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Investigating the effects of excitotoxic stimuli on the suprachiasmatic nucleus

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Keywords: suprachiasmatic nucleus (SCN), ischemic stroke, N-methyl-D-aspartate (NMDA), excitotoxicity

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Abstract

When neurons are overstimulated, the brain experiences ischemic trauma, which causes cell damage and eventually death: a phenomenon called excitotoxicity. But, not all regions of the brain are equally affected. The suprachiasmatic nucleus (SCN)—which houses the body's twenty-four-hour clock that regulates circadian rhythms—might be more resistant to excitotoxic shock than other brain regions. However, a systematic analysis of the degree of damage to the SCN compared to the hippocampus and cortex following an excitotoxic stimulus has not yet been explored. To investigate this phenomenon, tissues were isolated from different areas of the mouse brain including the SCN, hippocampus, and cortex and treated with NMDA to induce excitotoxicity. Then the slices were fixed and sectioned, and the relative amount of cell death was quantified using propidium iodide (PI), a cell death marker. The data show there is significantly less cell death in the SCN relative to the other tissues, indicating that the SCN might be intrinsically more resilient to ischemia-associated excitotoxicity compared to other brain regions. This lesser cell damage or death characteristic of the SCN, represented as lesser percentages of Grade 2 and Grade 3 cells compared to the total number of cells both before and after NMDA treatment (an excitotoxic stimulus), is sustained across all time points (Zeitgeber time (ZT) 6, ZT 12, ZT 16, ZT 22) with no apparent circadian rhythm, despite the SCN containing the primary circadian clock and exhibiting daily changes in cell signaling and morphology. Future investigation involves understanding the mechanisms underlying SCN excitotoxic resiliency, with a specific focus on relative damage/death across different cell types and the involvement of microglia in excitotoxic responses in the SCN, anterior hypothalamus (AH), hippocampus, and cortex. We would also like to add more replicates to each experiment to further confirm our results and perform a more comprehensive analysis regarding the

relationships between the SCN and other brain regions. We hope that these findings can lead to improved stroke treatments after an ischemic event.

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1. Introduction

In the United States, ischemic stroke is the primary cause of adult disability and fourth leading cause of death. In less developed nations, stroke incidence and mortality are continuously rising. Its main effects are termed the "Dreaded-D's," which include chronic disability, dementia, and death. Since the first two of these burdening consnequences often do not cause death until many years later, there is an extremely high prevalence (3% or approximately 7 million people) of stroke in developed nations such as the United States, where hospitals are better equipped to better control vascular risk factors and prevent death in these patients [11]. Due to the severity of its consequences and prominence in both undeveloped and industrial nations, it is important to understand the cellular mechanisms underlying stroke that induce cell damage and death as well as how those mechanisms differ across various brain regions such as the SCN, AH, hippocampus, and cortex.

Glutamate, the main excitatory neurotransmitter in the brain, primarily acts through binding to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors on neurons and glial cells in the brain to facilitate vital functions such as learning, memory, and behavior [6]. However, in an ischemic event such as a stroke, cerebral blood flow decreases significantly, inducing failure of neuronal Na^+/K^+ pumps and thus excessive glutamate release and NMDA receptor overactivation. This results in a rapid influx of calcium ions into postsynaptic neurons, activation of nitric oxide synthase, and production of nitric oxide that is eventually converted into toxic free radicals. These cellular events subsequent to the initial impaired blood flow generate cell damage and death, termed excitotoxicity, which spreads from the core ischemic zone to its periphery [2]. In the event of an ischemic stroke, patients' symptoms differ depending on which side of the brain the stroke is occurring. If in the

left side of the brain, the right side of the body may exhibit symptoms including, but not limited to right-side paralysis, Broca's or Wernicke's aphasia, cautious behavior, and memory loss. If in the right side of the brain, the left side of the body may exhibit symptoms including, but not limited to left-side paralysis, impaired vision, inquisitive behavior, and memory loss [1].

NMDA is an amino acid derivative of glutamate that mimics its excitotoxic effects by activating NMDA receptors and depolarizing neurons. When glutamate or NMDA binds to the highly calcium-permeable NMDA receptor, it can remain bound and activate it for hundreds of milliseconds, which allows for a rapid influx of Ca^{2+} ions [12]. If the excitatory signaling from glutamate release is enough to result in a postsynaptic membrane depolarization that causes the neuron to reach threshold, an action potential occurs. Unlike during normal brain function, excess NMDA receptor stimulation is deleterious to the brain. Previous studies have found that NMDA-induced lesions to the rat hippocampus—the brain region implicated in learning and memory—result in more errors and increased latency in reaching learning criterion in tasks assessing both reference and working memory in a W-track continuous spatial alternation task [8]. Similarly, a high extracellular concentration of NMDA induces excitotoxicity in mixed cortical cultures including both neurons and glial cells through interactions with somatic receptors at the synapse [15]. However, while previous works show that excess NMDA receptor activation induces significant hippocampal and cortical degeneration, a systematic investigation of NMDA-induced excitotoxicity across different brain regions has not been done [3].

There are two standard treatments for ischemic stroke: tissue plasminogen activator (tPA) and endovascular therapy. The first involves intravenous administration of tPA to restore blood flow by dissolving the clot that caused the stroke; however, it can result in further complications and more than half of treated patients either do not recover completely or die [5]. This treatment

must be given within 3 hours of stroke onset for the greatest benefit, though a patient's likelihood of survival and ability to remain independent is increased significantly as long as it is within 6 hours [14]. During an emergency endovascular procedure, physicians either deliver tPA directly to where the stroke is occurring through a catheter inserted into the femoral artery (intra-arterial thrombolysis) or remove the clot by inserting a stent retriever into the blocked blood vessel in the brain to trap and remove the clot, which is typically used for patients with large clots that cannot be completely dissolved with tPA and performed together with intravenous tPA treatment [17].

The purpose of this study was to compare excitotoxic responses in the SCN compared to the AH, hippocampus, and cortex, which would enable us to determine whether the SCN exhibits endogenous resilience to excitotoxic stimuli based on dosage and circadian time. We used NMDA to trigger neuronal overstimulation in brain slices containing the hippocampus, cortex, AH, and SCN and measured the degree of damage among the cells within each region. Then, we compared the percent of damaged or dead cells across the brain regions. As this is an ongoing investigation, we also hope to compare the effects of AMPA receptor activation across each of the aforementioned brain regions to determine whether there are more cellular processes through which excessive calcium influx induces neuronal excitotoxicity. Additionally, we would like to use different techniques for quantifying cell death intensity such as lactate dehydrogenase (LDH) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays to observe degree of damage through level of mitochondrial breakdown rather than nuclear envelope breakdown. Lastly, we want to explore whether SCN microglia differ from those in other regions in baseline of excitotoxicity-induced morphology. A more in depth understanding of the mechanisms underlying the SCN's endogenous resistance to excitotoxicity would contribute to

more successful ischemic stroke treatments. By allowing medical and health care professionals to mimic this specific cellular defense mechanism in other brain regions, they can potentially reduce or eliminate the deleterious effects of ischemic stroke in various parts of the brain and ensure a quicker, stronger, and overall more successful recovery.

2. Materials and Methods

2.1 Animals

All experiments used C57BL/6 wildtype (WT) mice from Engivo. All mice were housed in polycarbonate cages in the University of Tennessee, Knoxville animal facility in a 12h-light/12h-dark cycle (12L:12D) and fed and provided water ad libitum in a temperature-controlled room (23°C). Adult male mice aged 3-4 months old at the beginning of each experiment were used to obtain coronal cortex, hippocampus, and SCN/AH-containing brain slices. These experiments followed guidelines approved by the University of Tennessee, Knoxville Institutional Animal Care and Use Committee.

2.2 Brain slice and experimental preparation

2.2.1 Dose-Response experiments: Brain slices were made between ZT 0 and ZT 1.5 and maintained using standard protocols of the laboratory in Earle's Balanced Salt Solution (EBSS) medium until the time of drug treatment. At ZT 6 perfusion was stopped, and the medium in the tissue chamber was replaced with EBSS alone or EBSS containing either 50μM, 100μM, or 500μM NMDA. After 1 hour the medium was replaced with normal EBSS and perfusion was reinstated.

2.2.2 Time Dependence experiments: Brain slices were made between ZT 0 - ZT 1.5 (for slices treated at ZT 6 or ZT 12) or between ZT 10 - ZT 11.5 (for slices treated at ZT 16 or ZT 22)

and maintained using standard protocols. Slices were treated with EBSS alone or EBSS containing 50μM NMDA for 1 hour at ZT 6, ZT 12, ZT 16, and ZT 22 using the procedure described above.

2.3 Drug treatments

After a drug or control treatment, slices were maintained in normal EBSS for an additional 3 hours. Slices were then perfused with EBSS containing 4.6μg/μL PI for an additional 2 hours. Slices were then removed from the slice chamber and placed in cold 4% paraformaldehyde (PFA) for 10 minutes followed by 30% sucrose overnight. Tissue samples were then embedded in optimal cutting temperature (OCT) compound and frozen at -80°C until sectioning. Slices were sectioned using a cryostat (10μ thick) and cover slipped with DAPI-infused mounting media (VECTASHIELD).

2.4 Data analysis

All data analysis was conducted by individuals blind to the experimental conditions using ImageJ Analysis software. Images were split into the 4',6-diamidino-2-phenylindole (DAPI) channel, showing the total number of cells, and the PI channel, which only shows damaged cells. In the PI channel, fluorescence varies in intensity based on the degree of cell damage or death [10]. Specified regions of interest (ROIs) were drawn based on DAPI staining. The total number of cells were marked and counted using the DAPI channel only. Then, the marked cells were overlaid onto the PI channel, and the PI intensity was calculated for each cell. PI intensity thresholds were determined independently by 5 individuals, which separated cells into three categories: faint/diffuse PI intensity indicating minimal or no damage (Grade 0-1), moderate/less diffuse PI intensity indicating partial damage (Grade 2), intense, non-diffuse PI intensity

indicating dead/extremely damaged cells (Grade 3). See Figure 1 for example histology and ROIs.

2.5 Statistical analysis

Typically, an analysis of variance (ANOVA) test would be used in this study given the many different comparisons across experimental groups; however, there are not enough samples within each group to conduct this type of analysis. Therefore, to obtain an initial assessment of statistical significance of preliminary results, a series of student t-tests were conducted with the level of significance set at $p < 0.05$.

3. Results

3.1 The SCN exhibits the least amount of cell death

Fluorescent microscopy of control and NMDA-treated sections for all 4 brain regions reveal PI (red) relative to DAPI (blue) staining levels (Figure 1). The results of regional comparison experiments are displayed in Figures 2-5, showing that 50μM NMDA-bath applications ZT 6 (Figure 2), ZT 12 (Figure 3), ZT 16 (Figure 4), and ZT 22 (Figure 5) to brain slices generally induced a higher percentage of Grade 2 and Grade 3 cells across all regions compared to the SCN, although the AH was only different at ZT 22. However, more replicates are needed to confirm this relationship. These data confirm and extend previous studies showing an endogenous resilience to excitotoxicity characteristic of the SCN following NMDA treatment [4].

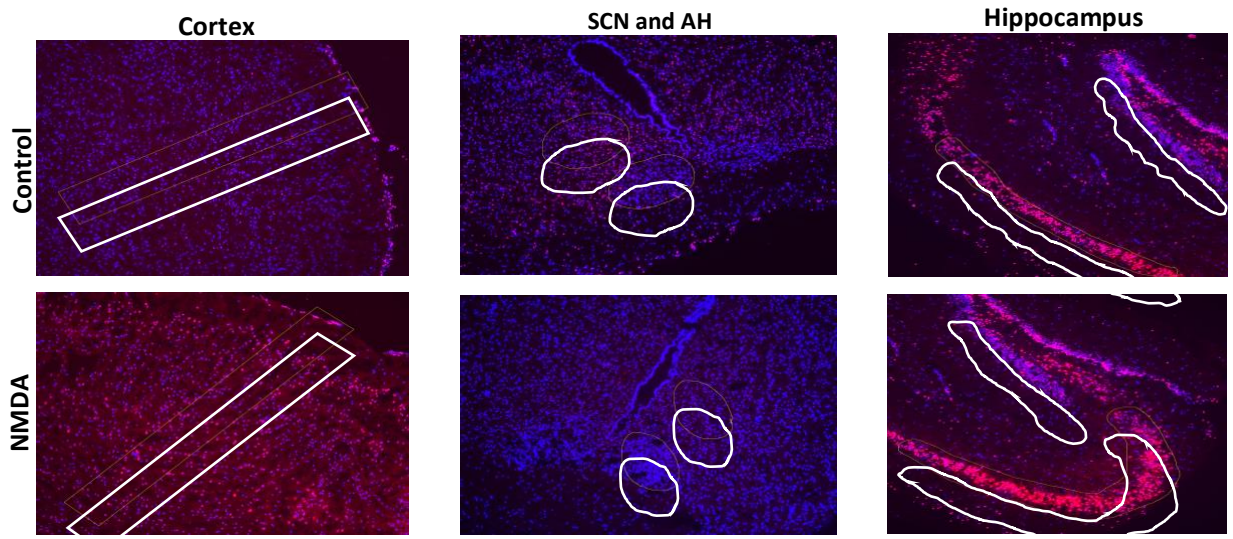

Fig. 1. Less cell death in the SCN compared to the AH, cortex, and hippocampus CA1 and CA3 regions (n = 3-4). Total number of cells is indicated by blue DAPI staining, which does not change based on degree of damage to the cell. Number of dead cells is indicated by the red PI staining, which varies in intensity based on degree of damage to the cell.

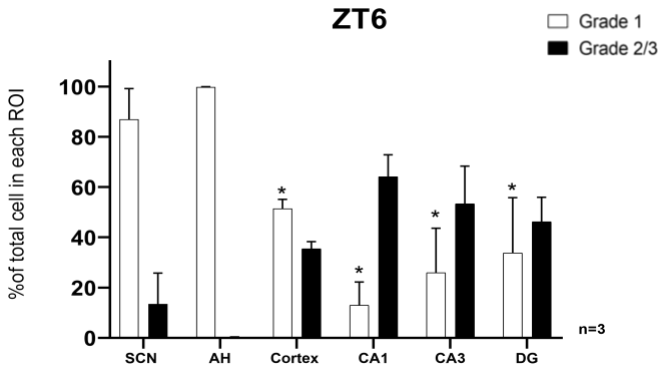

Fig. 2. Less cell death in the SCN at ZT 6 (n = 3) compared to the cortex and hippocampus dentate gyrus (DG), CA1, and CA3 regions. Data were analyzed using a student's t-test, where a * symbol indicates a statistically significant difference from SCN Grade 1 cells, with $p < 0.05$.

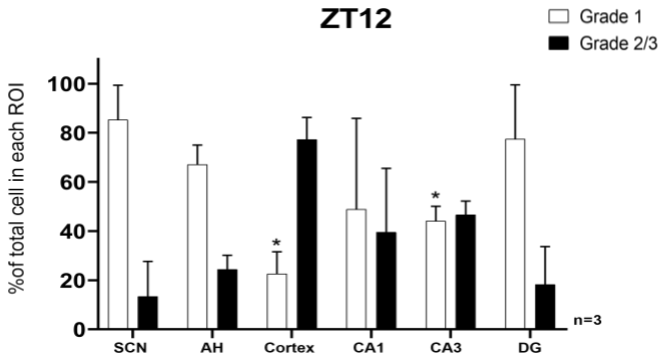

Fig. 3. Less cell death in the SCN at ZT 12 (n = 3) compared to the cortex, and hippocampus CA3 region. Data were analyzed using a student's t-test, with a * symbol indicating statistical significance at $p < 0.05$.

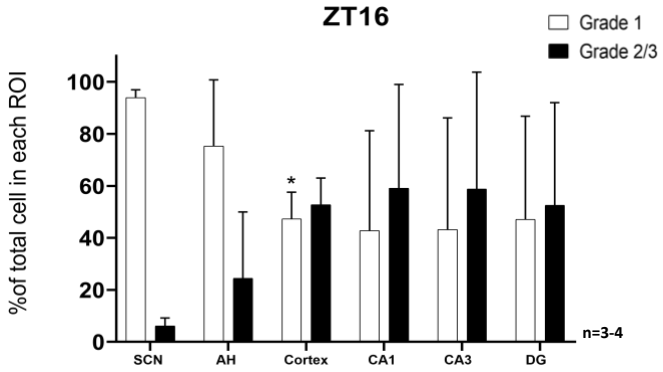

Fig. 4. Less cell death in the SCN at ZT 16 (n = 3-4) compared to the cortex. Data were analyzed using a student's t-test, with a * symbol indicating statistical significance at $p < 0.05$.

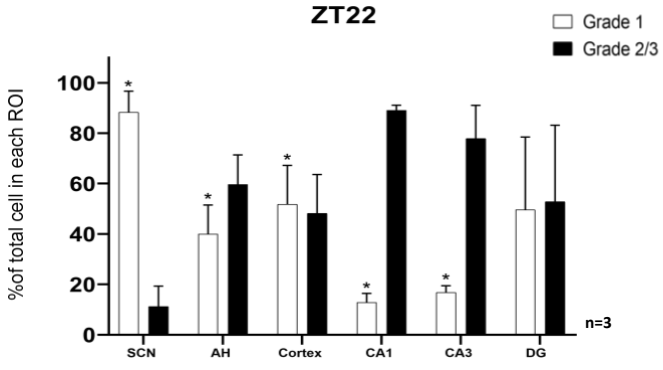

Fig. 5. Less cell death in the SCN at ZT 22 (n = 3) compared to the AH, cortex, and hippocampus CA1 and CA3 regions. Data were analyzed using a student's t-test, with a * symbol indicating statistical significance at $p < 0.05$.

3.2 Cell death in all brain regions does not exhibit circadian rhythms

The SCN is the locus of the primary mammalian circadian clock. As such, it is known that the molecular environment of the SCN regarding gene and cell-surface receptor expression changes based on circadian rhythms, including ion channels and their functions, transcription factor response elements, and CREB phosphorylation regulation [7, 9, 16]. Likewise, other brain regions exhibit a variety of circadian rhythms in cell activitiy due to inpyt from the SCN. Because of this, we investigated whether the resistance/susceptibility to excitotoxic damage varies with circadian time. We found that degree of cell damage resulting from in vitro NMDA treatment does not change based on the time of treatment administration in any of these regions. As seen in Figures 6-11, NMDA treatments at ZT 6, ZT 12, ZT 16, and ZT 22 resulted in roughly the same amounts of cell death, as evidenced by similar percentage values for Grade 1, Grade 2, and Grade 3 cells in each brain region.

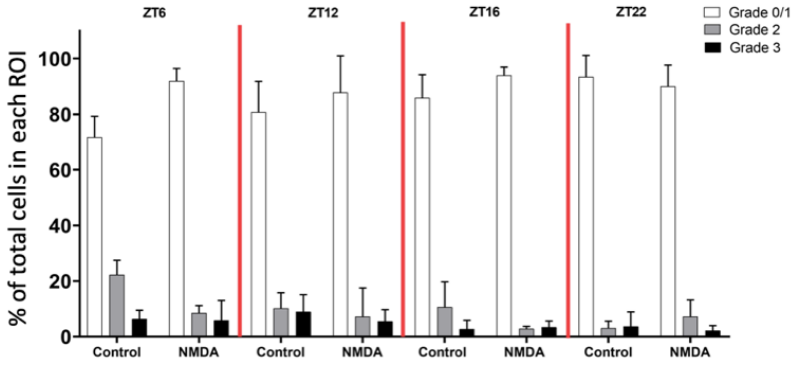

Fig. 6. Constant percentage of cell death relative to total number of cells in the SCN at ZT 6, ZT 12, ZT 16, and ZT 22 in both control and 50μM NMDA-treated slices (n = 3-5).

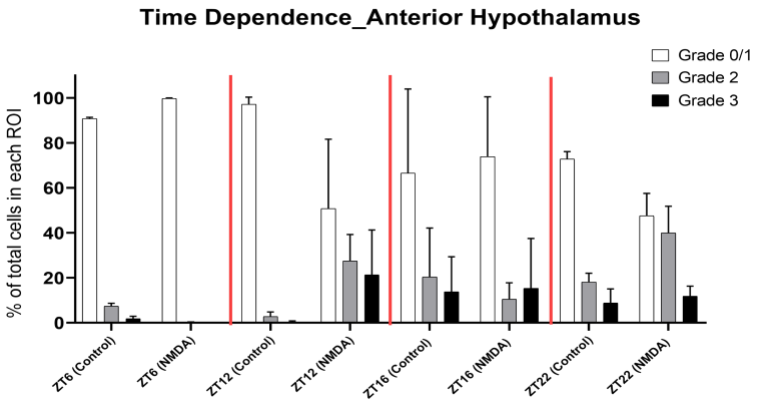

Fig. 7. Differences in percentage of cell death relative to total number of cells in the AH across ZT 6, ZT 12, ZT 16, and ZT 22 between control and 50μM NMDA-treated slices, with the least amount of NMDA-induced cell death occurring at ZT 6 and ZT 12 (n = 3-5).

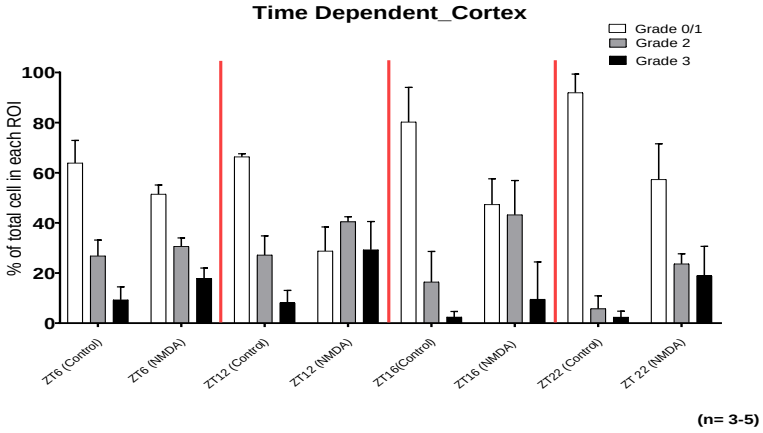

Fig. 8. Differences in percentage of cell death relative to total number of cells in the cortex across ZT 6, ZT 12, ZT 16, and ZT 22 between control and 50μM NMDA-treated slices, with the most amount of NMDA-induced cell death occurring at ZT 12 (n = 3-5).

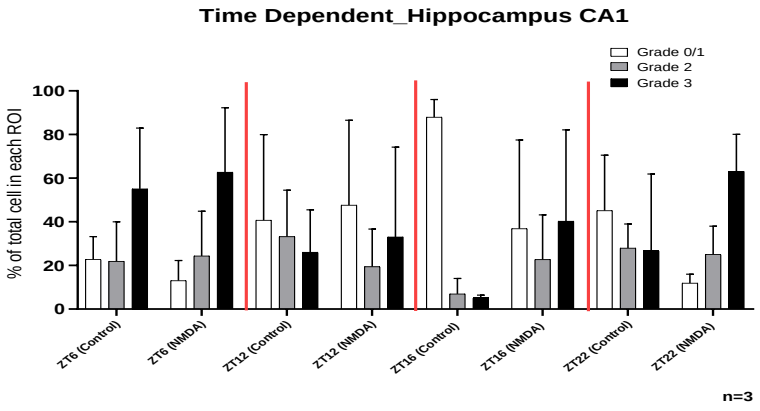

Fig. 9. Differences in percentage of cell death relative to total number of cells in the hippocampus CA1 region across ZT 6, ZT 12, ZT 16, and ZT 22 between control and 50μM NMDA-treated slices, with the most amount of NMDA-induced cell death occurring at ZT 6 and ZT 22 (n = 3).

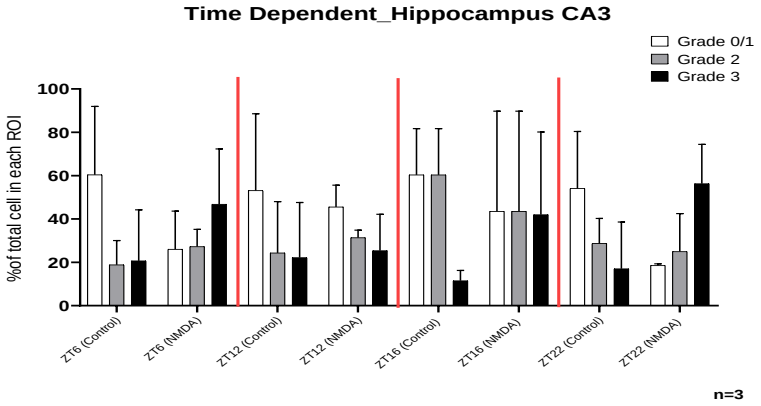

Fig. 10. Differences in percentage of cell death relative to total number of cells in the hippocampus CA3 region across ZT 6, ZT 12, ZT 16, and ZT 22 between control and 50μM NMDA-treated slices, with the least amount of NMDA-induced cell death occurring at ZT 12 (n = 3).

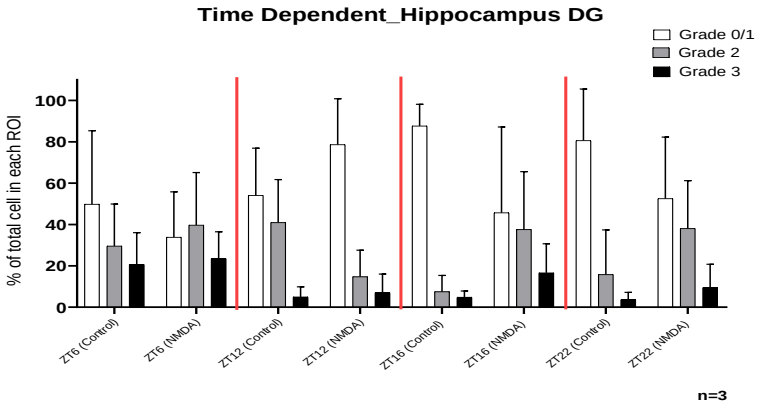

Fig. 11. Differences in percentage of cell death relative to total number of cells in the hippocampus DG across ZT 6, ZT 12, ZT 16, and ZT 22 between control and 50μM NMDA-treated slices, with the most amount of NMDA-induced cell death occurring at ZT 6 (n = 3).

3.3 Degree of cell death in the SCN is independent of NMDA concentration

The degree of cell damage or death in the SCN resulting from in vitro NMDA treatment does not change with increasing NMDA concentrations, which aligns with findings from

previous studies [4, 13]. The results of our dose-response experiments are displayed, showing that EBSS alone or EBSS containing 50μM, 100μM, and 500μM NMDA-bath applications at ZT 6 to brain slices prepared from a mouse induced minimal cell death, with no significant differences between each dosage as evidenced by constant percentage values for Grade 1, Grade 2, and Grade 3 cells across each treatment (Figure 12).

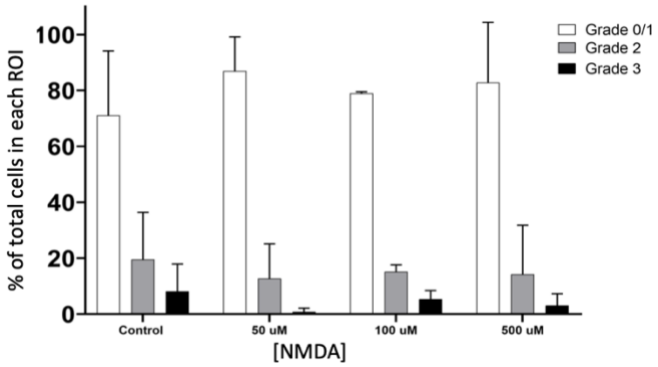

Fig. 12. Constant percentage of cell death relative to total number of cells in the SCN after 1 hour of exposure to increasing doses of NMDA from 0μM (control) to 50μM, 100μM, and 500μM (n = 3).

4. Discussion

Through these experiments, we have demonstrated that the SCN displays a unique endogenous resilience to NMDA-induced excitotoxicity that persists across time and with increasing concentrations of NMDA, rendering it more protected against overexcitation compared to other brain regions; however, the mechanism through which this occurs is still unknown. Understanding how the SCN resists excitoxic shock has various clinical implications that could potentially lead to advancements in ischemic stroke and neurodegenerative disease treatments as well as various other neurological disorders that disrupt circadian rhythms. Limitations for this study include the insufficient number of samples available in order to run proper statistical analyses (ANOVA), using only one method to assess the degree of cell damage

or death (PI staining), ImageJ analyses performed by multiple researchers, employing only one type of stressor (NMDA-induced excitotoxicity) where other methods might result in regional differences in resilience or susceptibility, and that these results are purely observational and thus do not provide functional information about the SCN or other brain regions. Further studies addressing this underlying mechanism behind the SCN's defense system could allow researchers and health care professionals to directly reproduce the cell machinery of this mechanism where the stroke took place in order to repair the damage or even prevent future events by treating at-risk patients before a stroke occurs.

However, more work is needed to further support our results and allow for future medical innovation. Such steps include increasing the number of samples to conduct an ANOVA test to more clearly define the relationship between the SCN and the other brain regions, and testing higher concentrations of NMDA to determine whether the SCN exhibits a response to excitotoxic stimuli using a more robust stressor. Additionally, since both AMPA and NMDA receptors are associated with the excitoxic response, we want to investigate whether AMPA induces the same effects—or lack thereof—on SCN tissue or whether it leads to the same levels of NMDA-induced cell damage and death in other brain regions. Then, to determine whether the SCN only displays resilience to excitoxicity or various types of cell death (i.e., metabolic or mitochondrial breakdown), we want to test SCN resilience under oxygen-glucose deprivation compared to that of other brain regions. We also want to use MTT and LDH assays to look at the SCN's excitotoxic response alone compared to that of other brain areas, which would indicate the level of cell viability by highlighting the presence of mitochondrial dysfunction characteristic of cell death. Since succinate dehydrogenase is a mitochondrial resident protein, the MTT assay allows for fluorescent visualizaton of mitochondrial breakdown; if succinate dehydrogenase is

present in the cytoplasm and thus converts MTT into MTT-formazan, then the mitochondrial membrane is no longer intact. Similarly, since LDH is also a resident mitochondrial protein, the LDH assay would allow for fluorescent visualization of pyruvate formed from cytoplasmic lactate if the mitochondrial membrane is broken down.

If these additional experiments also indicate an endogenous resilience unique to the SCN, then future studies should investigate the specific cellular mechanism(s) that give rise to this protective quality. One potential area of study involves intracellular calcium ion (Ca^{2+}) concentration following NMDA receptor activation in the SCN versus other brain regions. One possibility is that the SCN expresses a unique NMDA receptor subunit composition, which might restrict Ca^{2+} inflow into SCN cells relative to those in other brain areas and prohibit the excessive overstimulation implicated in cell damage and death. Additionally, microglia are known to play both neuroprotective and neurodegenerative functions in brain cells. Because of this these contradictory actions, the specific role of microglia in SCN resiliency should be investigated by comparing SCN microglia morphology and functional status post excitotoxic stimulus relative to that of other brain regions. This would determine whether the SCN's increased resilience to excitoxicity could be attributed to the presence of less reactive microglia that are more ramified—the typical physiological state of a healthy, resting central nervous system—and release fewer pro-inflammatory substances when the cell is overstimulated, whereas microglia in different brain regions would be more reactive and less ramified—a state of hypertrophy caused by acute injury.

In conclusion, excitotoxic stimuli induce a lesser effect on the SCN compared to other brain regions. The aforementioned experiments indicate that the SCN exhibits the least amount of cell death across time independent of circadian rhythm and NMDA concentration. While this

may be attributed to differential intracellular Ca^{2+} concentration due to unique NMDA receptor composition or microglia morphology and reactivity specific to SCN cells, it will be important to understand the exact endogenous mechanism through which the SCN is better protected against NMDA-induced cell damage and death. These results suggest that somehow, SCN cells employ a unique defense mechanism at the onset of excitotoxic stimuli that render them more able to resist its deleterious effects. Understanding the specific cell machinery behind this phenomenon can help us determine the SCN's role in neurological recovery after an event such as an ischemic stroke, which can potentially lead to innovation regarding treatment and prevention methods.

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