridization (ISH)

For qualitative validation of Aldh1l1-eGFP/Rpl10a mice, the RNAscope in situ hybridization (ISH) Fluorescent Multiplex Assay (cat# 320850; Advanced Cell Diagnostics; Newark, CA) was utilized. First, brains were rapidly removed, flash-frozen, and stored at -80C until further processing. Brains were then cryo-sectioned at 14 microns to isolate NAc containing sections, mounted on slides, and processed using the RNAscope Fluorescent Multiplex kit following the company provided protocol. Briefly, mounted tissue sections were fixed in 4°C chilled 10% normal buffered formalin for 30 minutes, followed by 1xPBS washing and a series of ethanol dehydration steps (50,70, & 100% EtOH) at room temperature. Following the addition of

an ImmEdge® hydrophobic barrier (Vector Labs; Burlingame, CA; RRID:AB_2336517), tissue sections were then hybridized with catalog probes against *Aldh1l1*, *eGFP*, and *Slc32a1* (ACD cat#405891, #400281-c3, & #319191-c2, respectively) at 40°C for 2 hours in the ACD HybEZ™ Hybridization Oven (Cat#310010). Amplification of signal was performed through 40°C incubation with sequential amplifiers AMP1-3 and AMP4 Alt-C was used to detect *Aldh1l1* in Atto 550, *eGFP* in Alexa 488, and *Slc32a1* (i.e., *Vgat*) in Atto 647. Sections were washed in the kit provided 1x wash buffer before being counterstained with DAPI and cover-slipped with ProLong™ Gold Antifade Mountant (#P36930; Invitrogen; Carlsbad, CA). Slides were stored at 4°C overnight protected from light before imaging using an Olympus Fluoview FV1200 confocal microscope (Olympus Corporation; Shinjuku, Tokyo, Japan). Images of the NAc were captured sequentially by frame at 60x magnification with 5-6 x 2.2 µm z-stacks, an 8.0 µs/pixel sampling speed, and 1024 x 1024 (pixel) image dimension. For representative images, the z-axis was collapsed into a single plane to show all signal across channels.

4.3.10 Lactate and GSH Assays

BMFL-NAc: GFAP-Cre and eGFP control mice were sacrificed across 2 times of day (ZT5 & ZT17) and the NAc was quickly micro-dissected before being snap-frozen on dry ice. NAc tissue was then used to measure levels of both L(+)-Lactate and glutathione (GSH) utilizing established and validated colorimetric and luminescent assays (Haskew-Layton et al., 2010; Baxter et al., 2015; Lerchundi et al., 2015; Muraleedharan et al., 2020). For measurement of NAc lactate concentration, 75 µg supernatant from homogenized NAc tissue was processed through the BioVision Lactate Colorimetric/Fluorometric Assay (BioVision; Milpitas, CA), as per the supplied

manufacturer instructions. Samples were run in triplicate in a clear 96-well plate using an Epoch microplate spectrophotometer (BioTek; Winooski, VT). In this assay, sample lactate is the substrate for an enzyme mix that generates a product, which then reacts with a probe to produce a color detected by measuring absorbance (OD 570 nm). Lactate concentrations were calculated using a standard curve and manufacturer-provided formula. For measurement of NAc GSH concentration, 75 µg of supernatant from homogenized NAc tissue (pre-treated with 2mM EDTA and 1mg/ml heparin) was processed through the GSH-Glo™ Glutathione Luminescence Assay (Promega; Madison, WI), as per manufacturer instructions. Samples were run in triplicate in an opaque 96-well plate using an FLx800 fluorescence-luminescence microplate reader (BioTek; Winooski, VT). In this assay, a luciferin derivative is converted to luciferin in the presence of sample GSH, catalyzed by glutathione-S-transferase. The luciferin generated is the substrate for luciferase, whereby the light generated is directly proportional to the amount of GSH. GSH concentrations were calculated using a standard curve. For both lactate and GSH, levels are reported as concentrations (nmol) divided by the input (µg).

4.3.11 Statistical Analyses

GraphPad Prism 9 software (GraphPad Software; San Diego, CA, USA; RRID:SCR_002798) was utilized for all statistical data analyses. For statistical analysis of more than two groups, one-way, two-way, or three-way analysis of variance (ANOVA) was utilized with significant interactions followed by Bonferroni *post-hoc* tests corrected for multiple comparisons. Repeated measures were employed where appropriate. Statistical analysis of just two groups was performed using a Student's t-test. Across all data analyses, outliers were tested for

using the Grubb's test. Statistical analyses tested for sex as a variable; throughout, males and females are grouped together unless a main effect of sex was detected, in which case males and females are displayed separately. Z-normalization (i.e., Z-scoring) of behavioral data from exploratory drive assays was performed as published (Guilloux et al., 2011), whereby relevant parameters from each of the assays were converted to Z-scores, indicating how many standard deviations the observations were above or below the control mean. Unless otherwise noted, all data are expressed as mean ± SEM with $\alpha = 0.05$ considered statistically significant.

4.4 Results

4.4.1 The astrocyte transcriptome is highly rhythmic in the Nucleus Accumbens

Our lab has previously demonstrated whole NAc rhythmicity at both the cellular and molecular levels (Logan et al., 2015). These findings are further supported by a recent study demonstrating the whole NAc displays circadian variation at the transcriptome level (Brami-Cherrier et al., 2020). However, the rhythmicity of the NAc has yet to be studied in a cell-type-specific manner. Specifically, no studies to date have investigated NAc astrocyte rhythmicity. To investigate this, we utilized the *Aldh1l1-eGFP/Rpl10a* mouse model (Doyle et al., 2008; Sakers et al., 2017; Sapkota et al., 2019), wherein astrocytes exclusively express an eGFP tag on their ribosomal complex subunit RpL10a that allows for immunoprecipitation (IP) and isolation of astrocyte-specific ribo-associated mRNA (**Figure 11A**). We performed IP against eGFP with whole NAc tissue harvested across 6 times of day utilizing a previously published method (Sanz

et al., 2009; Chandra et al., 2015); this allowed for the isolation of astrocyte-specific mRNA, in that *eGFP* is highly-colocalized with *Aldh1l1,* a pan-astrocyte marker (Cahoy et al., 2008; Yang et al., 2011), and not with *Vgat*, a GABA transporter expressed in the GABAergic MSNs of the NAc (**Figure 11B**). Isolated mRNA was then processed for RNA-sequencing analysis, resulting in the detection of 12,739 genes identified in the NAc. Ingenuity Pathway Analysis and Metascape analysis (Zhou et al., 2019a) revealed core astrocyte-associated biological processes and canonical pathways to be enriched among the top 200 expressed genes identified (**Figure 11C**), further validating this method. Notable enriched astrocyte functions include glycolysis and lactate synthesis, oxidative phosphorylation (ATP generation), glutamate and GABA receptor signaling, neurotransmitter uptake (Glutamate), and neuroinflammation (NF-kB) signaling. These processes are historically considered among astrocytes' top functions and further underscore their potential importance in the NAc.

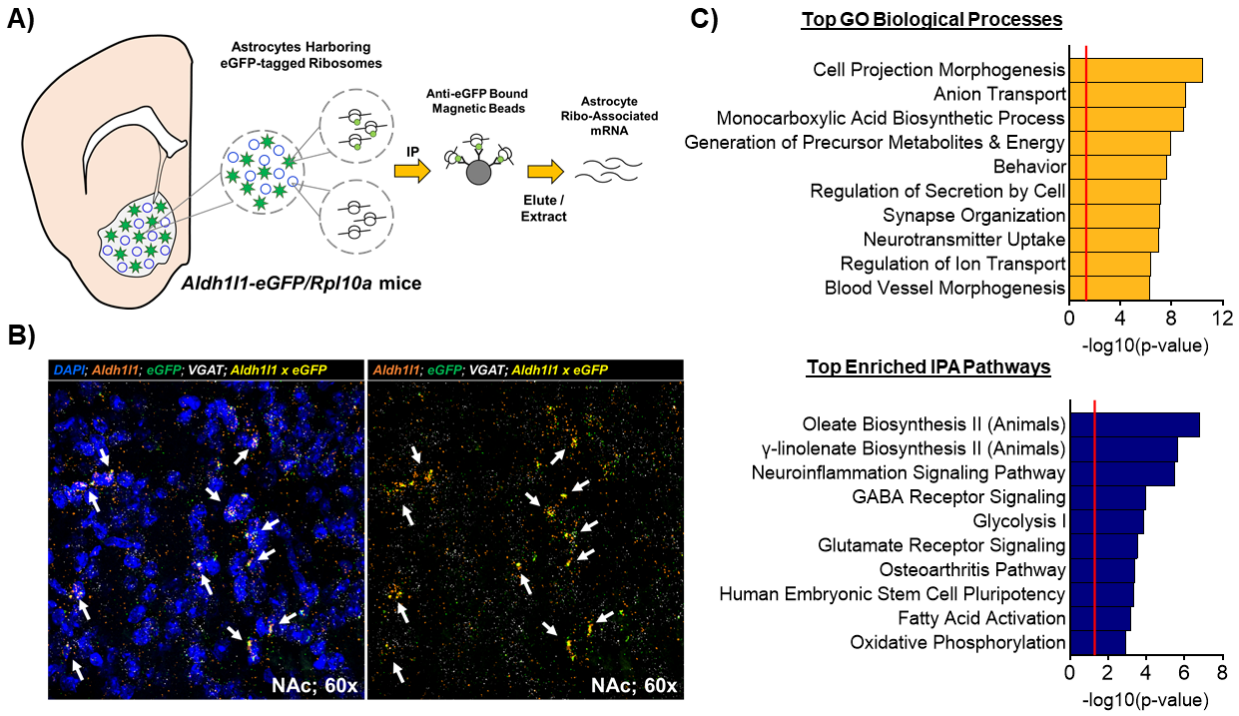

Figure 11. Aldh1l1-eGFP/Rpl10a mice allow for isolation of astrocyte-specific ribo-associated mRNA. (**A**) Schematic illustrating the immunoprecipitation (IP) of astrocyte-specific ribo-associated mRNA from whole NAc tissue of Aldh1l1-eGFP/Rpl10a mice that express an eGFP tag on the ribosomal subunit RpL10a. (**B**) The fluorophore eGFP is expressed specifically in cells expressing the pan-astrocyte marker *Aldh1l1*, but not in cells expressing the neuronal GABA transporter *VGAT,* as determined by RNAscope*;* arrows indicate cells with co-expression of *Aldh1l1* and *eGFP*. (**C**) Following RNA-seq, the top canonical pathways and biological processes enriched among the top 200 expressed genes in NAc astrocytes include key metabolic and synaptic neurotransmission functions generally attributed to astrocytes. Red line in bar graphs indicates significance threshold of $p<0.05$ or -log10(p-value) > 1.3.

In addition to looking at the top expressed transcripts in NAc astrocytes, transcripts with circadian variation in expression across the 6 times of day sampled were identified using the non-parametric statistical algorithm, JTK_Cycle (Hughes et al., 2010). Out of the 12,739 genes identified in our NAc astrocyte-specific RNA-seq dataset, we found that approximately 43.3% of transcripts display circadian rhythmicity, or 5,527 genes at the Bonferroni-adjusted p-value cutoff

of p<0.05 (**Figure 12A**). Using the most stringent statistical cutoff, we still see approximately ~9% of transcripts display circadian rhythmicity, or 1,141 genes at the Benjamini-Hochberg q-value cutoff of q<0.0001 (**Figure 12A**). Interestingly, through both radar plot and heatmap visualizations of circadian acrophase (time of day at which rhythmic transcripts show peak expression), the majority of rhythmic genes peak between ZT14 and ZT22 (9pm - 5am), coinciding with the mouse's active phase (**Figure 12B**). In addition to IPA and Metascape pathway enrichment analyses of top rhythmic transcripts (q<0.0001), including *circadian rhythm signaling* and *circadian regulation of gene expression* (**Figure 12C**), many of the top 20 rhythmic transcripts are known core circadian genes (e.g *Arntl or Bmal1*, *Per2, Nr1d1 or Rev-erbα, Ciart or Chrono, Dbp*) and their expression patterns align with previously established expression relationships (**Figure 12D**). Interestingly, alongside circadian pathways and processes, IPA and Metascape analyses also revealed important metabolic-relevant functions to be enriched among the top rhythmic genes (q<0.0001), including NRF2-mediated oxidative stress response, regulation of ATPase activity, and glucocorticoid receptor signaling (**Figure 12C**). Taken together, these data highlight the many functions astrocytes may contribute to NAc function and underscore the high degree of circadian regulation governing the NAc astrocyte transcriptome across time of day.

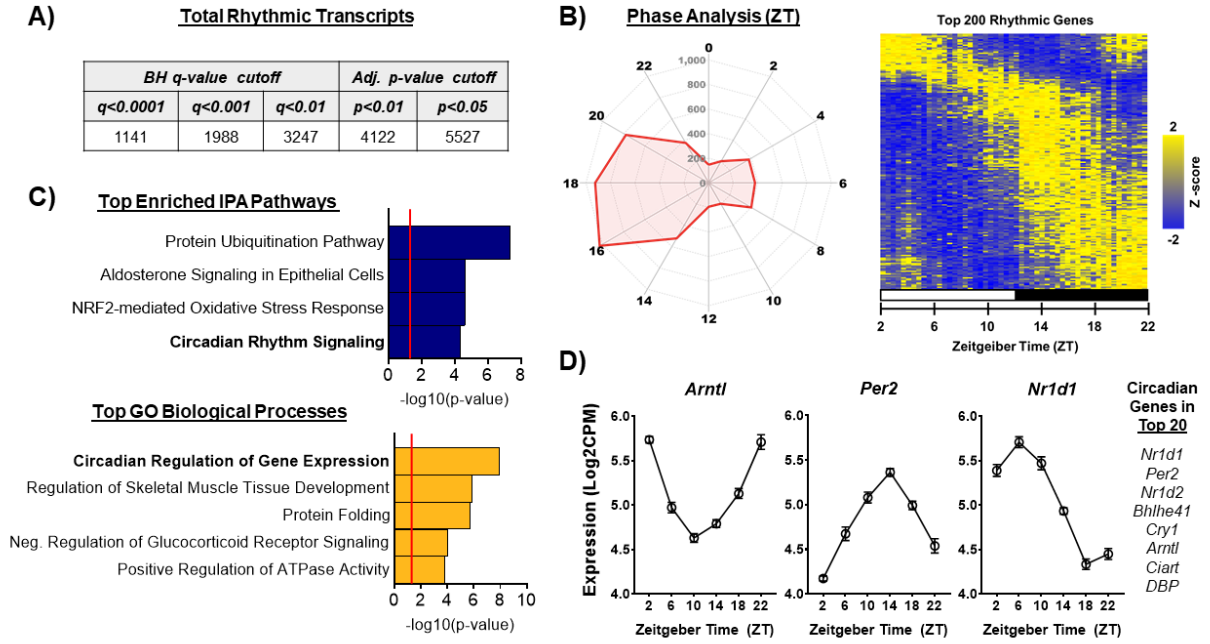

Figure 12. Nucleus accumbens astrocytes are highly rhythmic at the transcriptome level. (**A**) Among the 12,739 genes identified in NAc astrocytes, approximately 43.3% of genes exhibited rhythmic expression across the 6 ZTs sampled (ZT2,6,10,14,18,22; n=4-5 per ZT, JTK_Cycle, cutoff p<0.05). (**B**) Phase analysis through radar plotting of top rhythmic transcripts (p<0.05) and heatmap visualization of the top 200 rhythmic genes reveal a majority of rhythmic transcripts peak between ZT14 and ZT18, coinciding with the dark phase / active phase. The radar plot perimeter axis represents ZT, while the inner axis is the number of genes with peak expression at a given ZT. Heatmap displays Z-transformed expression for each of the top 200 statistically rhythmic genes (as determined by JTK_Cycle), ranked in descending order along the Y-axis (rows) and each subject ordered by ZT along the x-axis (columns). The white bar indicates lights on (ZT2,6,10) and the black bars indicate lights off (ZT14,18,22). (**C**) IPA pathway analysis and Metascape Gene Ontology (GO) Biological Process analysis revealed both circadian and metabolic-related processes enriched among the top rhythmic genes (q<0.0001). Red line in bar graphs indicates significance threshold of p<0.05 or -log10(p-value) > 1.3. (**D**) Core circadian genes are among the top rhythmic genes and show expression patterns that align with previously established functional relationships (i.e., *Arntl* anti-phasic with *Per2* and *Nr1d1*).

4.4.2 Loss of BMAL1 function in NAc astrocytes increases locomotor response to novelty

Our lab has previously demonstrated circadian molecular clock function in the NAc to be important for reward-relevant behaviors in mice through studies investigating NPAS2 (Ozburn et al., 2015, 2017; Parekh et al., 2019; DePoy et al., 2020), the core molecular clock protein highly-enriched in D_1-containing MSNs in the NAc (Garcia et al., 2000; Ozburn et al., 2015). However, no studies to date have investigated the functional relevance of astrocyte molecular clock function in the NAc. To assess this, *Bmal1* floxed mutant mice (*Bmal1*$^{f/f}$) were stereotaxically injected into the NAc with an adeno-associated virus (AAV) expressing Cre recombinase under the astrocyte-specific promoter *Gfap*; in the presence of Cre, the basic helix-loop-helix (bHLH) functional domain of BMAL1 is deleted and consequently results in loss of molecular clock function and rhythmicity (**Figure 13A,B**) (Storch et al., 2007; Tso et al., 2017; Lananna et al., 2018). To test the functional relevance of NAc astrocyte function for reward-related behavior, mice were first run through the locomotor response to novelty task (**Figure 13C**) – a behavior established as a predictor of drug-seeking and addiction-like behavior vulnerability (Stead et al., 2006; Flagel et al., 2010; Zhou et al., 2019b). Interestingly, mice with a loss of BMAL1 function in NAc astrocytes show a significantly higher locomotor response to novelty (LRN), relative to GFAP-eGFP control mice (**Figure 13C**) (Virus: $F_{(1, 36)} = 5.68$, * $p=0.02$; Time: $F_{(11, 396)} = 40.41$, $p<0.0001$; Time x Virus: $F_{(11, 396)} = 0.92$, $p=0.51$). This increase was observed both as a main effect of the virus and in the total locomotor activity in the first hour of the two-hour task (**Figure 13C** and inset) ($t_{(35)}=2.32$, * $p=0.02$). Notably, this was not seen in the second hour of the task, and this increased LRN was not due to any differences in habituation to the novel environment or differences in total

activity across the full task (**Figure 13D** and inset) (Virus: $F_{(1, 36)} = 0.0003$, n.s. p=0.98; Time: $F_{(11, 396)} = 16.66$, p<0.0001; Time x Virus: $F_{(11, 396)} = 0.26$, p=0.99; inset, $t_{(35)} = 0.46$, p=0.64).

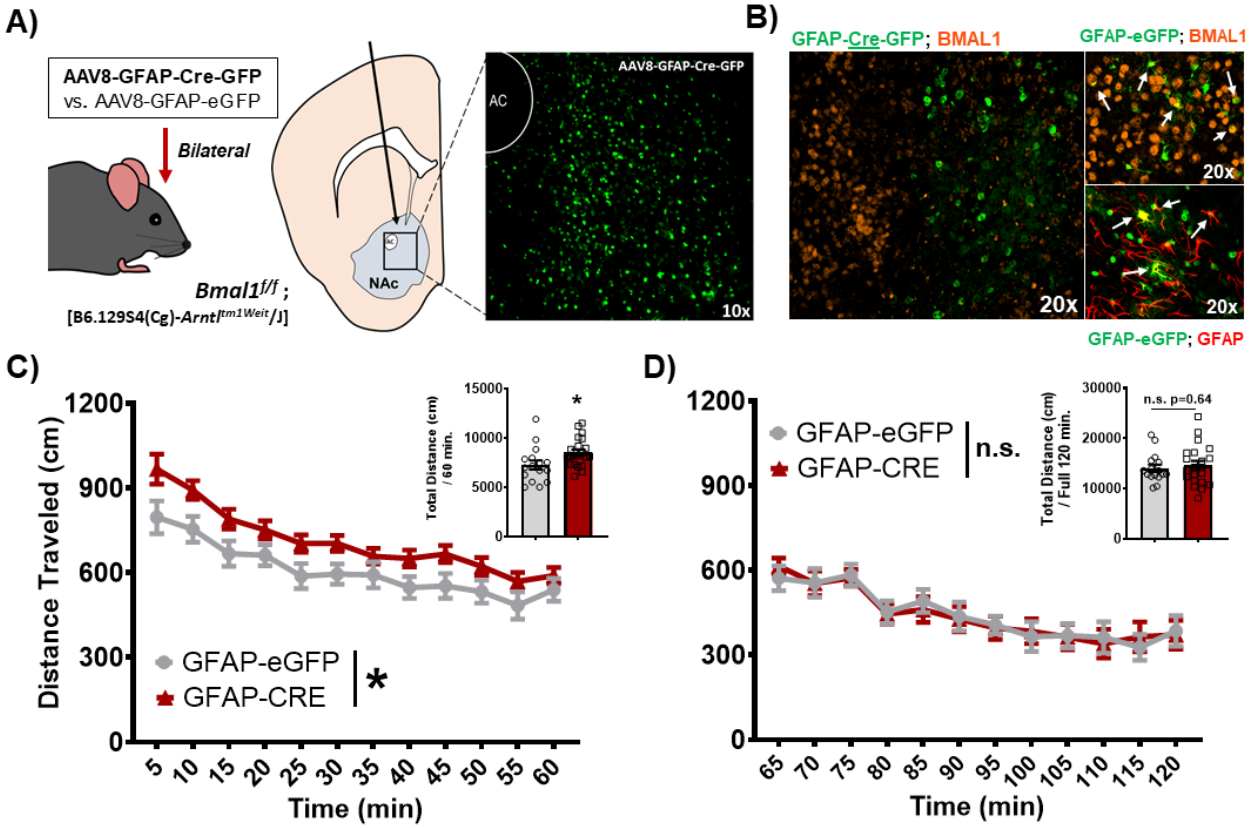

Figure 13. Loss of BMAL1 function in NAc astrocytes increases locomotor response to novelty in mice. (**A**) Schematic illustrating the bilateral stereotaxic injection of either AAV8-GFAP-Cre-GFP or GFAP-eGFP virus into the NAc of *Bmal1* floxed mutant mice; injection at AP: +1.5, ML: ± 1.5, and DV: -4.4 microns resulted in a region-specific expression of the virus in the NAc. (**B**) GFAP-Cre-GFP virus resulted in a NAc-specific loss of BMAL1 detection by immunofluorescence (IF), specifically in GFAP+ cells (i.e., astrocytes). Arrows indicate cells with co-expression. (**C**) In the locomotor motor response to novelty (LRN) task, mice with a loss of BMAL1 function in NAc astrocytes (GFAP-Cre) show a significantly greater LRN relative to control mice. (**D**) This increase in LRN is not driven by differences in habituation or overall locomotor activity across the full task (inset). Mean ± SEM; n=16-22; * p<0.05, n.s. = not significant.

4.4.3 Loss of BMAL1 function in NAc astrocytes increases exploratory drive behavior

In addition to the locomotor response to novelty, increased exploratory drive and novelty-seeking behavior are also established predictors of vulnerability for drug-seeking and addiction-like behaviors in rodent models (Stead et al., 2006; Bush and Vaccarino, 2007; Flagel et al., 2010, 2014; Dickson et al., 2015; Wingo et al., 2016). Given this association and the observed increase in LRN, we wanted also to investigate whether circadian astrocyte function in the NAc was necessary for exploratory drive. To test this, mice were run through a battery of exploratory drive / anxiety-related behavioral tasks, including open field (OF), light-dark box test (L/D), elevated plus maze (EPM), and novelty suppressed feeding (NSF). Strikingly, across all four behavioral tasks, mice with a loss of BMAL1 function in NAc astrocytes exhibit significantly elevated exploratory drive relative to controls (**Figure 14**). GFAP-Cre mice exhibit increased number of center entries in the OF (**Figure 14A**; $t_{(18)} = 1.91$; # p=0.07), greater percent time spent in the light chamber of the L/D task (**Figure 14B**; $t_{(19)} = 2.37$; * p=0.02), greater percent time spent in the open arms of the EPM (**Figure 14C**; $t_{(18)} = 2.35$; * p=0.03), and a shorter latency to eat food in the NSF task (**Figure 14D**; $t_{(22)} = 1.9$; # p=0.06). Further supporting these findings, exploratory drive was also found to be significantly greater in GFAP-Cre mice following z-normalization of the 4 complementary behaviors (**Figure 14E**; $t(11) = 8.14$, **** $p<0.0001$), a method previously shown to increase sensitivity, reliability, and robustness of behavioral phenotyping (Guilloux et al., 2011).

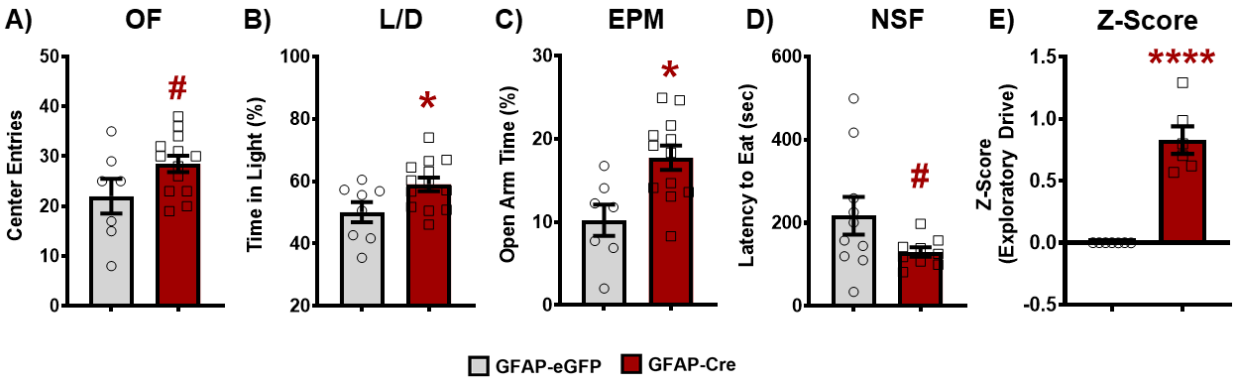

Figure 14. Loss of BMAL1 function in NAc astrocytes increases exploratory drive in mice. Mice were run through a panel of behavioral assays testing exploratory drive. (**A**) In the open field (OF) task, BMFL-GFAP-Cre mice show an increased number of center entries relative to eGFP controls. (**B**) In the light-dark box test (L/D), GFAP-Cre mice spend a significantly greater percentage of time in the brightly lit chamber of the arena. (**C**) GFAP-Cre mice also spent significantly more time in the open arms of the elevated plus maze (EPM). (**D**) Following an overnight food restriction, GFAP-Cre mice had a shorter latency to eat food in the novelty suppressed feeding (NSF) task. (**E**) Across the behavioral panel, Z-normalization of the 4 behavioral tasks revealed a loss of BMAL1 function in NAc astrocytes significantly increases exploratory drive behavior relative to control mice. Mean ± SEM; n=7-13; # $p \leq 0.07$, * $p < 0.05$, **** $p < 0.0001$.

Given that GFAP-Cre mice show a significant increase in LRN, it was important also to measure activity during the exploratory drive behavioral assays to ensure any differences seen are not being driven by locomotor differences. Notably, relative to controls, GFAP-Cre mice show no differences in locomotor activity during the OF test (**Figure 15A**; $t_{(18)} = 0.14$, p=0.88), no differences in total number of entries in the L/D test (**Figure 15B**; $t_{(16)} = 0.78$, p=0.44), and no differences in locomotor activity during the EPM task (**Figure 15C**; $t_{(18)} = 0.28$, p=0.77). Given that mice are food restricted overnight prior to the NSF task, it was important also to measure post-test food consumption to ensure any differences in latency to eat are not being driven by the

mouse's drive/motivation to eat. In the home-cage post-test food consumption assessment, GFAP-Cre mice show no differences in grams of food consumed relative to control mice (**Figure 15D**; $t_{(17)} = 0.63$, p=0.53) – suggesting the decreased latency to eat is not attributed to differences in hunger/motivation to eat. Together, these data further confirm the findings that loss of BMAL1 function in NAc astrocytes significantly increases exploratory drive in mice.

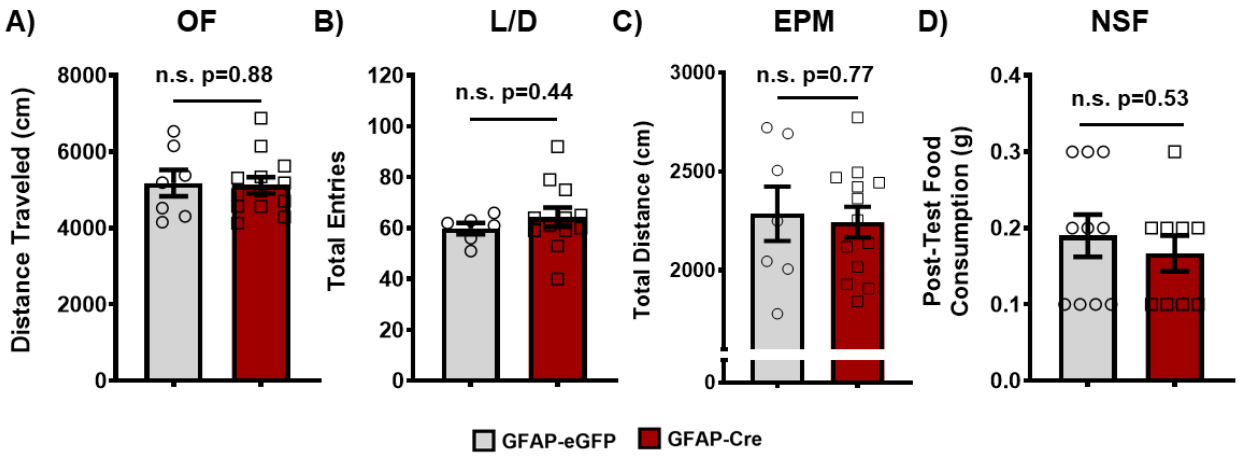

Figure 15. Increases in exploratory drive are <u>not</u> attributed to differences in locomotor activity or drive to eat. While the loss of BMAL1 function in NAc astrocytes leads to an increase in exploratory drive, (**A**) mice do not show any significant differences in locomotor activity in the open field (OF) test, (**B**) total number of entries in the light-dark box (L/D) test, (**C**) distance traveled in the elevated plus maze (EPM), or (**D**) food consumed in the home-cage post-test food consumption assessment following the novelty suppressed feeding (NSF) task. Mean ± SEM; n=7-13; n.s. = not significant.

4.4.4 Loss of BMAL1 function in NAc astrocytes increases food self-administration and motivation

While LRN and exploratory drive have been established as reward-relevant behaviors, we next wanted to investigate reward-regulation and motivation specifically. To investigate this, mice were trained in an operant food self-administration task in which mice discriminate between two levers (active versus inactive) to receive a food pellet. Mice were first trained on a fixed ratio 1 (FR1) schedule whereby mice 1 *active* lever press is rewarded with 1 food pellet, and *inactive* lever presses result in no pellets (**Figure 16A**). On the FR1 schedule, all mice successfully learned the task and reached acquisition criteria (≥ 25 pellets for 3 sessions) (Main effect of Session: $F_{(6, 60)} = 59.63$, $p<0.0001$). While no significant differences were detected between GFAP-Cre and eGFP control mice by 3-way ANOVA (**Figure 16B**; Virus: $F_{(1, 10)} = 1.68$, $p=0.22$), a main effect of sex was detected in that male mice exhibit a higher response rate than females (Sex: $F_{(0.4, 4)} = 37.11$, ** $p=0.006$; Session x Sex: $F_{(3.5, 35.2)} = 8.52$, *** $p<0.0001$); this finding is consistent with previous literature showing sex differences in operant appetitive learning (Mishima et al., 1986; van Haaren et al., 1990; McDowell et al., 2013).

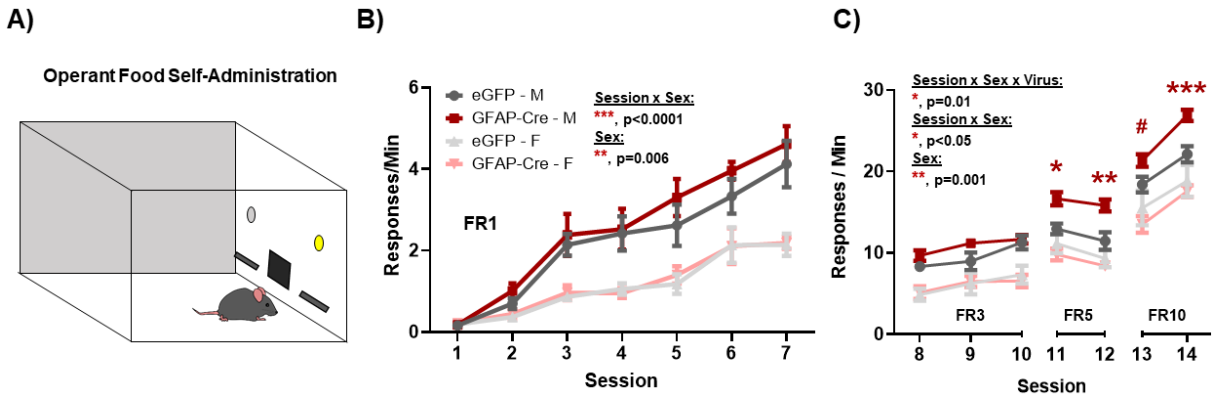

Figure 16. Loss of BMAL1 function in NAc astrocytes increases operant food self-administration and motivation. To assess natural reward and reward-motivation, mice were trained to self-administer food pellets in an operant food self-administration task. (**A**) Mice were trained to discriminate between an active and an inactive lever, whereby active lever pressing resulted in a fixed ratio (FR) of food pellets. (**B**) On an FR1 schedule (i.e., 1 lever press : 1 pellet), BMFL-GFAP-Cre mice successfully learn to self-administer food, but show no significant differences relative to controls. In both virus groups, males show a significantly greater response rate relative to females. (**C**) To test reward motivation, mice were tested across increasingly difficult FR schedules (i.e., FR3, 3 presses : 1 pellet; FR5, 5 presses : 1 pellet; FR10, 10 presses : 1 pellet). GFAP-Cre male mice not only show a robust maintenance, but also a significant increase in food self-administration rate across FR schedules, relative to control counterparts. No effect of the virus was seen in female mice. Mean ± SEM; n = 6; # p=0.07, * p<0.05, ** p<0.01, *** p=0.0001.

Following FR1 training, food self-administration motivation was tested through the escalation of progressively more behaviorally demanding FR schedules; i.e., mice on an FR3 schedule must press the lever 3 times for 1 pellet, mice on an FR5 schedule must press the lever 5 times for 1 pellet, and so on. Strikingly, across progressively harder FR schedules (FR3, FR5, and FR10), a significant Session x Sex x Virus interaction emerged in that male GFAP-Cre mice demonstrate a robust increase in food self-administration in spite of the increasingly more demanding task (**Figure 16C**; $F_{(6, 59)} = 2.96$, * p=0.01); this is further supported by a Sex x Virus interaction ($F_{(1, 10)} = 5.09$, * p<0.05) and a main effect of sex ($F_{(0.6, 6)} = 34.92$, ** p=0.001).

Surprisingly, even on the FR10 schedule where mice must press the lever 10 times to receive just 1 pellet, male GFAP-Cre mice show a significant increase in food self-administration relative to control counterparts (*** $p<0.001$). Taken together, these findings further support the role of astrocytes in regulating reward-related behavior in that loss of BMAL1 function in NAc astrocytes significantly increases both food self-administration response rate and motivation to self-administer.

4.4.5 Loss of BMAL1 function in NAc astrocytes disrupts metabolic homeostasis

Given that astrocytes play a crucial role in regulating neurometabolic homeostasis (**Figure 11C**) and several of these key metabolic functions seem to be under circadian regulation (**Figure 12C**), we were particularly interested in investigating how loss of BMAL1 function in NAc astrocytes affected factors important for regulation of cellular metabolic state in the whole NAc. We first utilized RT-qPCR in whole NAc tissue from both BMFL-GFAP-Cre mice and eGFP controls to assess expression of key astrocyte and metabolic-state relevant genes (**Figure 17A**). No significant difference was seen in expression of the pan-astrocyte marker *Aldh1l1* ($t_{(24)} = 0.09$, $p=0.92$). However, loss of BMAL1 function in NAc astrocytes leads to a significant upregulation of *Gfap* ($t_{(23)} = 3.74$, ** $p=0.001$) and *Nrf2* ($t_{(23)} = 2.87$, ** $p=0.008$) in whole NAc tissue relative to controls, established markers of astrocyte reactivity and oxidative stress induced anti-oxidant response, respectively (Liddelow and Barres, 2017; Vomund et al., 2017). Moreover, loss of BMAL1 function in NAc astrocytes also leads to significant downregulation of *Gclc* ($t_{(23)} = 2.15$, * $p<0.05$), *Pgc1α* ($t_{(24)} = 2.13$, * $p<0.05$), *Ldha* ($t_{(22)} = 2.75$, * $p=0.01$), *Mct1* ($t_{(24)} = 2.18$, * $p<0.05$)

and *Mct2* ($t_{(24)}$ = 1.77, # p=0.08) in the NAc, all genes relevant for glutathione production, mitochondrial function, and lactate synthesis and shuttling.

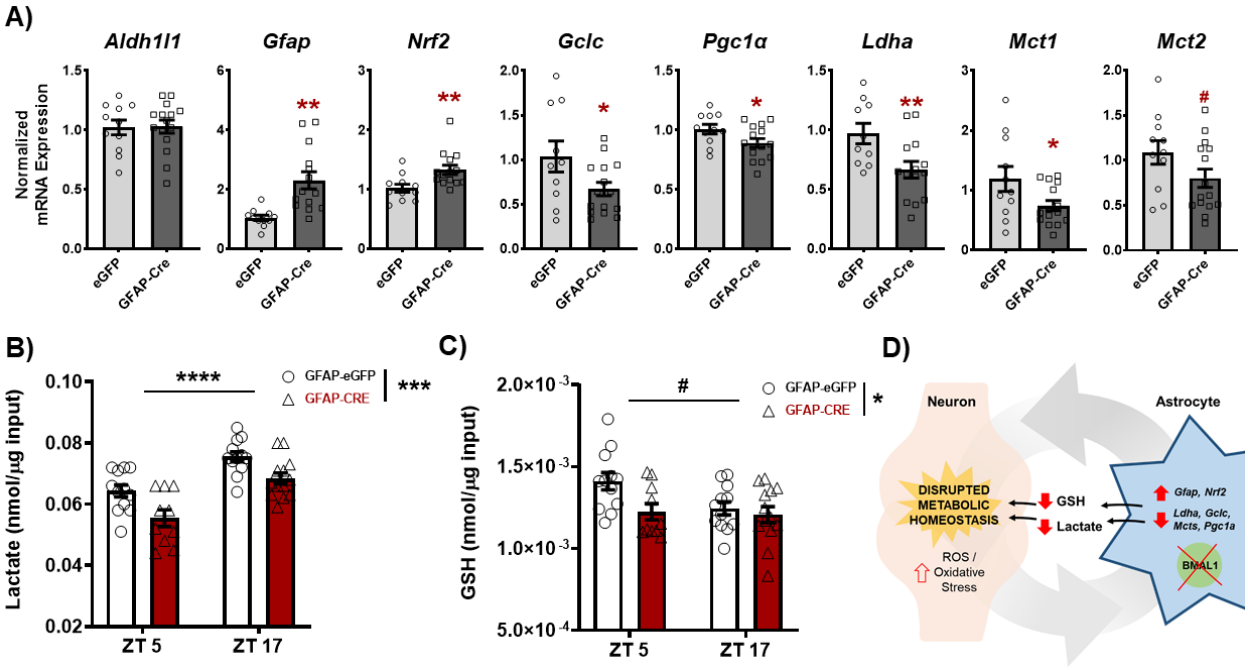

Figure 17. Loss of BMAL1 function in NAc astrocytes disrupts NAc metabolic homeostasis. NAc tissue from BMFL-GFAP-Cre mice was processed through a range of molecular assays assessing factors relevant for regulation of cellular metabolic state. (**A**) Through reverse transcriptase quantitative PCR (RT-qPCR), a panel of metabolic genes was assessed in the whole NAc of GFAP-Cre and eGFP control mice. While there were no differences in the astrocyte marker Aldh1l1, GFAP mice showed significantly elevated expression of *Gfap* and *Nrf2*, markers of astrocyte activation and oxidative stress antioxidant response. GFAP-Cre mice also showed significant reductions in genes relevant for glutathione (GSH production (*Gclc*), mitochondrial biogenesis (*Pgc1a*), and lactate synthesis and shuttling (*Ldha*, *Mct1, Mct2*). (**B**) Moreover, loss of BMAL1 function in NAc astrocytes significantly reduces the concentration of lactate (main effects of virus*** & time****) and (**C**) GSH (main effects of virus* & time#) in the whole NAc across time of day. (**D**) The effects on key metabolic genes, lactate, and GSH are hypothesized to disrupt neurometabolic homeostasis, potentially leading to the downstream effects on NAc-regulated behavior. Gene expression normalized to 18s. Mean± SEM. n=10-15. # p=0.06; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

In addition to measuring the expression of genes relevant for metabolic state, we next wanted to investigate the concentration of lactate and GSH in the NAc following loss of BMAL1 function in NAc astrocytes. Astrocytes synthesize and shuttle both lactate and GSH to neurons to help support their metabolic demands and prevent oxidative stress, respectively uptake (Bélanger et al., 2011; Bolaños, 2016; Gonçalves et al., 2018). To first measure lactate levels, NAc tissue from BMFL-GFAP-Cre mice was run through a colorimetric enzyme-linked immunosorbent assay (ELISA) in which the presence of L(+)-Lactate reacts with an enzyme mix and probe to produce a color measured by spectrophotometry. Alongside the reductions in expression of lactate synthesis and shuttling genes *Ldha*, *Mct1*, and *Mct2* (**Figure 17A**), loss of BMAL1 function in NAc astrocytes significantly reduces lactate concentration in the NAc relative to controls (**Figure 17B**). NAc lactate concentration exhibits diurnal variation in eGFP controls (Time: $F_{(1, 43)} = 35.76$, **** $p<0.0001$) which was disrupted in GFAP-Cre mice through a significant reduction across ZT (Virus: $F_{(1, 43)} = 15.79$, *** $p=0.0003$). However, no significant Time x Virus interaction was observed ($F_{(1, 43)} = 0.20$, $p=0.65$). Alongside measuring lactate, NAc tissue from BMFL-GFAP-Cre mice was also run through a GSH luminescent ELISA in which a luciferin derivative is converted to luciferin, catalyzed by glutathione-s-transferase, and serves as a substrate for the light-emitting luciferase enzyme. Much like lactate levels, loss of BMAL1 function in NAc astrocytes significantly reduces GSH concentration in the NAc relative to controls (**Figure 17C**). Control mice exhibit diurnal variation in NAc GSH concentration (Time: $F_{(1, 43)} = 3.56$, # $p=0.06$) that is significantly reduced in BMFL-GFAP-Cre mice (Virus: $F_{1, 43)} = 5.31$, * $p=0.02$) with no significant Time x Virus interaction ($F_{(1, 43)} = 2.36$, $p=0.13$). Taken together, these findings

illustrate the role astrocytes play in regulating cellular metabolic state and the importance of the circadian molecular clock in orchestrating these metabolic processes through astrocytes.

4.5 Discussion

SUDs have long been associated with disruptions in sleep and circadian rhythms; conversely, circadian rhythms and the genes that regulate them are thought to play an important role in regulating reward processes relevant for SUD vulnerability. Accumulating evidence from pre-clinical research suggests the NAc exhibits a high degree of circadian rhythmicity (Castañeda et al., 2004; Li et al., 2013; Logan et al., 2015; Brami-Cherrier et al., 2020). However, no studies to date have investigated this circadian regulation in a cell-type-specific manner, and especially not in glial cells. Here we demonstrate for the first time that astrocytes in the NAc are highly rhythmic, and this circadian function is important for the regulation of both metabolic homeostasis and reward-related behavior. Through cutting-edge genetic tools, we were able to characterize the NAc astrocyte transcriptome across time of day and test its functional relevance for NAc function and NAc-regulated behaviors. Pathway analysis of NAc astrocyte-specific mRNA sequencing data revealed enrichment of many processes historically associated with astrocytes, including regulation of synaptic organization and morphology, ion and anion transport, glutamate uptake and receptor signaling, generation of precursor metabolites and energy (i.e., oxidative phosphorylation, glycolysis, lactate synthesis, etc.), neuroinflammation, and blood vessel morphogenesis. Interestingly, JTK_Cycle analysis of the RNA-seq data across 6 times of day revealed approximately ~43% of detected genes in the NAc astrocyte transcriptome show

rhythmicity. Moreover, pathway analyses revealed circadian rhythm signaling, circadian regulation of gene expression, NRF2-mediated oxidative stress, ATPase Activity, and Glucocorticoid Receptor Signaling to be enriched processes among the most significantly rhythmic genes. We were able to test the functional signification of this circadian regulation through a NAc astrocyte-specific loss of BMAL1 function in a genetic mouse model. Strikingly, the loss of BMAL1 function in NAc astrocytes significantly increased locomotor response to novelty, increased exploratory drive, and a sex-specific increase in both food self-administration and motivation – all reward-related behaviors relevant to the understanding of SUDs. Moreover, at the molecular level, loss of BMAL1 function in NAc astrocytes leads to a disruption of metabolic homeostasis in the NAc through decreased concentrations of lactate and GSH across time of day, as well as reduced expression of the genes that regulate these processes. Loss of BMAL1 function in NAc astrocytes also yielded significant upregulation of *Gfap* and *Nrf2*, markers of astrocyte reactivity and oxidative stress, respectively, and potentially serving as indicators of oxidative stress and metabolic dysfunction in the NAc as a result of our experimental manipulation. Overall, these results highlight the functional significance of astrocytes in the NAc and the degree to which their circadian regulation is essential for NAc function and NAc regulated behaviors.

Previous work from our lab has extensively demonstrated that core circadian genes in the NAc regulate aspects of reward processing and reward-related behavior. Within the NAc, NPAS2 has been shown to play an integral role in regulating synaptic plasticity, cocaine reward sensitivity, and reward-related behavior through its enriched expression in D_1-MSNs (Ozburn et al., 2015, 2017; Parekh et al., 2019; DePoy et al., 2020). While this previous work illustrates the importance of circadian molecular clock function for reward processing, it fails to account for non-neuronal cell types, namely astrocytes, in this regulation of reward. As we demonstrated here, loss of

BMAL1 function in NAc astrocytes yields a significant increase in locomotor response to novelty, exploratory drive, and operant food self-administration and motivation. One unexplored mechanism by which this phenotype arises may be through uncoupling circadian regulation of neurons from astrocytes. Astrocytes in the SCN have previously been shown to play an integral role in regulating neuronal rhythmicity through circadian controlled uptake/release of glutamate and modulation of both GABAergic tone and receptor signaling (Barca-Mayo et al., 2017; Brancaccio et al., 2017). Moreover, loss of BMAL1 function in SCN astrocytes causes a lengthening of the circadian period (i.e., an inability to keep rhythms consolidated to ~24 hours) in both SCN molecular rhythms and overall behavior rhythms (Tso et al., 2017). This is particularly relevant for the NAc given that much like the SCN, the NAc consists primarily of GABAergic neurons, and astrocytes play a crucial role in modulating their function through regulation of extracellular glutamate (Fellin et al., 2007; Parpura and Verkhratsky, 2012; Scofield and Kalivas, 2014). Similar to how SCN astrocytic regulation of extracellular glutamate in a circadian manner is important for regulating circadian rhythms (Brancaccio et al., 2017), astrocytic regulation of glutamate in the NAc is thought to be important for reward-regulation and drug addiction vulnerability (Kalivas, 2009; Scofield and Kalivas, 2014; Reissner et al., 2015; Scofield et al., 2015, 2016a). This is further supported in our study through pathway analyses revealing glutamate receptor signaling, GABA receptor signaling, and neurotransmitter uptake (glutamate) pathways/processes to be enriched among the top 200 expressed genes in NAc astrocytes. While unstudied here, future investigation is needed into how the loss of astrocyte rhythmicity in the NAc affects glutamate homeostasis and/or its effects on neuronal rhythmicity, with particular focus on downstream implications for reward regulation.

Alongside regulating glutamate levels, astrocytes have been well-established to play a central role in the maintenance of neuronal metabolic homeostasis, including synthesis and shuttling of both lactate and GSH (Danbolt, 2001; Malarkey and Parpura, 2008; Bolaños, 2016; Gonçalves et al., 2018). The astrocyte-to-neuron lactate shuttle (ANLS) hypothesis proposes that increased neuronal activity and subsequent glutamate uptake by astrocytes triggers astrocytic intake of glucose from blood capillaries, increased production of lactate (one of the primary energy substrates of the brain) from glycolysis-generated pyruvate, and then increased shuttling of lactate via monocarboxylate transporters (MCTs) to neurons (Pellerin and Magistretti, 1994, 2012; Magistretti and Pellerin, 1996; Magistretti and Allaman, 2018). Moreover, much like the ANLS hypothesis, it is thought that activation of NRF2 in astrocytes, the master antioxidant transcription factor, is coupled with glutamatergic neurotransmission of neurons whereby glutamate triggers activation of the NRF2 antioxidant signaling pathway (Habas et al., 2013; Jimenez-Blasco et al., 2015; Bolaños, 2016; McGann and Mandel, 2018). NRF2 is also activated directly by the presence of reactive oxygen species (ROS), whereby activation of NRF2 induces the expression of enzymes crucial for GSH biogenesis (GCLC, GCLM, GSH synthetase, etc.) and many other antioxidants (Ma, 2013; Baxter and Hardingham, 2016; Tu et al., 2019) that remove ROS (e.g., hydrogen peroxide, superoxide, etc.) that would otherwise damage DNA, RNA, proteins, and lipids, also known as oxidative stress (Schieber and Chandel, 2014). Importantly, our study further supports these concepts in that pathway analyses revealed monocarboxylic acid biosynthesis (i.e., lactate), glycolysis, oxidative phosphorylation, and generation of precursor metabolites & energy pathways/processes to be enriched among the top 200 expressed genes in NAc astrocytes. Moreover, circadian analysis of the NAc astrocyte transcriptome revealed NRF2-mediated oxidative stress response (i.e., GSH and antioxidant production), regulation of ATPase Activity,

and glucocorticoid receptor signaling to be enriched among the top rhythmic transcripts. Most notably, these functional roles are clearly supported by this present study revealing a loss of BMAL1 function in NAc astrocytes significantly downregulates lactate and GSH levels in the NAc, as well as expression of genes important for regulation of these processes and overall metabolic state (*Gclc*, *Ldha*, *Mct1*, *Mct2*, and *Pgc1a*). These findings highlight both the functional significance of astrocytes in the NAc, while also revealing the contribution of the circadian molecular clock to the regulation and maintenance of their functioning.

In addition to the circadian molecular clock contributing to astrocytic regulation of metabolic homeostasis in the NAc, this regulation of metabolic state has many implications for regulation of reward-related behavior and contributions to drug addiction vulnerability. Substances of abuse, especially psychostimulants like cocaine and amphetamine, significantly disrupt the regulation of cellular metabolic state and lead to increased oxidative stress and damage through reactive oxygens species (ROS) accumulation (Cunha-Oliveira et al., 2013; Uys et al., 2014; Jang et al., 2015; Pavlek et al., 2020). Interestingly, the ROS themselves are thought to even contribute to the reinforcing properties of substances of abuse. Studies in rats have demonstrated cocaine and methamphetamine increases oxidative stress, and treatment with a non-specific ROS scavenger or a superoxide-selective scavenger not only decreases oxidative stress effects, but also attenuates cocaine/methamphetamine locomotor sensitization, decreases cocaine/methamphetamine self-administration, and significantly downregulates the enhancement of dopamine release typically seen with cocaine or methamphetamine administration (Numa et al., 2008; Jang et al., 2015, 2017). These effects on behavior are thought to be due to oxidative stress-induced changes to glutathione redox status and S-glutathionylation signaling (Uys et al., 2011, 2014; Womersley and Uys, 2016), and treatment with the GSH precursor, N-acetylcysteine, has proven to be effective in attenuating

and/or blocking addiction-like behaviors in rodents (Dean et al., 2011; Kupchik et al., 2012; Womersley et al., 2019). Interestingly, a recent study by the Musiek lab demonstrated loss of BMAL1 function in cortical astrocytes induces activation of astrocytes and significant suppression of S-glutathionylation signaling (Lananna et al., 2018). In our current study, loss of BMAL1 function in NAc astrocytes significantly downregulated GSH levels in the NAc, accompanied by increased *Gfap* and *Nrf2* expression, markers of astrocyte activation and oxidative stress, respectively. Given the increased reward-seeking behavior alongside these molecular perturbations induced by loss of BMAL1 function in NAc astrocytes, future studies will explore the role of S-glutathionylation in this model and if circadian regulation of S-glutathionylation in the NAc is necessary for reward-related behavior. Moreover, with a handful of studies suggesting astrocyte activation itself may contribute to reward-seeking behavior (Bull et al., 2014; Kang et al., 2020), future studies will also parse apart this circadian regulation of astrocyte activation and its importance for reward processing in the NAc.

Finally, loss of BMAL1-function in NAc astrocytes significantly downregulated lactate concentrations in the NAc across time of day, as well as expression of genes encoding the lactate synthesis enzyme LDHA and the lactate shuttle proteins MCT1 and MCT2. This is of particular interest for reward regulation given the interconnectivity of circadian rhythms, neuronal metabolism, and drug reward (Freyberg and Logan, 2018). Lactate is one of the primary energy substrates of the brain, and the shuttling of lactate to neurons is central to the maintenance of their energetic needs (Magistretti and Allaman, 2018; Rabinowitz and Enerbäck, 2020). Lactate synthesis and shuttling are directly regulated by the circadian molecular clock in that expression of LDHA, MCTs, and extracellular lactate levels all show circadian variation (Ahlersová et al., 1981; Rutter et al., 2001; Naylor et al., 2012; Henriksson et al., 2017; Milićević et al., 2020;

Wallace et al., 2020). While the role of the circadian variation of lactate levels in reward regulation in the NAc remains to be studied, two studies have shown blocking lactate shuttling in the amygdala can attenuate cocaine CPP and/or self-administration that can later be rescued with lactate administration (Boury-Jamot et al., 2016a, 2016b; Zhang et al., 2016). In the NAc, a study published just this year found that glucocorticoid-mediated lactate release may regulate the conditioned response to morphine in a conditioned place preference (CPP) task (Skupio et al., 2020). This is particularly interesting in that not only is there extensive literature suggesting a dynamic interaction between the circadian system and glucocorticoid system with relevance for reward regulation (Perreau-Lenz and Spanagel, 2015; Becker-Krail and McClung, 2016), but also here we show that regulation of glucocorticoid receptor signaling is an enriched pathway among the top rhythmic genes in NAc astrocytes and astrocyte-specific loss of BMAl1 function significantly reduces lactate levels in the NAc and expression of genes relevant for its regulation. Future investigation is needed into both this relationship and/or the direct contribution of lactate to the phenotype seen with loss of BMAL1 function in NAc astrocytes.

Collectively, the results of this study demonstrate for the first time that NAc astrocytes exhibit a great degree of circadian rhythmicity, with nearly half of the genes identified in their transcriptome exhibiting significant circadian variation across time of day. In addition to the many well-established astrocyte functions enriched among the top expressed NAc astrocyte genes, we also show several metabolic relevant pathways and processes to be among the top significantly rhythmic genes. Most notably, our data reveal that this circadian astrocyte function is not only important for the maintenance of metabolic homeostasis, but also for downstream regulation of reward-relevant behavior, including novelty induced locomotion, exploratory drive, and operant food self-administration and motivation. Ultimately, these data further our understanding of the

relationship between circadian rhythms, metabolic state, and reward circuity, and reveal a fundamental role of astrocyte rhythmicity in regulating NAc function.

4.6 Acknowledgments

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5.0 General Discussion and Future Directions

5.1 Summary of Findings

Taken together, the studies outlined in this dissertation aimed to test the hypothesis that reward is regulated in the NAc through interactions between the circadian molecular clock and cellular metabolic state, which is mediated at both the cellular level by astrocytes and at the molecular level by circadian interfacing redox sensors. More specifically, these studies investigated (1) the interaction between the circadian transcription factor NPAS2 and the NAD+-dependent deacetylase SIRT1 at the molecular level and their role in reward-regulation, and (2) the circadian regulation of astrocytes and how their circadian function may be important for reward-related behavior and maintenance of metabolic homeostasis. Through these investigations, we not only highlight the complexity of reward-regulation and the degree to which circadian and metabolic processes influence the processing of reward, but also underscore the significance of studying the contribution of non-neuronal cell-types in the regulation of reward circuitry. The summaries of our findings and future directions are discussed below.

Through the studies described in **Section 3**, we not only investigated how cocaine exposure affects the diurnal variation of key regulators of circadian molecular clock function and metabolic state, but also the interaction between the circadian transcription factor NPAS2 and the metabolic sensing deacetylase SIRT1and how together they may regulate reward. Ultimately, these studies revealed that cocaine exposure significantly alters the diurnal expression of *Npas2*, *Sirt1*, and the NAD+ salvage pathway enzyme *Nampt*, whereby upregulation of these genes results in a phase-

shifting of acrophase from dark to light phase (i.e., shift from mouse's active phase to inactive phase). We also detected diurnal variation of the redox coenzyme NAD+ in the NAc, and this diurnal variation is altered by cocaine exposure upregulating NAD+ and shifting acrophase to the dark phase (i.e., mouse's active phase). Additionally, our studies are the first to demonstrate that NPAS2 and SIRT1 interact in the NAc and through ChIP-seq analyses, we revealed many metabolic, dopamine, and reward-relevant pathways/processes to be enriched among their shared gene targets in the NAc. Finally, through NAc-specific knockdown of *Npas2*, we demonstrated NPAS2 is necessary for the increase in cocaine preference seen with SIRT1 activation by resveratrol. Overall, these studies indicate that NPAS2 and SIRT1 interact in the NAc to regulate reward-relevant processes and ultimately reward-regulated behavior. This interaction also introduces a mechanism by drugs' effects on cellular metabolic state may affect the circadian regulation of the NAc and downstream reward function.

Through the studies outlined in **Section 4**, we sought out to investigate the circadian regulation of astrocytes in the NAc and the functional importance of their circadian regulation for reward-related behaviors and metabolic homeostasis. Ultimately, through NAc astrocyte-specific RNA-sequencing, we revealed enrichment of many processes/pathways historically associated with astrocytes, including regulation of synaptic organization and morphology, glutamate uptake and receptor signaling, neuroinflammation, and generation of precursor metabolites and energy (i.e., oxidative phosphorylation, glycolysis, lactate synthesis, etc.). Through rhythmic analysis of the sequencing data, NAc astrocytes were determined to be highly circadian regulated, with approximately 43% of the genes identified in their transcriptome exhibiting significant rhythmicity, and pathway analyses revealing circadian and metabolic processes to be enriched among the top rhythmic genes. Finally, viral-mediated loss of BMAL1 function in NAc astrocytes

revealed the circadian regulation of NAc astrocytes to be important for not only regulating reward-related behavior (e.g., exploratory drive and food self-administration), but also metabolic homeostasis through mediating lactate and GSH levels in the NAc. Overall, these studies demonstrate that astrocytes are highly rhythmic in the NAc and highlight the functional significance of this circadian regulation for NAc function and -regulated behaviors.

5.2 Proposed Mechanism

In testing the hypothesis that reward is regulated in the NAc through interactions between the circadian molecular clock and cellular metabolic state, both at the cellular level by astrocytes and at the molecular level by circadian interfacing redox sensors (e.g., NPAS2 and SIRT1), the studies outlined in this dissertation reveal an intriguing mechanism by which substances of abuse and other stressors may influence reward regulation and increase vulnerability to addiction-like behavior (**Figure 18**). Disruptions to astrocyte function in the NAc, mediated by internal and/or external factors (e.g., substances of abuse, stress, circadian gene mutations, etc.), may directly impact the neurometabolic coupling between astrocytes and neurons in the NAc and have downstream effects on reward regulation. As shown in **Section 4**, disruptions to circadian astrocyte function directly impact cellular metabolic homeostasis through downregulation of lactate and GSH shuttling, and this is coupled with mice demonstrating increased reward-seeking behavior. This loss of circadian astrocyte function in the NAc was also associated with increased *Gfap* and *Nrf2* expression, which is known to be upregulated by increased ROS and oxidative stress (Morgan et al., 1997; Ma, 2013; Daverey and Agrawal, 2016; Tu et al., 2019). These changes in

neurometabolic homeostasis and reward-related behaviors are particularly interesting in light of the findings presented in **Section 3**. Here we show cocaine exposure significantly upregulates NAD+ levels in the NAc, which may be partly driven by increased ROS and oxidative stress known to be associated with cocaine exposure (Dietrich et al., 2005; Bashkatova et al., 2006; Jang et al., 2015); through the peroxiredoxin cycle, increased ROS accumulation has been shown to directly induce upregulation of NAD+ levels (Ying, 2008; O'Neill and Reddy, 2011; Edgar et al., 2012; Baxter and Hardingham, 2016). Previous studies in our lab and others have shown changes in NAD+ can directly influence circadian molecular clock function relevant for driving reward-related behavior through SIRT1 (Kong et al., 2018; Logan et al., 2019), and this is further supported by our findings that SIRT1 and NPAS2 interact in the NAc to regulate reward. Taken together, perhaps disruptions to circadian astrocyte function (e.g., by substances of abuse, genetic mutations, etc.) and subsequent effects on neurometabolic homeostasis may influence reward-regulation through ROS induced changes in NAD+ and downstream effects on NPAS2 and SIRT1 in the NAc – increasing vulnerability to addiction-like behavior. While not directly tested here, accumulating evidence has revealed that substances of abuse directly affect astrocyte morphology/function specifically in the NAc (Knackstedt et al., 2010; Scofield et al., 2016b; Testen et al., 2018; Jarvis et al., 2019; Siemsen et al., 2019) - though it remains to be seen whether cocaine and other substances of abuse affect circadian astrocyte function specifically. However, as highlighted in this dissertation, accumulating evidence of cocaine's effects on circadian molecular clock function, cellular metabolic state / ROS accumulation, and astrocyte morphology and function, all provide support of this proposed mechanism and warrant its future investigation.

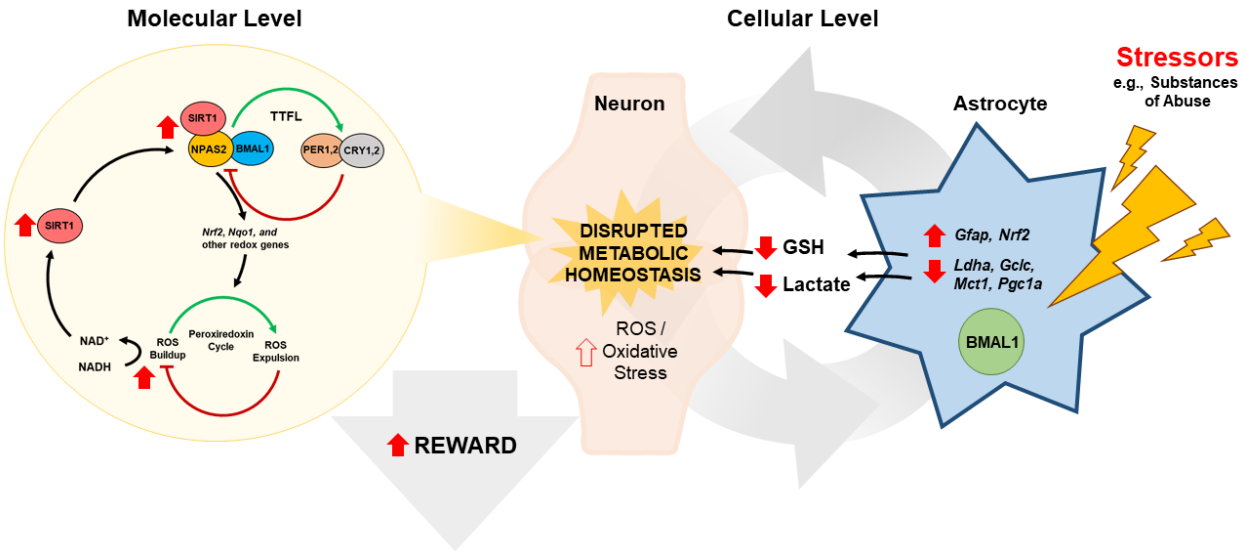


Figure 18. Substances of abuse may influence reward through interactions between circadian molecular clock function and regulation of cellular metabolic homeostasis in the NAc. Schematic synthesizing findings within this dissertation illustrating how disrupting neurometabolic coupling between astrocytes and neurons may ultimately increase oxidative stress and disrupt metabolic homestasis, this in turn feeding into circadian molecular clock function via circadian redox sensors and driving reward-relevant and addiction-like behaviors.

5.3 Relevance to Hypotheses of SUDs and Addiction-like Behavior

The "dopamine hypothesis" for the development of SUDs posits that substances of abuse, like alcohol, cocaine, nicotine, opiates, and marijuana, act directly on the brain's reward system to increase mesolimbic DA release and promote both reward and subsequent reward-seeking behavior (Koob, 1992; Willuhn et al., 2010; Volkow and Morales, 2015). Previous work in our lab has demonstrated that circadian genes, like *Clock* and *Npas2*, can influence dopaminergic activity, dopaminergic receptor signaling, synaptic plasticity, and subsequent drug reward-related behavior (Ozburn et al., 2015; Parekh and McClung, 2015; Parekh et al., 2015, 2019; Logan et al., 2019).

The studies outlined in **Section 3** expand on these findings and highlight a novel role for NPAS2 and SIRT1 in regulating reward in the NAc. Notably, DA transport and pre-synaptic membrane synaptic transmission were enriched processes among the shared gene targets between NPAS2 and SIRT1 in the NAc. Considering that psychostimulants like cocaine or amphetamine act directly at NAc synapses to increase the concentration of extracellular DA available either by blocking dopamine transporter (DAT) mediated dopamine reuptake or promoting greater DA release, respectively (Kalivas, 2007), the regulation of dopamine transport by NPAS2 and SIRT1 in the NAc (potentially together) presents a novel mechanism by which the circadian and metabolic systems may contribute to the mechanism of action of drugs of abuse. However, future investigation into this mechanism and its relevance for reward is still necessary.

While not studied in this dissertation, a study published this year also implicates a novel role for astrocytes in the regulation of dopamine synaptic function in the NAc. In this recent study, astrocytes in the NAc were found to also respond to dopamine release through the expression of their own D1 receptors, whereby dopamine activation of astrocytes induced a Ca^{2+} dependent synaptic release of ATP/adenosine that resulted in a depression of excitatory synaptic transmission through the expression of presynaptic neuronal adenosine A1 receptors (Corkrum et al., 2020). Moreover, amphetamine was also found to produce this response, suggesting astrocytes may be key elements in dopamine signaling in the NAc, both modulating and mediating drugs' actions (Corkrum et al., 2020). This is further supported by recent findings in the dorsal striatum showing activation of the astrocytic release of adenosine and downstream receptor signaling facilitates the transition from habitual to goal-directed reward-seeking behavior in mice (Kang et al., 2020). Together, these recent findings may uncover an intriguing mechanism by which circadian regulation of astrocytes may also play a role in dopamine signaling in the NAc, in that both

dopamine receptor expression and astrocyte ATP release is known to be directly regulated by the circadian molecular clock (Marpegan et al., 2011; Ozburn et al., 2015). Furthermore, considering the direct association between lactate synthesis and the subsequent generation of ATP through OXPHOS (Rabinowitz and Enerbäck, 2020), our studies demonstrating a loss of BMAL1 function in NAc astrocytes leading to a down-regulation of *Ldha* and lactate levels in the NAc may suggest evidence for alterations to ATP synthesis and downstream signaling. While not explored in this dissertation, perhaps disrupting circadian astrocyte function disrupts the dopamine dependent activation of astrocyte gliotransmission (e.g., via decreased dopamine receptor expression and/or decreased ATP release) and disrupts the subsequent downregulation of excitatory synaptic transmission – together contributing to the increased reward-seeking behavioral phenotype seen with loss of BMAL1 function in NAc astrocytes. However, future investigation into this mechanism is still needed.

In addition to dopamine signaling being central to reward processing, there is significant evidence suggesting dysregulation of glutamate homeostasis, heavily attributed to astrocyte dysfunction and downregulation of glutamate transporters, may underlie the development of SUDs, also known as the "glutamate homeostasis hypothesis" (Kalivas, 2009). Several studies show rats exposed to cocaine self-administration and extinction have significant NAc-specific reductions in expression and function of astrocyte-specific glutamate transporters, resulting in decreased glutamate clearance and uptake velocity (Knackstedt et al., 2010; Fischer-Smith et al., 2012; LaCrosse et al., 2017), and pharmacological restoration of glutamate transporter expression and function improves glutamate homeostasis and significantly attenuates reinstatement of cocaine-seeking behavior in rats (Baker et al., 2003; Knackstedt et al., 2010; Trantham-Davidson et al., 2012; Fischer et al., 2013; Reissner et al., 2014). This is supported in this dissertation by our

findings that both glutamate receptor signaling and neurotransmitter uptake (glutamate) pathways/processes are enriched among the top 200 expressed genes in NAc astrocytes. While glutamate homeostasis was not investigated in this dissertation following loss of circadian astrocyte function in the NAc, there are several lines of evidence to suggest this may be a mechanism worth future investigation.

First, astrocytes are central to the regulation of glutamate homeostasis and synaptic transmission, through the uptake of glutamate, conversion to glutamine, and subsequent shuttling of glutamine to neurons to be used to restore intracellular glutamate levels for signaling (Schousboe et al., 2014; Mahmoud et al., 2019). This maintenance of glutamate homeostasis is not only essential to the regulation of glutamate neurotransmission, but also many metabolic processes, including the tricarboxylaic acid (TCA) cycle (Schousboe et al., 2014). Second, several studies have shown that glutamate and its glutamate transporters are under direct and indirect circadian regulation (Castañeda et al., 2004; Beaulé et al., 2009; Chi-Castañeda and Ortega, 2018); e.g., mutations in core clock genes *Clock* or *Npas2* in astrocytes significantly reduces glutamate transporter expression and glutamate uptake velocity (Beaulé et al., 2009). Moreover, astrocytes actually regulate circadian rhythmicity in the SCN through circadian regulation of glutamate homeostasis (Brancaccio et al., 2017), and circadian regulation of astrocyte glutamatergic signaling alone in the SCN is sufficient to restore rhythmicity in the absence of *Per2* (Brancaccio et al., 2019). Third, the neurometabolic coupling of astrocytes to neurons directly depends on glutamate uptake, in that the lactate and GSH shuttles are directly coupled to neuronal glutamatergic transmission and subsequent uptake of glutamate by astrocytes (Bolaños, 2016). Our findings that both lactate and GSH levels are reduced in the NAc following loss of circadian astrocyte function may be indicative of altered glutamatergic neurotransmission and/or decreased

glutamate uptake by astrocytes; i.e., decreased expression of glutamate transporters and/or decreased glutamate uptake resulting in decreased glutamate-dependent initiation of lactate and GSH synthesis/shuttling. Finally, glutamate release from neurons activates metabotropic glutamate receptors (mGluRs) expressed on astrocytes, inducing Ca^{2+} dependent release of gliotransmitters, like ATP/adenosine, D-serine, Glutamate, etc. (D'Ascenzo et al., 2007; Covelo and Araque, 2018). Interestingly, transgenic mice unable to release gliotransmitters from astrocytes show a significant attenuation of cue-induced reinstatement to both cocaine CPP and cocaine self-administration (Turner et al., 2013); while pharmacologically inducing release of glutamate from NAc astrocytes significantly blocks reinstatement of cocaine-seeking in rats (Scofield et al., 2015). Additionally, activation of astrocyte gliotransmitter release (e.g., ATP/adenosine) in the dorsal striatum facilitates the transition from habitual to goal-directed reward-seeking behavior through modulation of MSN activity (Kang et al., 2020). Notably, as mentioned above, this release of ATP is also known to be tightly regulated by the circadian system, in that gliotransmission of ATP displays daily oscillations and require a functional molecular clock (e.g., requiring a functional *Clock* and *Bmal1*, both *Per1* and *Per2*, and both *Cry1* and *Cry2*) (Marpegan et al., 2011). Given that (1) astrocytes play a critical role in regulating glutamate homeostasis, (2) glutamate uptake is central to the neurometabolic coupling of astrocytes and neurons, (3) glutamate signaling contributes to the activation of gliotransmitter release, and (4) these processes are all tightly integrated and regulated by the circadian molecular clock, it is incredibly likely that loss of BMAL1 function in NAc astrocytes significantly disrupts regulation of glutamate homeostasis in the NAc and drives the downstream metabolic and reward-promoting behavioral phenotype seen in our model (e.g., via downregulation of glutamate transporters / uptake, decreased astrocytic gliotransmitter release and subsequent neuromodulation, and/or an

overall uncoupling of astrocytes from neuronal activity). However, since these potential mechanisms were not explored in this dissertation, future investigation into how circadian astrocyte function and the regulation of glutamate homeostasis play a role in reward-regulation is still necessary.

In addition to the astrocyte findings from **Section 4** being particularly relevant in the context of the glutamate hypothesis of SUDs, NPAS2 and SIRT1 have also been shown to play a role in glutamate signaling and synaptic function. Recent work from our lab has demonstrated that NPAS2 regulates the glutamatergic input onto D1-containing MSNs, in that *Npas2* knockdown in D1-MSNs blocked cocaine-induced enhancement of synaptic strength and glutamatergic transmission specifically onto D1-MSNs (Parekh et al., 2019). This is specifically important for reward regulation in that the NAc integrates an abundance of glutamatergic input from the PFC, hippocampus, and BLA, all of which contribute to reward-value, -salience, -memory, -decision-making, and ultimately, -motivation (Scofield et al., 2016a). Additionally, SIRT1 has been shown to regulate expression of the glutamate receptors *Gria1* and *Gria2*, important for cocaine-mediated synaptic plasticity; more specifically, *Sirt1* overexpression in the NAc leads to a significant increase in *Gria1* and a cocaine-mediated increase in *Gria2* (Ferguson et al., 2013). While not studied in this dissertation, it is possible that together NPAS2 and SIRT1 regulate aspects glutamate signaling and plasticity that contribute to cocaine-reward and the cocaine-mediated long-term changes in physiology – especially given our findings here demonstrating cocaine increases expression of *Npas2* and *Sirt1* in the NAc and together they interact in the NAc to regulate reward. However, future investigation into the shared role of NPAS2 and SIRT1 in regulating glutamatergic transmission and related plasticity in the NAc is necessary.

Taken together, our findings from **Section 3** and **Section 4** are especially relevant in the context of both the dopamine and glutamate hypotheses of SUDs, and together further expand our understanding of the potential circadian, metabolic, and astrocytic contributions to the regulation of these neurotransmitter systems in the NAc and their significance for reward.

5.4 NPAS2 and SIRT1 in Astrocytes

While not mechanistically investigated in this dissertation, the studies outlined in **Section 3** and **Section 4** may share a degree of overlap through the regulation of astrocyte metabolic functions by NPAS2 and/or SIRT1. Confirmed both through the Barres lab's Brain RNA-seq database (Zhang et al., 2014b; Clarke et al., 2018) and in our NAc astrocyte-specific RNA-seq dataset, *Npas2* and *Sirt1* are expressed in astrocytes and display a diurnal variation in expression (**Figure 19**). This is further confirmed by *Npas2* being among the top 20 upregulated genes during astrocyte development (Cahoy et al., 2008). Despite their enriched expression in astrocytes and their previously shown roles in regulating reward, an investigation into the role of NPAS2 or SIRT1 in astrocytes, especially in the NAc, remains understudied.

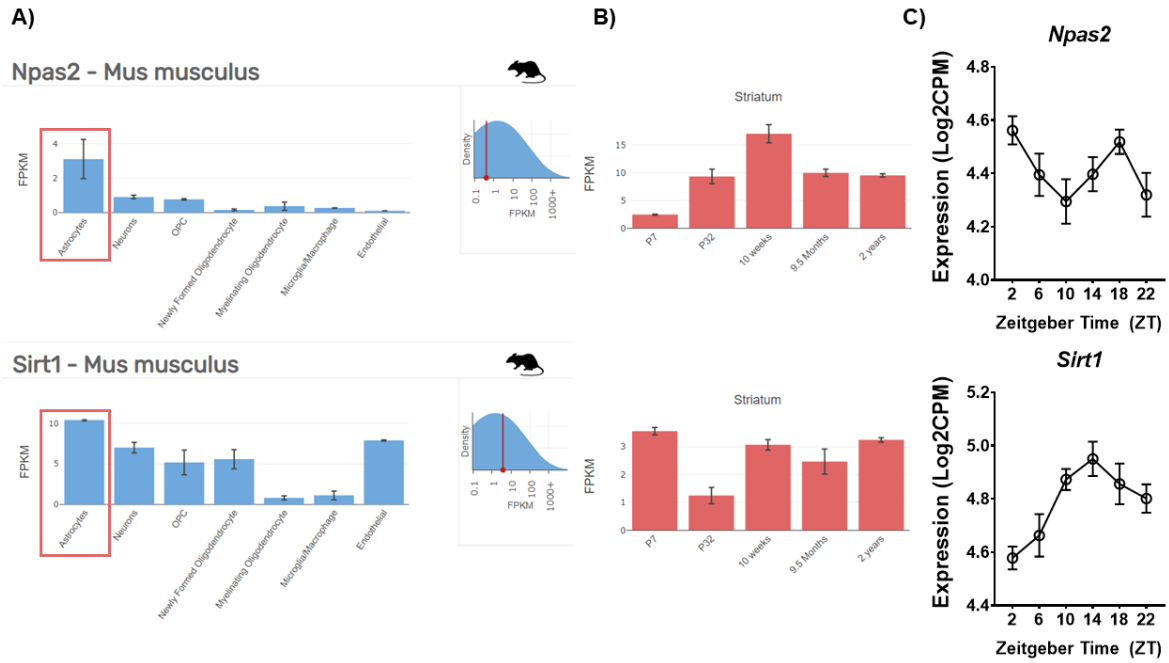


Figure 19. *Npas2* and *Sirt1* are expresssed in astrocytes. (**A**) Utilizing the Barres Lab Brain RNA-seq database, astrocytes show enriched expression of *Npas2* and *Sirt1* in mouse brain tissue, and (**B**) more specifically within the striatum throughout development. (**C**) Our astrocyte-specific RNA-seq dataset also confirms the expression of Npas2 and Sirt1 in astrocytes specifically, and this expression shows diurnal variation.

Though the roles of NPAS2 and SIRT1 in astrocytes have not been investigated in the context of reward regulation, a few studies have investigated the role of NPAS2 and SIRT1 in the regulation of core astrocyte functions. Regarding NPAS2, only 2 studies have investigated its functional role in astrocytes. Notably, while *Npas2* does not appear to be necessary for the maintenance of astrocyte molecular rhythms *in vitro* (Marpegan et al., 2011), NPAS2 does appear to regulate astrocyte glutamate uptake; interestingly, in astrocyte cultures from *Npas2* mutant mice, *Npas2*$^{-/-}$ astrocytes exhibit a 50% reduction in expression of the glutamate transporter GLAST (i.e., EAAT1) that correlated with a 20% reduction in glutamate uptake velocity (Beaulé et al., 2009). Given the importance of glutamate uptake for both reward-relevant NAc function

(Kalivas, 2009; Scofield et al., 2016a) and activity-driven metabolic processes in astrocytes (Bolaños, 2016; Gonçalves et al., 2018), coupled with our findings presented in **Section 3**, future investigation into the role of NPAS2 in NAc astrocyte function may prove relevant to the study of SUDs neurobiology and addiction-like behavior. Regarding SIRT1, several studies have shown SIRT1 to play a role in astrocytes' regulation of neurometabolic homeostasis. First, in primary astrocyte culture studies, glucose deprivation leads to a significant upregulation of both NAD+ levels and *Sirt1* expression, and this was determined to have antioxidant and neuroprotective effects through SIRT1's upregulation of Forkhead box protein O4 (FOXO4) mediated transcription of the antioxidants SOD2 and catalase (Cheng et al., 2014). This is particularly relevant given that the association between SIRT1 and FOXO3 is thought to mediate cocaine's actions in the nucleus accumbens (Ferguson et al., 2015), though this has not been studied in astrocytes. In addition to interacting with FOXO4, SIRT1 mediated activation of the NRF2 pathway in primary astrocyte culture both promotes cell viability and mitigates oxidative stress through increased acetylation-dependent nuclear transport of NRF2 and a significant upregulation of SOD activity (Xia et al., 2017). Finally, alongside regulating antioxidant production, SIRT1 in astrocytes has also been shown to regulate cellular metabolism. Through Seahorse analysis of astrocyte mitochondrial oxygen consumption rate and nuclear magnetic resonance (NMR) spectroscopy of cortical tissue slices, activation of SIRT1 by resveratrol was found to increase glycolysis and subsequent lactate production, respectively (Rowlands et al., 2015). However, in other tissues, SIRT1 has been found to be a negative regulator of glycolysis through its interactions with peroxisome proliferator-activated receptor α (PPARα) and PGC-1α (Chang and Guarente, 2014). The known interaction between SIRT1 and the PPAR family of nuclear receptors is also particularly interesting, because in addition to being essential for regulating metabolic processes,

a recent study found PPARγ activation to be central to the cocaine-mediated circadian reprogramming of the NAc transcriptome (Brami-Cherrier et al., 2020); however, the role of SIRT1 and/or astrocytes was not investigated in this study. Given the above studies and our findings in **Section 3** and **Section 4** separately illustrating circadian astrocyte function and NPAS2:SIRT1 in the NAc to be important for the regulation of both reward and metabolic function, future investigation into how these observations are related may afford greater appreciation of the interconnectivity of these processes and further reveal a mechanism by which drugs may influence metabolic homeostasis and downstream reward regulation.

5.5 Limitations and Considerations

Several key considerations must be made in the evaluation of the data presented in this dissertation. First and foremost, behavioral experiments throughout are modeling reward-related behavior in mice and not the complexities associated with human SUDs. While rodent models of addiction-like behavior offer excellent face validity in modeling many aspects of drug reward and drug-seeking behavior (Spanagel, 2017), they fail to capture the many experiential and psychosocial factors of SUDs in humans that contribute to the disease's complexity (Venniro et al., 2020). Furthermore, the studies outlined in this dissertation only test reward-related behavior, including cocaine conditioned place preference, locomotor response to novelty, exploratory drive, and operant food self-administration. While all of these behaviors test relevant aspects of reward processing that often correlate with operant drug/reward-seeking behavior (Piazza et al., 1989; Hooks et al., 1991; Stead et al., 2006; Blanchard et al., 2009; Flagel et al., 2010, 2014; Spanagel,

2017; Zhou et al., 2019b), future studies will benefit from implementing operant intravenous self-administration of drugs of abuse (e.g., cocaine, amphetamine, morphine, etc.). This is especially true in light of recent findings from the Kalivas lab highlighting differences in natural and cocaine mediate reward-seeking behavior, whereby mice trained to self-administer cocaine and sucrose show distinct and different neuronal ensembles recruited for the coding of reward (Bobadilla et al., 2020). Nevertheless, our findings illustrate a unique mechanism by which the circadian molecular clock and metabolic state regulate reward, both globally in the NAc and through astrocytes specifically, warranting future investigation in the context of vulnerability to addiction-like behavior. Taken together, in spite of the limitations, before we can begin to appreciate the deep complexities associated with aberrant reward processing in SUDs, it is important to understand the basic neurobiology underlying reward-regulation and reward-related behavior.

Secondly, while many of the molecular studies outlined in this dissertation were investigated across time of day, the behaviors outlined in both **Section 3** and **Section 4** only tested reward-relevant behaviors at one time of day. Given that many reward-relevant behaviors show diurnal variation, whereby rodents exhibit higher reward-motivated behaviors during their active phase (i.e., the dark phase) (Baird and Gauvin, 2000; Akhisaroglu et al., 2004; Webb et al., 2009), future investigation into the regulation of reward by NPAS2:SIRT1 or circadian astrocyte function in the NAc is necessary. This is particularly true in light of recent findings from our lab showing sex- and time-dependent modulation of cocaine intravenous self-administration *Npas2* mutant mice (DePoy et al., 2020). While Npas2 mutant males and females both show robust increases in cocaine self-administration during the mouse's inactive phase (i.e., light phase), *Npas2* mutant females show amplified differences during the mouse's active phase (i.e., dark phase) that are abolished in ovariectomized females. This is incredibly interesting considering the sex-specific

differences seen in operant food self-administration in male mice with loss of BMAL1 function in NAc astrocytes, the downregulation and disruption of diurnal variation in lactate and GSH levels in the NAc, and the phase-shifting effects of cocaine on circadian and metabolic gene expression in the NAc. Investigating these findings across time of day will allow us to fully appreciate the circadian regulation of these processes and guide future investigation into the circadian contribution of these processes to the regulation of reward and/or metabolic processes. Nonetheless, the differences we see in reward-relevant behavior emphasize the importance of circadian genes and circadian astrocyte function for reward-relevant behaviors and warrant future investigation.

Additionally, while our findings from **Section 3** illustrate for the first time an interaction between NPAS2 and SIRT1 in the NAc and together they may regulate reward-relevant processes through shared gene targets, the cross comparison of ChIP-seq datasets to reveal shared gene targets between NPAS2 and SIRT1 has two main limitations that must be considered in the interpretation of these data. This cross comparison took advantage of two independently generated and previously published ChIP-seq datasets investigating the genes bound by NPAS2 (Ozburn et al., 2015) and SIRT1 (Ferguson et al., 2015) in the NAc. The first study by our lab was designed to compare genes bound by NPAS2 in the NAc across 6 times of day (ZT 2, 6, 10, 14, 18, & 22), relative to CLOCK, and ultimately identify novel NPAS2-specific gene targets (Ozburn et al., 2015). The SIRT1 study conducted by the Nestler lab was designed to compare genes bound by SIRT1 in the NAc in saline versus cocaine treated mice, with the ultimate goal of identifying novel cocaine-mediated SIRT1 gene targets (Ferguson et al., 2015). Notably, in the latter study, investigation into SIRT1's gene targets was conducted only at one time of day (~ZT 2). The first main limitation is that the shared gene targets between NPAS2 and SIRT1 identified are only at

the one time of day (ZT2) the two datasets had in common. Given their known circadian functions and their diurnal expression patterns, future investigation into shared targets between NPAS2 and SIRT1 across time of day is still necessary. This would be especially relevant considering NPAS2 had twice as many unique gene targets in the dark phase at ZT 18, than in the light phase at ZT2 (Ozburn et al., 2015). The second main consideration for interpreting these data is that the SIRT1 gene targets were identified in mice injected with saline, while the NPAS2 dataset was generated in mice not receiving any injections. Since injections, even with saline, can be stressful for mice (Lapin, 1995; Drude et al., 2011), investigation into shared gene targets between NPAS2 and SIRT1 in non-injected mice is still necessary. An immediate next step will be designing a more controlled and comprehensive investigation into the shared gene targets between NPAS2 and SIRT1 in the NAc across time of day and without injection stress; such an experiment may even uncover additional reward-relevant shared gene targets between the two not seen in this dissertation. Moreover, considering cocaine was shown to increase NPAS2 and SIRT1 expression in the NAc and alter diurnal variation, future studies may also explore how cocaine affects their shared gene targets across time of day. Nonetheless, the findings presented in **Section 3** reveal a shared regulation of interesting reward-relevant processes that warrant future investigation into how NPAS2 and SIRT1 together regulate reward in the NAc.

Finally, the studies in **Section 3** utilized resveratrol as a means of activating SIRT1. This choice was based on previous findings from our lab and the Nestler lab illustrating a reward promoting effect of resveratrol through SIRT1 in the NAc (Renthal et al., 2009; Logan et al., 2019). While resveratrol has been extensively shown to activate/promote SIRT1 activity (Knutson and Leeuwenburgh, 2008; Mohar and Malik, 2012; Price et al., 2012; Lakshminarasimhan et al., 2013; Hou et al., 2016), resveratrol has also been found to have other off-target interactions and effects

(Salehi et al., 2018; Saqib et al., 2018; Shaito et al., 2020). Though these findings may confound interpretation of NPAS2 being necessary for the reward promoting effects of SIRT1 activation by resveratrol, in that the non-specificity of resveratrol cannot be ruled out, it's important to note our lab and others have further validated the reward promoting effects of SIRT1 activation specifically through SIRT1 overexpression or the upregulation of NAD+ by its precursor NMN or the NAD+ salvage pathway enzyme NAMPT (Ferguson et al., 2013; Kong et al., 2018; Logan et al., 2019). While the studies outlined in **Section 3** convincingly reveal a shared role of NPAS2 and SIRT1 in regulating reward through an interaction in the NAc, future investigation of this reward regulation using more specific SIRT1 activators and/or viral and genetic tools may help further elucidate the full extent of this regulation and/or reveal the full potential of this interaction through the removal of potential off-target confounds.

5.6 Conclusion

Ultimately, the studies outlined in this dissertation reveal a multifaceted and highly integrated relationship between circadian rhythms, metabolic state, and reward-regulation in the NAc at both the molecular level through an interaction between NPAS2 and SIRT1, and at the cellular level through the circadian regulation of astrocyte functioning. Together, these studies provide a potential mechanism by which drug-related alterations to either circadian and/or metabolic state may influence NAc-dependent reward-regulation and a means by which aberrant functioning of either system may drive vulnerability to addiction-like behavior. In a field long dominated by neuron-centric perspectives and hypotheses, astrocytes and other glial cells are now

emerging as critical contributors to the maintenance and regulation of healthy neurotypical functioning, as well as potential players in aberrant and/or pathophysiological processes. We hope that through the studies outlined in this dissertation, we not only further our appreciation of the many interconnected circadian, metabolic, and cell-type-specific variables that may contribute to reward regulation and vulnerability to SUDs onset, but also inspire investigation and development of novel therapeutic targets for the more efficacious treatment of SUDs.

Appendix A NPAS2 regulates exploratory drive across time of day

Appendix A.1 Introduction

Previous work in our lab has demonstrated the circadian transcription factor NPAS2 to be important for reward-related behaviors through its enriched expression in the NAc (Ozburn et al., 2015, 2017; Parekh et al., 2019; DePoy et al., 2020). NPAS2 was specifically shown to regulate exploratory drive and/or anxiety-like behavior, whereby *Npas2* null mutant mice exhibit increased exploratory drive or decreased anxiety-like behavior compared to wild-type mice; Npas2 mutant mice show increased locomotor response to novelty, increased open arm entries in the EPM task, decreased latency to enter the brightly lit side in the L/D task, and a trending increased distance traveled and time spent in the center of the OF arena (Ozburn et al., 2017). However, these behavioral effects were only measured during the mouse's inactive phase (i.e., at ZT 2 during the light phase). Given that mice are most active during the dark phase (ZT 12 - 23) and the known time-of-day effects seen with *Npas2* mutant mouse cocaine self-administration behavior (DePoy et al., 2020), here we wanted to investigate the *Npas2* null mutant exploratory drive behavioral phenotype during the dark/active phase.

Appendix A.2 Methods

Appendix A.2.1 Animals

Experiments utilized either male *Npas2* mutant and wild-type (WT) littermates (maintained on a C57BL/6J background) from the Weaver lab (Dallmann et al., 2011), ages 8-12 weeks. *Npas2* mutant mice carry a mutation resulting in NPAS2 lacking its functional domain and rendering it incapable of binding BMAL1 (Garcia et al., 2000). All mice were maintained on a 12:12 reverse light-dark cycle (lights on at 0700, zeitgeber time (ZT) 0, and lights off at 1900, ZT12) with food and water provided *ad libitum*. Behavioral testing occurred between ZT14-17 under red light, with at least 30 minutes of habituation to the room. Animal use was conducted in accordance with the National Institute of Health guidelines, and all procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Appendix A.2.2 Behavioral Testing

A battery of behavioral tests assessing exploratory drive / anxiety-like behavior was conducted with both *Npas2* null mutant and wild-type mice.

Locomotor Response to Novelty (LRN). Mice were placed individually in 25 x 45 cm plexiglass boxes equipped with photo beams (Kinder Scientific Smart Cage Rack System; Poway, CA). Distance traveled (cm) was recorded for 2 hours in 5-minute bins, as well as overall total distance.

Open Field (OF). Under 20 lux lighting, mice were placed individually in a large black plexiglass arena (52 x 52 x 25 cm) and allowed to explore for 10 min freely. Behavior was recorded and analyzed using Ethovision XT 13 (Noldus; Leesburg, VA), calibrated by blind hand scoring. Time spent in the center of the arena (24 x 24 cm square in the center), center entries, and distance traveled were recorded.

Light / Dark Box (L/D). Mice were placed individually in a 25 x 45 cm plexiglass arena equipped with photo beams, divided into two equally sized chambers (Kinder Scientific Smart Cage Rack System; Poway, CA) – one side brightly lit (~880 lux) and the other containing a black opaque box. Mice were placed on the dark side for 2 min before a door opened, allowing mice to explore both sides for 20 minutes freely. Distance traveled, light entries, and time spent in the light side were all recorded.

Elevated Plus Maze (EPM). Under 20 lux lighting, mice were placed in the center of an elevated plus maze (81 cm off the ground), consisting of 2 open arms and 2 enclosed arms oriented perpendicularly (each 30 x 5 cm). Mice started facing an open arm and were allowed to explore the maze for 10 minutes freely. Behavior was recorded and analyzed using Ethovision XT 13, calibrated by blind hand scoring. Arm entries (both open and closed), time spent in the open arm, and distance traveled were recorded.

Appendix A.2.3 Statistical Analyses

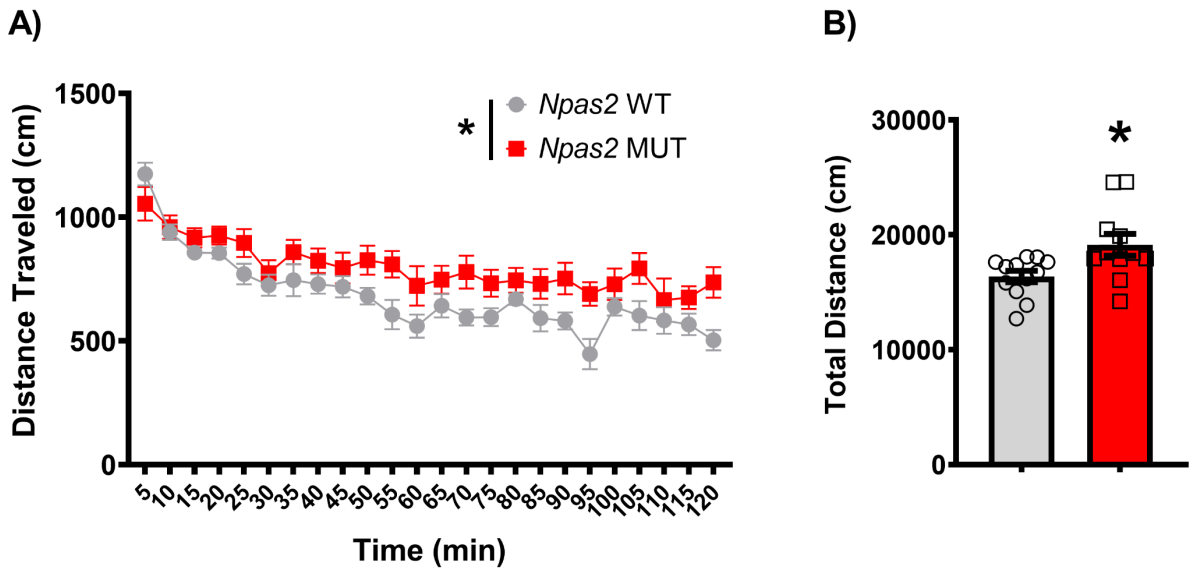
For all statistical analyses, GraphPad Prism 9 software was used (GraphPad Software; San Diego, CA, USA; RRID:SCR_002798). Statistical analysis of locomotor data was performed using a repeated measures two-way analysis of variance (ANOVA). Otherwise, statistical analysis of

just two groups was performed using a Student's t-test. Across all data sets, outliers were tested for using the Grubbs' test. Data throughout are expressed as mean ± SEM with $\alpha = 0.05$ considered statistically significant.

Appendix A.3 Results

Expanding on our characterization of exploratory drive / anxiety-like behavior during the light phase (Ozburn et al., 2017), to determine if functional NPAS2 is important for exploratory drive across time of day, we assayed *Npas2* null mutant and WT mice littermates in a battery behavioral tests during their active phase (i.e., the dark phase). This was assessed through conducting the locomotor response to novelty, open field, light/dark, and elevated plus maze assays at ZT 14-17 under red light. *Npas2* mice were first run through the locomotor response to novelty task (**Appendix Figure 1**) – a behavior established as a predictor of drug-seeking and addiction-like behavior vulnerability (Stead et al., 2006; Flagel et al., 2010; Zhou et al., 2019b). Interestingly, unlike in the light phase where Npas2 mutant mice shown an increased locomotor response to novelty primarily in the first half of the 2 hour task (Ozburn et al., 2017), in the dark phase, *Npas2* mutant mice show an increase in locomotor activity during the second half of the task (**Appendix Figure 1A**). This increase is seen through both main effects of genotype and time and a significant interaction, in a repeated measures two-way ANOVA (Genotype: $F_{(1, 21)} = 6.85$, * p=0.01; Time: $F_{(9, 195)} = 17.99$, p<0.0001; Time x Genotype Interaction: $F_{(23, 483)} = 1.72$, * p=0.02). However, it is unclear whether this increase is driven by an overall increase in locomotor

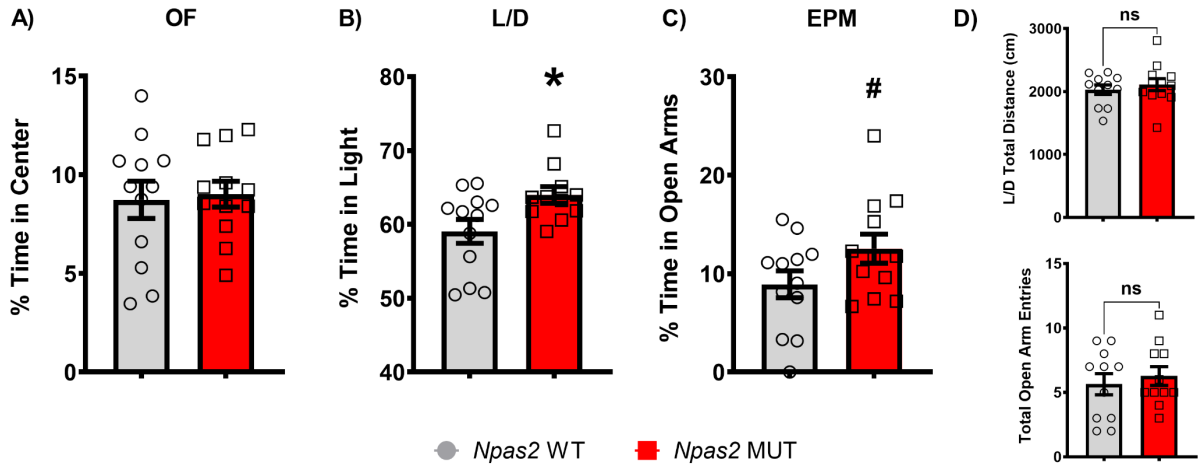
activity, in that *Npas2* mutant mice show a significant increase in total distance traveled across the task ($t_{(21)} = 2.62$, * p=0.01) (**Appendix Figure 1B**).



Appendix Figure 1. *Npas2* mutant mice show increased locomotor activity in the dark phase. (**A**) In the locomotor motor response to novelty (LRN) task, *Npas2* mutant mice show a significantly greater locomotor response during the dark phase, relative to WT controls, as measured by main effects of time, genotype (* |), and a significant interaction. (**B**) However, this increased locomotor response may be driven by an overall increase in activity, in that *Npas2* mutant mice show a significant increase in total distance traveled during the task. Mean ± SEM; n=11-12; * p<0.05.

In addition to the locomotor response to novelty, increased exploratory drive and novelty-seeking behavior are also established predictors of vulnerability for drug-seeking and addiction-like behaviors in rodent models (Stead et al., 2006; Bush and Vaccarino, 2007; Flagel et al., 2010, 2014; Dickson et al., 2015; Wingo et al., 2016). Following the LRN task, *Npas2* mice were then run through a panel of exploratory drive / anxiety-like behavior assays during the dark phase. While *Npas2* mutant mice show no significant differences in the open field task (**Appendix Figure**

2A), *Npas2* mutant mice exhibit significantly greater percent time spent in the light chamber of the L/D task (**Appendix Figure 2B**; $t_{(21)} = 2.46$; * p=0.02) and a greater percent time spent in the open arms of the EPM (**Appendix Figure 2C**; $t_{(22)} = 1.81$; # p=0.08). Notably, this increased exploratory drive was not attributed to any significant locomotor differences during the three tasks, in that *Npas2* mutant mice show no differences in OF distance traveled (data not shown; $t_{(22)} = 0.26$, p = 0.79), no differences in L/D total distance traveled (**Appendix Figure 2D**: **Top**; $t_{(21)} = 0.67$, ns, p = 0.51), and no differences in total open arm entries (**Appendix Figure 2D: Bottom**; $t_{(20)} = 0.58$, ns, p = 0.57).



Appendix Figure 2. *Npas2* mutant mice show increased exploratory drive in the dark phase. Mice were run through a panel of behavioral assays testing exploratory drive. (**A**) In the open field (OF) task, *Npas2* mice show no sifnicant differneces. (**B**) In the light-dark box test (L/D), *Npas2* mutant mice spend a significantly greater percentage of time in the brightly lit chamber of the arena, relative to controls. (**C**) *Npas2* mutant mice also spent a greater percentage of time in the open arms of the elevated plus maze (EPM). (**D**). These differences in exploratory drive were not attributed differences in L/D total distance traveled (top) or EPM total number of entries (bottom); Mean ± SEM; n=11-12; # p=0.08, * p<0.05, ns = not significant.

Appendix A.4 Discussion

Disruptions to circadian rhythms and the circadian molecular clock have been shown to play an important role in the regulation of reward and reward-related behavior in mice. Previous work in our lab has demonstrated the circadian transcription factor NPAS2 to be important for reward-related behaviors through its enriched expression in the NAc (Ozburn et al., 2015, 2017; Parekh et al., 2019; DePoy et al., 2020). NPAS2 was previously shown to regulate exploratory drive / anxiety-like behavior during the light phase, whereby *Npas2* null mutant mice show increased locomotor response to novelty and increased exploratory drive across multiple behavioral assays (Ozburn et al., 2017). Here we show NPAS2 may regulate these behaviors across time of day, in that *Npas2* null mutant mice show similar changes in behavior during the dark phase. Much like during the light phase, *Npas2* mutant mice also show increased exploratory drive (i.e., decreased anxiety-like behavior) during the dark phase, as measured by the greater percent time in both the light chamber of the light/dark assay and the open arms of the elevated plus maze. However, while *Npas2* null mutant mice show increased locomotor response to novelty during the light phase, during the dark phase Npas2 mutant mice demonstrated an overall increase in locomotor activity during the same task. Notably, these differences in locomotor activity did not contribute to the differences seen in the exploratory drive behavioral panel, in that no significant differences were seen in total distance traveled or total number of entries in those tasks. Taken together, these data suggest NPAS2 contributes to the circadian regulation of reward-relevant behaviors, regulating exploratory drive across phase/time of day. These findings are further corroborated by our recent study demonstrating *Npas2* mutant mice also show increased intravenous cocaine self-administration across time of day (DePoy et al., 2020). However, while

other studies from our lab have shown much of the *Npas2* null mutant phenotype can be recapitulated either through NAc specific knock-down of *Npas2* or even through D1-MSN specific knock-down of *Npas2* in the NAc (Ozburn et al., 2015, 2017; Parekh et al., 2019), no studies have investigated the role of NPAS2 in the NAc for these behaviors across time of day. Future investigation is needed into how NPAS2 in the NAc regulates reward-related behavior across time of day, and through which cell types these mechanisms are mediated. Nonetheless, these findings contribute to our understanding of the role NPAS2 plays in regulating reward-related behavior and further underscores the functional significance of the circadian molecular clock.

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