Determining Parvalbumin-expressing Interneuronal Loss in Dp16 Down Syndrome Model Mice

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ABSTRACT

Studies have shown that individuals living with Down Syndrome (DS) are prone to experiencing sleep disturbances as well as the early-onset development of neurodegenerative disease. Although scientists are not entirely sure why individuals with DS express the sleep phenotypes they do, research has shown that a lack of sufficient sleep is linked to the development of neurodegenerative disorders in the future. To further investigate the mechanisms behind these sleep disturbances, this study utilized a DS mouse model to explore the phenotypic effects of reducing the *Rcan1* gene from three copies to the typical two copies via a process known as dosage correction. This study also seeks to compare the quantity of parvalbumin-expressing interneurons (PV+) found in three genotypic groups; the first being wildtype, the second being the down syndrome model Dp16, and the third group also stemming from Dp16; however, only two copies of the *Rcan1 gene* are present instead of three. A significant difference in PV+ cells was found following the first quantification, however, an increase in the sample size contradicted this significance after the second quantification, indicating that some inconsistencies may have been introduced between stains. Because a power calculation still deemed the amount of data insufficient for accurate results, we are currently in the process of conducting a third quantification where all staining and quantifying will be done at the same time, simultaneously achieving sufficient power, and mitigating potential technical confounds.

INTRODUCTION

Down Syndrome and Sleep

Trisomy of *Homo sapiens* chromosome 21 (HSA21), or the presence of an extra 21st chromosome, results in a collection of clinical traits known as Down Syndrome (DS). 206,366 individuals in the United States were estimated to be living with DS in 2010, making this condition the most common and one of the most survivable complex genetic disorders of intellectual disability. While common manifestations and deeper mechanisms of DS have not been extensively explored, researchers do know that approximately 60% of the DS population displays sleep disturbances (Fan et al., 2017). Though obstructive sleep apnea (OSA) resulting from abnormal facial structure is one cause of sleep disturbance in DS individuals, additional studies show that sustained sleep disruption does exist independently of OSA (Nisbet et al., 2015). These disruptions are characterized by an increased latency to non-rapid eye movement (NREM) sleep, sleep fragmentation, and reduced REM sleep (Andreou et al., 2002; Fernandez and Edgin, 2013; Hamaguchi et al., 1989), each of which can play a large role in cognitive development and maintenance. In addition, DS individuals have altered electroencephalography (EEG) oscillations associated with cognitive deficits (Lopez-Loeza et al., 2016; Politoff et al., 1996; Smigielska-Kuzia et al., 2005; Velikova et al., 2011), revealing a potential biomarker for cognitive impairment in DS (Levenga et al., 2018; Salem et al., 2015; Velikova et al., 2011). In order to further explore these observations, the Hoeffer lab has performed a series of experiments in an effort to help elucidate potential molecular mechanisms backing DS phenotypes.

Previous studies

In May of 2022, the Hoeffer lab published a paper that investigated the role of Regulator of calcineurin 1 (*Rcan1*) in the maintenance of diurnal and circadian rhythms with implications for Down syndrome (DS), Alzheimer's disease (AD), and aging (Wong et al., 2022). *Rcan1* is known to be overexpressed in all three contexts and abnormal levels have been linked to memory deficits and neurodegenerative pathophysiological characteristics of these conditions (Ermak & Davies, 2013; Rachidi et al., 2007). Anomalous rest-activity patterns and circadian rhythm disruptions are common features in DS, AD, and aging, contributing to cognitive decline and disease progression (Lee & Silva, 2009; Rachidi et al., 2007). However, the influence of *Rcan1* dysregulation on these circadian disturbances has not been extensively explored.

Using mouse models with *Rcan1* deficiency and overexpression, various parameters of diurnal and circadian locomotor activity in both young and aged mice were examined. The results revealed that balanced *Rcan1* expression is essential for normal circadian locomotor activity rhythms, with *Rcan1*-null and *Rcan1*-overexpressing mice displaying lengthened endogenous circadian periods similar to mouse models of AD and aging (Wong et al., 2022). Additionally, these mice exhibited hypoactivity, fragmented rest-wake patterns, and attenuated circadian activity rhythms, resembling the characteristics observed in DS, AD, and aging. Further investigation using the Dp(16)1Yey/+ (Dp16) mouse model for DS, which expresses three copies of Rcan1, demonstrated reduced wheel running activity and rhythmicity akin to *Rcan1*-overexpressing mice. More importantly, restoring Rcan1 to two copies in Dp16 mice either partially or fully rescued these diurnal and circadian deficits, indicating a direct link between *Rcan1* dosage and circadian disturbances in DS (Wong et al., 2022).

Another important finding was that *Rcan1* deficiency altered protein levels of the clock gene Bmal1 in the suprachiasmatic nucleus (SCN), a key regulator of circadian rhythms, suggesting a mechanism by which *Rcan1* may influence circadian clock function. In conclusion, the collective data suggests that both loss and aberrant gain of *Rcan1* contribute to abnormal diurnal and circadian activity patterns reminiscent of DS, AD, and potentially aging. These data underscore the importance of *Rcan1* in circadian regulation, a valuable finding that helped inspire the structure and notions behind this thesis.

In conjunction with the aforementioned 2022 publication, data from the lab's current manuscript, in which this research will be included, also provides evidence that supports an association between *Rcan1* and the disturbances observed in DS individuals. Levenga et al. (2018) demonstrated that aged Dp16 mice spend more time awake and less time in non-rapid eye movement (NREM) sleep compared to wild-type (WT) mice. Based on this study, we conducted an experiment that would explore differences in sleep patterns between WT, Dp16, and *Rcan1* dosage corrected Dp16 mice (Rcan1^{2n}). Interestingly, reducing *Rcan1* protein levels in aged Dp16 Rcan1^{2n} mice normalized sleep architecture to a pattern similar to WT mice. Additionally, aged Dp16 Rcan1^{2n} mice exhibited less difficulty initiating and maintaining sleep than aged

Dp16 mice. These findings imply that decreasing *Rcan1* levels in DS individuals could enhance NREM bout length, potentially improving sleep maintenance.

Regulator of calcineurin 1

Rcan1 is known to modulate the activity of calcineurin (CaN), a key enzyme involved in various physiological processes including circadian rhythmicity and sleep (Fuentes-Pardo et al., 1995; Rothermel et al., 2000). CaN activity regulates the expression of clock genes, contributing to the entrainment and phasing of circadian rhythms (Dyar et al., 2015; Huang et al., 2012; Katz et al., 2008; Kweon et al., 2018; Nakai et al., 2011; Sachan et al., 2011). Furthermore, CaN dephosphorylates nuclear factor of activated T cell (NFAT) transcription factors, which play a role in circadian clock expression (Aramburu et al., 2000; Dyar et al., 2015; Lee et al., 2019; Sachan et al., 2011). Dysregulation of *Rcan1* may disrupt CaN activity and subsequent NFAT signaling, potentially contributing to DS-related sleep disturbances. NFAT signaling involves the translocation of NFAT transcription factors from the cytoplasm to the nucleus, where they regulate the expression of target genes involved in various cellular processes (Aramburu et al., 2000). In the context of circadian rhythms, NFAT-mediated gene transcription is crucial for coordinating the expression of clock genes, such as Bmal1 and Clock, which form the core molecular machinery of the circadian clock (Dyar et al., 2015; Lee et al., 2019; Sachan et al., 2011). Disruptions in NFAT signaling, possibly induced by dysregulated RCAN1-CaN interactions, may therefore lead to alterations in clock gene expression and perturbations in circadian rhythmicity, contributing to the sleep phenotypes observed in DS and other neurodevelopmental disorders.

Parvalbumin-expressing Interneurons in the Hippocampus

In addition to the previous and current research regarding *Rcan1* and its relationship to the molecular circadian clock, other studies have highlighted interesting findings that led to the project at hand. More specifically, findings that allude to parvalbumin-expressing interneurons (PV+) in the hippocampus (HPC) being a potential mechanism for sleep disturbances observed in DS. The motivation to explore the HPC is attributed to the HPC's multifaceted role in memory formation, spatial navigation, and its intricate relationship with sleep patterns. Studies have consistently shown that hippocampal function is impaired in individuals with Down syndrome (DS), characterized by deficits in spatial learning, memory consolidation, and synaptic plasticity (Belichenko et al., 2009; Costa & Grybko, 2005; Fernandez et al., 2007; Vorhees & Williams, 2006), illustrating a variety of neuronal function.

Moreover, emerging research suggests that the HPC plays a significant role in regulating sleep-wake cycles and sleep-related memory processes as the HPC exhibits distinct patterns of neural activity during different stages of sleep (Buzsáki, 2015). Additionally, hippocampal neurons exhibit place cell firing patterns during both wakefulness and sleep, suggesting a role in spatial memory consolidation during sleep (Wilson & McNaughton, 1994), highlighting another process that highly depends on an individual’s ability to initiate and maintain sufficient sleep.

Understanding how hippocampal dysfunction contributes to both cognitive deficits and sleep disturbances in DS could provide valuable insights into the underlying pathophysiology of the disorder and inform potential therapeutic interventions.

In order to identify potential differences in the neuronal architecture of the HPC, we referred to previous research that has underscored the pivotal role of parvalbumin-expressing (PV+) interneurons in the intricate regulation of wake-sleep cycles and the generation of sleep-related electroencephalogram (EEG) oscillations (Brown et al., 2012; Roopun et al., 2006; Saper and Fuller, 2017). An earlier study also revealed a reduction in the population of PV+ interneurons, specifically in the stratum oriens of the HPC in Dp16 mice. This implicates PV+ interneuronal loss in the manifestation of sleep and EEG irregularities observed in aged Dp16 mice (Raveau et al., 2018). Based on these studies, our lab encouraged a potential link between diminished PV+ interneurons and aberrant sleep patterns in Dp16 mice. To explore these implications further, our investigation supplementally delved into assessing the abundance of PV+ interneurons in various brain regions known to play critical roles in the regulation of sleep-wake cycles and the generation of EEG rhythms. While the research and data detailed in this thesis will solely focus on results generated by the HPC, it is important to note that this experiment also explores the reticular nucleus of the thalamus (RT) and medial septum of the forebrain (MS) as these regions have been shown to have well-established functions in sleep-wake regulation and EEG rhythmogenesis (Brown et al., 2012).

The Dp(16)1Yey/+ (Dp16) Mouse Model

Today, mouse models of DS are helping to reveal the behavioral and molecular backings behind the clinical manifestations associated with DS. DS mouse models are produced via chromosomal engineering of mouse chromosomes 10 (~39 orthologous genes), 16 (~113 orthologous genes), and 17 (~19 orthologous genes), three chromosomes that are either partially or majorly syntenic to human chromosome 21. In this study, *Dp(16)1Yey/+* (Dp16) was the choice of model as mouse chromosome 16 covers the majority of genes present in human chromosome 21. In the case of Dp16 model mice, the chromosome is triplicated by replicating and attaching a “third arm” onto one of the pre-existing chromosomes. This model is widely acknowledged as “Dp16” because it represents a duplication of all genes on the 16^{th} chromosome rather than the addition of a third 16^{th} chromosome. When paired with a typical WT 16^{th} chromosome, the Dp16 model produces three copies of each gene located on the chromosome.

METHODS

Animals

Dp(16)1Yey/+ (Dp16) mice (Yu et al., 2010) on a C57BL/6J background were bred with *Rcan1* heterozygous mice (Vega et al., 2003) to generate wildtype (WT), Dp16, and Dp16 littermates carrying only two copies of the *Rcan1* gene ($Rcan1^{2n}$) (Figure 1). Based on PCR testing performed a few weeks after birth, mice were categorized by genotype and maintained

individually in plastic cages measuring 31x18x18 cm with a temperature of 22-24°C. Food and water were available *ad lib*. All mice were aged eight months or greater prior to perfusion, and both female and male mice were utilized to account for potential sex differences between counterparts.

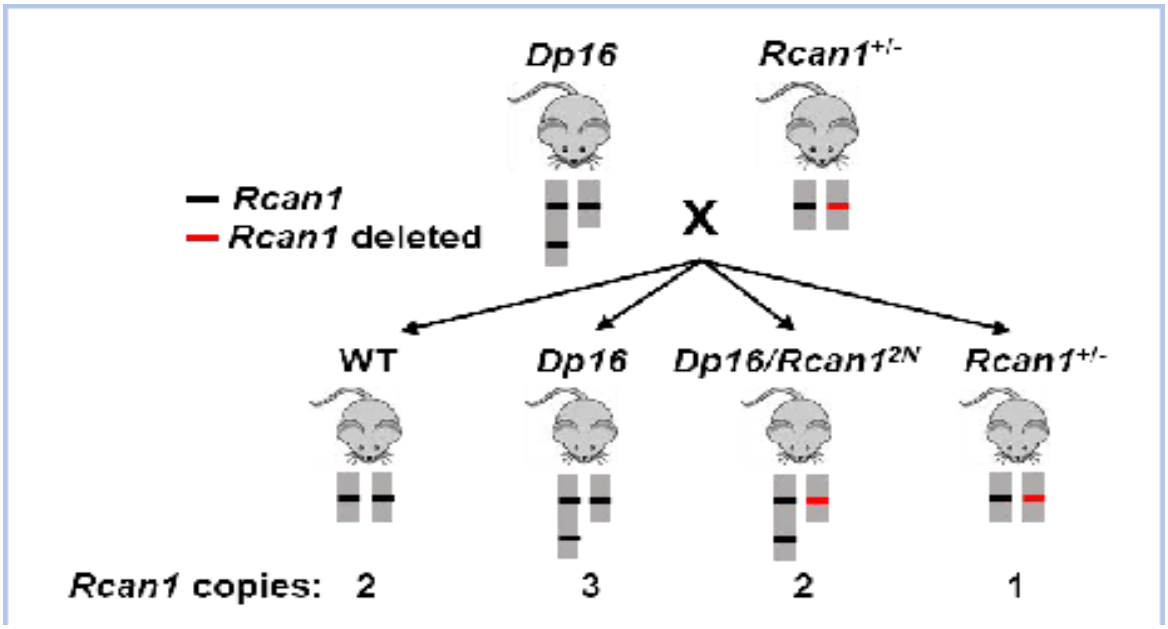

Fig 1: Breeding scheme to generate Dp16 mice with *Rcan1* dosage correction

Slicing and Staining

Fluorescent immunostaining was conducted on fixed brain sections obtained from WT, Dp16, and Dp16 Rcan1^{2n} mice following previously established protocols (Levenga et al., 2017). Mice were acclimated for at least an hour in the same holding room before undergoing transcardial perfusion with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). After tissue was deemed fixed, brains were extracted and immersed in 4% PFA for 24 hours at 4°C. They were then transferred to 30% sucrose in PBS for a minimum of 24 hours at 4°C after which they were sliced into 30 µm coronal sections using a cryostat (Leica) and stored free-floating in cryoprotectant solution (30% sucrose/30% ethylene glycol in phosphate buffer) at −20°C. Sections encompassing all strata and sub-regions of the dorsal hippocampus (HPC), reticular nucleus (RT) of the thalamus, and medial septum (MS) of the basal forebrain, were rinsed with PBS and then blocked at room temperature in staining buffer comprising 0.05 M Tris pH 7.4, 0.9% NaCl, 0.25% gelatin, 0.5% TritonX-100, and 2% donkey serum prior to immunostaining for parvalbumin (PV). PV expression was detected using a primary antibody from Millipore (MAB1572) at a concentration of 1:1000, followed by Alexa Fluor 647-conjugated anti-mouse IgG1 secondary antibody (Invitrogen A21240) at a dilution of 1:500. Co-staining for NeuN (Novus NBP1-92693) at 1:1000 was performed, with detection carried out using Alexa Fluor 555-conjugated anti-mouse IgG2B secondary antibody (Invitrogen A21147) at

1:500. Hoechst was utilized at a concentration of 1:3000. The sections were incubated with primary antibodies at 4°C for 24 hours followed by secondary antibodies and Hoechst at room temperature for 2 hours.

PV+ Neuron Imaging and Quantification in the HPC

Stained sections were then mounted, coverslipped with Mowiol, and imaged using the Nikon A1R confocal microscope. Z-stacks taken at 20x were captured through the entire thickness of the slice and microscope parameters were maintained throughout imaging. Neurons positive for parvalbumin (PV+) in at least three slices were blindly quantified using a bioimage informatics interface known as ICY. Using the same program, a hand traced 2D region of interest (ROI) was drawn around the left and right HPCs. Fluorescing cells were manually labeled with 2D markers. Labeled cells were totaled and divided by the highlighted area to produce a standardized reading for cell density.

Statistical Analysis

All statistical analyses performed for this project were done so using Excel (Microsoft Corp., WA), Statisty (Graz, Austria), and GraphPad/Prism. Excel was utilized for the student's t-test and Pearson's correlation test while Satisty provided values for the one-way ANOVAs. An alpha level of 0.05 indicated a significant difference between groups and outliers were removed via ROUT in Graphpad. PV+ cells and hippocampal area were manually counted and recorded via ICY Bioimage Analysis Software.

RESULTS

PV+ cell count is altered in the Dp16 model

Dorsal hippocampal slices containing PV+ cells were manually quantified using ICY Bioimage Analysis Software to determine whether there might be a difference across genotypes. After analyzing the raw cell counts for wildtype (mean = 121.9167) and Dp16 (mean = 106.25) model mice, a student's t-test value of $p = 0.04629$ indicated a significant difference in cell count, showing that the number of PV+ cells is altered in the Dp16 model mice compared to their wildtype counterparts (Fig 2A). However, after plotting the datasets, the Dp16 and WT females appeared to have a much lower PV+ cell count compared to the males, suggesting that there may be a difference in HPC size depending on sex. To correct for this, all cell counts were divided by HPC area, resulting in a much more even distribution among sex cohorts while maintaining significance (Fig 2B).

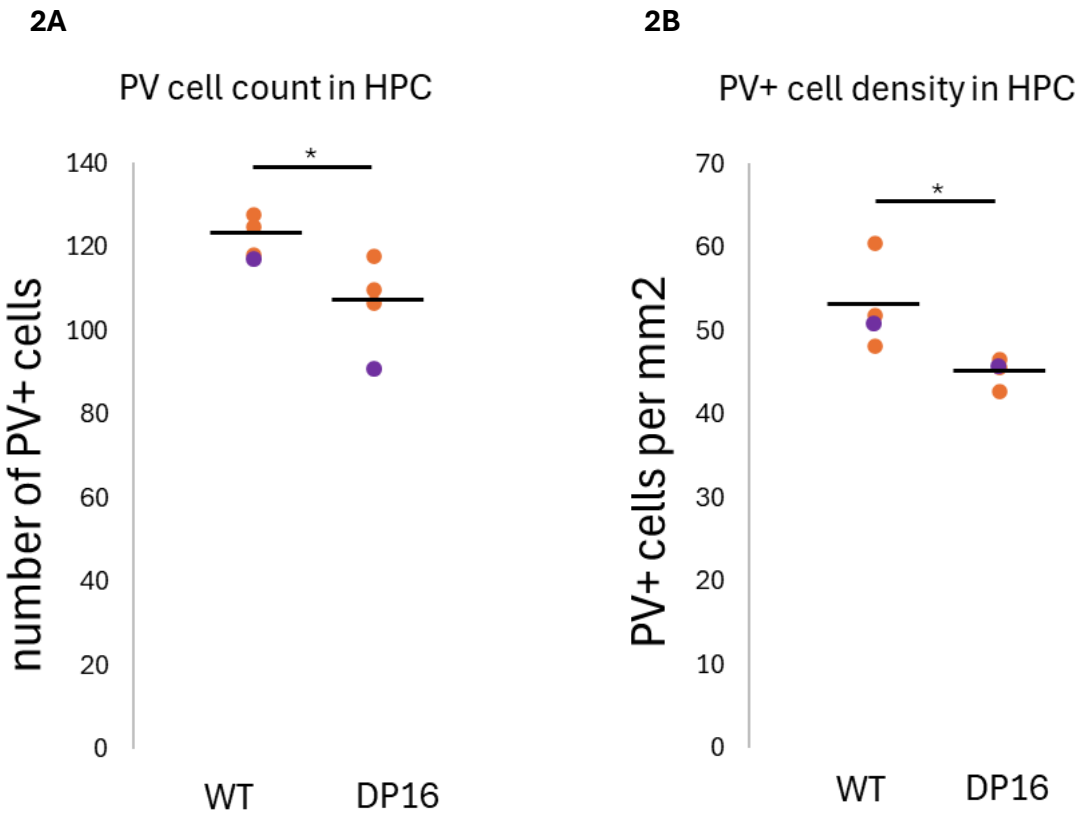

Fig. 2*: Raw PV+ cell counts and density in the HPC of wildtype and DS model mice.* ***(A)*** *Raw PV+ cell counts in WT and Dp16 model mice, with both cohorts containing data from one female (purple) and three males (orange) (WT n=4 and Dp16 n=4).* ***(B)*** *HPC area was accounted for by dividing the PV+ cell counts by the area of the HPC. This correction resulted in a more even distribution of male versus female data points and appeared to correct for any potential difference in HPC size.*

Addition of *Rcan1*^{2n} data contradicts significance found after first quantification

After discovering a partial rescue in the sleep/wake patterns of *Rcan1*^{2n} model mice (Figure 3A), we were curious to see if *Rcan1* might also play a role in hippocampal PV+ cell count. This led to the addition of a second experimental group and subsequent rounds of staining and quantification (Figure 3B). This time, no significant difference in PV+ cell density was found across genotypes (WT mean = 51.84108 ± 2.598481, Dp16 mean = 45.00974 ± 1.958042, and *Rcan1*^{2n} mean = 46.21189 ± 1.842265). This was confirmed with a one-way ANOVA ($p = 0.098526$). Groups were originally balanced (n=7), but an outlier in *Rcan1*^{2n} was identified and removed via ROUT in Graphpad.

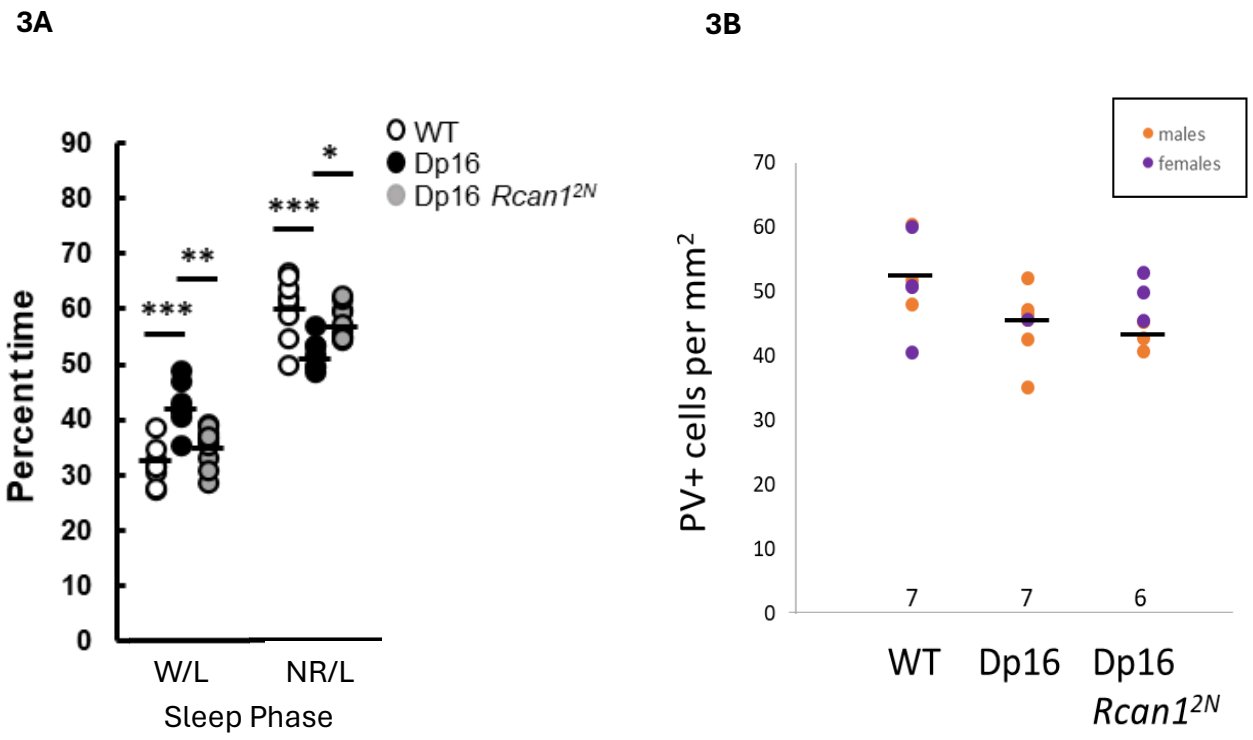

Fig. 3*: Partial rescue of Rcan1^{2n} sleep phenotype does not translate to hippocampal PV+ cell count.* ***(A)*** *Percentage of time awake during the light phase (W/L) and of non-rapid eye movement (NREM) sleep during the light phase (NR/L). WT n=9; Dp16 n=7; Dp16 Rcan1^{2n} n=9.* *******p<0.05;* ********p<0.01;* ********* *p<0.001. During typically inactive phases for nocturnal animals (light), Dp16 mice portrayed a decreased ability to sleep and maintain NREM sleep. Restoring Rcan1 to two copies showed a significant rescue, highlighting the gene's seemingly prominent role in sleep.* ***(B)*** *Hippocampal PV+ cell density in WT, Dp16, and Rcan1^{2n} model mice. WT n=7, Dp16 n=7, Rcan1^{2n} n=6. Females are represented by purple data points and males are represented by orange.*

Significant correlations found between PV+ cell density and HPC size as well as animal age

Pearson correlation tests in Excel revealed that upon removal of an outlier (cell density = 20.86656 cells/mm^2), the correlation between cell density and HPC size is significant (p = 0.0476) (Figure 4A). Another correlation test performed between cell density and age also proved to be significant (p = 0.0026) when excluding two additional older outliers (two females aged 21.9 and 20.5 months) (Figure 4B). Outliers were identified and removed via ROUT in Graphpad.

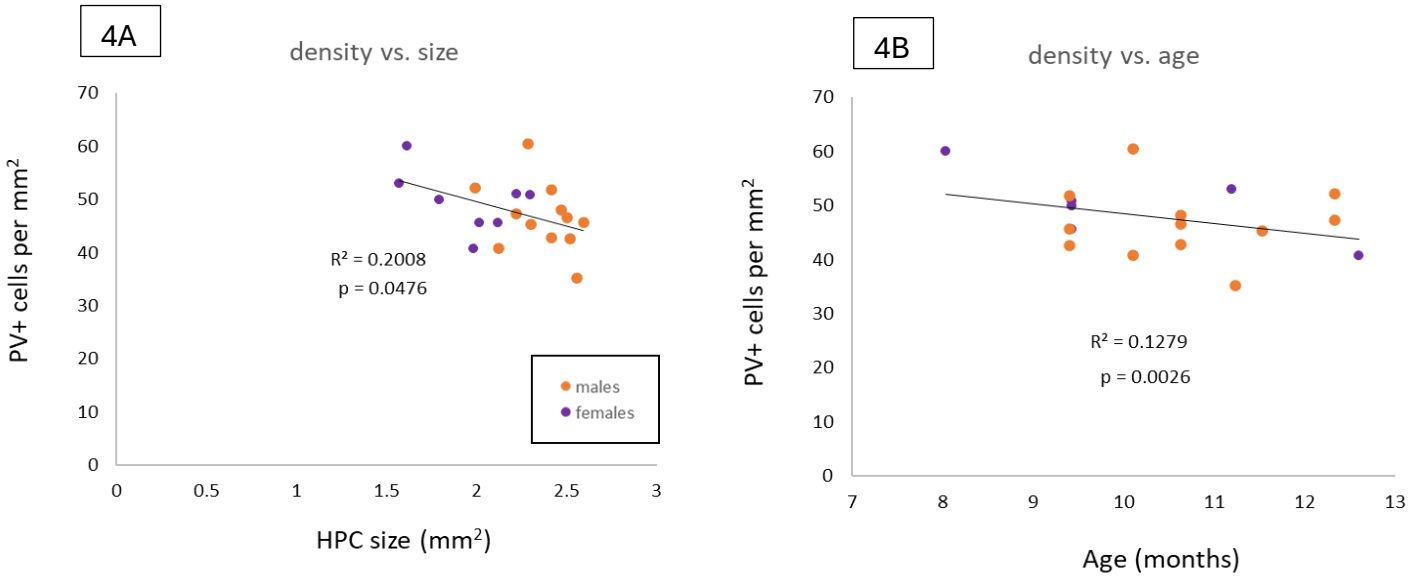

***Fig. 4**: Significant correlations found between PV+ cell density, HPC size, and animal age. **(A)** Correlation between hippocampal PV+ cell density (cells/mm^2) and HPC size (mm^2). R^2 = 0.2008, p = 0.0476. WT n=7, Dp16 n=7, Rcan1^{2n} n=6. Females are represented by purple data points and males are represented by orange. **(B)** Correlation between hippocampal PV+ cell density (cells/mm^2) and animal age (mo). WT n=6, Dp16 n=7, Rcan1^{2n} n=5. Females are represented by purple data points and males are represented by orange.*

ANOVA reveals a significant effect of batch number on PV+ cell count and HPC area

A one-way ANOVA test revealed that both PV+ cell counts (p = 0.005) and hippocampal area (p = 0.017) were significantly affected by staining batch number, however, PV+ cell density was not. Because the ANOVA results indicate notable discrepancies between group-specific quantifications performed during the first and second round of staining, some potentially confounding variables may have been introduced between stains.

DISCUSSION

Upon the first quantification, our data supported the initial hypothesis that there is likely a discrepancy in the number of PV+ hippocampal cells found in WT and Dp16 model mice. Although a student's t-test did show the finding to be significant, the females in each cohort appeared to have a much lower neuron count than their male counterparts. In order to prevent hippocampal size from skewing results, cell counts were divided by the area of the hippocampus so the resulting value would represent cell density rather than count, as shown in Figure 2B. This calculation corrected the uneven distribution between sexes observed in Figure 2A while maintaining significance.

However, as we expanded our sample size and included data from the *Rcan1*^{2n} group during a second quantification, significance was no longer observed. Because the two stains and quantifications were performed at different moments in time, the question was raised on whether or not we could compare the two separately stained batches of slices accurately. In order to determine whether there were significant differences among separately stained slices, we performed three one-way ANOVAs, two of which indeed confirmed a significant difference caused by batch number.

Despite the lack of statistical significance, calculations further revealed that the study was still underpowered, indicating room for further investigation. In terms of future directions, the study could include a larger cohort of animals to improve statistical power and allow for more robust conclusions regarding the relationship between *Rcan1* levels, PV+ cell density, and sleep phenotypes. This would involve increasing the sample size of each group and including age-matched WT controls to better compare sleep patterns and PV+ cell populations. To address this issue, we are currently in the process of increasing our sample size and completely balancing sex cohorts. This ongoing effort provides a glimmer of hope that we have yet to record significant differences in PV+ cell count between Dp16, Dp16/ *Rcan1*^{2n}, and WT mice. In addition, we are hoping that performing a one-time collective stain will elucidate a more specific effect on the *Rcan1* dosage-corrected mice, as well as shed light on any unaddressed sex differences.

If significance is established in future analyses, it is equally important to recognize that the identification of altered PV+ composition alone does not elucidate its role in the sleep disturbances observed. Understanding the causal relationship between PV+ cell count and sleep disturbances requires more complex and in-depth experimentation. More specifically, experimentation that would require the manipulation of PV+ neurons. While these kinds of experiments have not been conducted in the context of Dp16 model mice, they represent a promising avenue for future research to explore the mechanistic underpinnings of sleep disturbances in the context of Down Syndrome. Additional molecular mechanisms underlying the interaction between *Rcan1* and PV+ interneurons in the HPC could be explored by examining the effects of *Rcan1* manipulation on calcium signaling pathways, synaptic transmission, and neuronal excitability in PV+ interneurons (Belichenko et al., 2009; Costa & Grybko, 2005; Fernandez et al., 2007; Vorhees & Williams, 2006).

While our research only pertains to mouse model Dp16 at this stage, the implications of our findings have the capacity to extend beyond the laboratory setting. If significant differences in PV+ cell count are confirmed, this could pave the way for novel therapies targeting PV+ cell density or activity as well as *Rcan1* gene expression. Targeting *Rcan1* pharmacologically could potentially benefit individuals with DS by addressing the dysregulated calcium signaling and associated neurological deficits observed in this population (Dierssen, 2012; Ermak & Davies, 2002; Lee & Silva, 2009; Rachidi et al., 2007). One potential pharmacological approach is to develop *Rcan1* inhibitors to restore normal calcium homeostasis and mitigate downstream effects on neuronal function (Belichenko et al., 2009; Costa & Grybko, 2005; Fernandez et al., 2007; Vorhees & Williams, 2006).

However, there are several factors to consider regarding the potential side effects of targeting *Rcan1* pharmacologically. Firstly, *Rcan1* is involved in multiple cellular processes beyond calcium signaling including cell proliferation, apoptosis, and gene transcription (Dierssen, 2012; Ermak & Davies, 2002). Therefore, pharmacological inhibition of *Rcan1* may have off-target effects that could lead to unintended consequences, such as altered cell growth or increased susceptibility to apoptosis (Rachidi et al., 2007). Additionally, *Rcan1* is expressed in various tissues throughout the body, not just in the brain (Dierssen, 2012). Therefore, systemic administration of *Rcan1* inhibitors may affect physiological processes in other organs, potentially resulting in adverse systemic side effects (Rachidi et al., 2007). For example, *Rcan1* has been implicated in cardiac function, and its inhibition could potentially lead to cardiac arrhythmias or other cardiovascular complications (Belichenko et al., 2009; Costa & Grybko, 2005). Given *Rcan1's* involvement in numerous biological processes, the timing and duration of *Rcan1* inhibition may be crucial to minimize side effects. Chronic inhibition of *Rcan1* could disrupt normal physiological processes and lead to long-term complications (Dierssen, 2012). Therefore, careful dosing and administration protocols would need to be established to ensure efficacy while minimizing side effects (Fernandez et al., 2007; Vorhees & Williams, 2006).

Additionally, continuing to investigate the effects of *Rcan1* manipulation in mouse models, specifically within the HPC, could provide further insights into its role in regulating PV+ interneurons and sleep-wake cycles. Potential genetic manipulation could involve techniques such as viral-mediated gene delivery using adeno-associated viruses (AAVs) engineered to express specific gene constructs or RNA interference (RNAi) strategies targeting *Rcan1* expression within the HPC. First, researchers would design AAV vectors containing short hairpin RNA (shRNA) sequences targeting *Rcan1* mRNA. These vectors would then be stereotaxically injected into the hippocampal region of Dp16 model mice. This approach allows for the selective reduction of *Rcan1* expression within the HPC while leaving expression levels in other regions unaffected.

In terms of translational research, pharmacological interventions could involve the administration of small molecule inhibitors or antisense oligonucleotides (ASOs) specifically designed to inhibit *Rcan1* mRNA translation or protein activity within the HPC. For instance, researchers could utilize pharmacological compounds known to cross the blood-brain barrier and selectively target *Rcan1* expression. If executed properly, these approaches could work to achieve *Rcan1* modulation without producing off-target effects in other regions of the brain or body.

Overall, a multidisciplinary approach combining behavioral, neurophysiological, molecular, and pharmacological techniques will be essential for fully understanding the role of *Rcan1* in DS-related sleep disturbances and developing targeted therapeutic interventions to alleviate the sleep disturbances observed in DS individuals.

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