Circadian Modulation of Astrocyte Morphology and Synaptic Transmission

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Circadian Modulation of Astrocyte Morphology and Synaptic Transmission

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Abstract

The circadian rhythm affects behavior and physiology in many animal species. Our previous work in mice showed that within the hippocampus, a subordinate circadian oscillator, astrocytes change their proximity to excitatory synapses. Here we obtain 3D reconstructions of biocytin-filled hippocampal astrocytes processed with protein retention expansion microscopy and imaged using two-photon laser scanning microscopy. Our findings identify the subtle changes in astrocyte morphologies that encode information about time-of-day in hippocampal astrocytes. These findings shed light on fundamental mechanisms that allow the function of hippocampal circuits to adapt to different times of the day. This will help us understand how structural changes in astrocytes affect inhibition as we also consider the effects of circadian rhythms on GABAergic inhibition. Our experiments on GABAergic inhibition show changes in tonic inhibition due to circadian rhythms. Furthermore, we explore changes in dendritic spines in response to Aβ42-AAV injections and the functional implications of Aβ42-AAV at glutamatergic synapses.

Keywords: Circadian rhythm, Astrocytes, GABAergic inhibition, Alzheimer’s Disease
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Introduction

Circadian rhythms regulate biological and physiological processes such as body temperature, hormone release, sleep, and wakefulness. The suprachiasmatic nuclei (SCN) of the hypothalamus are described as the main circadian master clock. The internal rhythms of the SCN are greatly affected by environmental cues, particularly light, and 19% of the genes in the SCN are regulated by circadian rhythms. Light impacts the SCN through the glutamatergic connections in the retino-hypothalamic tract, which cause an increase in the firing rate of SCN neurons. The primary role of the SCN is in synchronizing circadian rhythms throughout the body by controlling the activity of subordinate circadian oscillators (Brancaccio et al., 2017; Gerstner et al., 2009).

The hippocampus is a subordinate circadian oscillator where more than 10% of its genes and proteins show circadian fluctuations. The hippocampus is composed of the dentate gyrus, the hippocampus proper, and the subiculum. In the hippocampus proper we can identify three brain regions: CA1, CA2 and CA3. Previous research in the hippocampus shows that functional and structural changes, like synaptic remodeling, drive learning and memory (Mu & Gage, 2011). The hippocampus is implicated in learning and memory formation, which are regulated by circadian rhythms as well. This is confirmed by the fact that the hippocampus shows molecular changes with respect to light and dark phases of circadian rhythms even in cell culture from tissues isolated from the hippocampus. There is reduced long term potentiation (LTP) at hippocampal Schaffer collateral synapses in slices prepared during the D-phase. LTP describes when frequency activation strengthens synaptic connections between neurons (McCauley et al., 2020).

Astrocytes are star-shaped glial cells with a function in neurotransmission and synaptogenesis, which have been described as autonomous circadian oscillators (Brancaccio et al., 2019). In the SCN, astrocytes are active in the dark phase where they regulate extracellular
glutamate levels and subsequently the activity of SCN neurons. Glutamate release activates α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and following frequent activation of AMPA receptors, the membrane is depolarized. Membrane depolarization causes the voltage block on N-methyl-D-aspartate (NMDA) receptors to be removed. By removing the voltage block, NMDA receptors, and by proxy the cell, become more sensitive to glutamate. The increased sensitivity of the postsynaptic cell to glutamate makes the synapse stronger as a result of NMDA receptor dependent LTP (McCauley et al., 2020). Previous research has shown that pharmacologically interfering with glutamate release from astrocytes suppresses electrical and molecular circadian oscillations, reaffirming the role of astrocytes in the circadian cycle (Brancaccio et al., 2017).

Astrocytes have been shown to have a role in regulating glutamate release in response to circadian cycles. When studying astrocytes from the hippocampus, we expect to see changes in astrocyte morphology corresponding with light and dark phases of circadian rhythms. Previous research using confocal images of biocytin-filled astrocytes suggests that astrocytes lose some fine processes in the D-phase. The functional impact of a loss of fine astrocytic processes is a reduction in the clearance of neurotransmitters from the synaptic cleft. Specifically, less glutamate is removed from the extracellular space by astrocytes which leads to the degradation of LTP (McCauley et al., 2020).

γ-aminobutyric acid (GABA) is a neurotransmitter in the central nervous system (CNS), which acts through multiple subtypes of target receptors referred to as GABA_A, GABA_B and GABA_C receptors (Semyanov et al., 2004). GABA_A are ionotrophic receptors permeable to the chloride anion. When negative chloride anions flow into the cell through the GABA_A receptor, the cell membrane is hyperpolarized, which makes action potential firing less likely. GABA_A receptors
are activated either transiently or persistently; transient activation mediates phasic inhibition while persistent activation mediates tonic inhibition (Brickley & Farrant, 1996). Hippocampal pyramidal cells show a tonic GABA-A mediated current early in development but not in adult tissue (Semyanov et al., 2004). Here we investigate changes in the modulation of GABAergic inhibition when looking at CA1 pyramidal neurons taken from the Light (L) and Dark (D) phase of the circadian cycle.

Astrocytic processes localize near dendritic spines where they regulate extracellular neurotransmitter levels and propagate calcium signals. Dendritic spines are small protrusions growing from dendrites that receive glutamatergic innervations. The primary function of dendritic spines is communication. Spines are dynamic and can change their shape rapidly in response to neuronal activity. Increases in spine size are correlated with LTP while decreases in spine size correspond to long term depression (LTD). Changes in spine morphology and spine density are associated with learning and memory formation (Haber et al., 2006; McCann & Ross, 2017).

Spine morphology can be categorized into four groups: mushroom, thin, stubby, and filopodia. NMDA and AMPA receptors can be found in the plasma membrane of dendritic spines, both receptors bind glutamate. Glutamate binding leads to the influx of calcium and sodium ions which trigger signaling cascades important for neuronal plasticity (Kasai et al., 2010; McCauley et al., 2020). In Alzheimer's Disease (AD), the hippocampus shows shrinkage and elimination of dendritic spines (Boros et al., 2017). The hippocampus is a brain region implicated in memory formation and one of the hallmark symptoms of AD is a progressive loss of memory. The biological basis for AD is rooted in the presence of extracellular amyloid plaques. Amyloid plaques form following the accumulation of the amyloid beta peptide (Aβ). In the healthy brain, the Aβ protein is removed by glial cells before it can accumulate into plaques. Furthermore, in the healthy
brain, the Aβ protein acts as a membrane protein with a role in neuronal growth and repair (Reiss et al., 2017). To contrast, the role of Aβ in AD is much more detrimental, Aβ accumulation can cause pore formation, disruption of cellular calcium balance, and synaptic loss (Bloom, 2014). Pyramidal cells in region CA1 of the hippocampus (CA1-PCs) are a major output of the hippocampus. Since previous research on AD describes functional and structural changes in the hippocampus, we decided to investigate changes in spine morphology in CA1-PCs of mice injected with Aβ42.
Materials and Methods

**Ethical Statement:** All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the State University of New York (SUNY) Albany and guidelines described in the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals.

**Mice:** C57BL/6J mice were group housed and kept in dark conditions for 12 hrs and then light conditions for 12 hrs (lights on at 7 AM, ZT0; lights off at 7 PM, ZT12). Times ZT0-12 are referred to as the L-phase, while the D-phase are times ZT12-24. For other experiments, mice were kept under constant darkness conditions using red dim light illumination. The intensity of red dim light matched that used for the D-phase of mice kept in 12 hrs of light and then 12 hrs of dark. However, even for mice maintained in constant darkness, times ZT0-12 are referred to as the L-phase while times ZT12-24 are the D-phase. Mice of both sexes received stereotaxic surgeries between P12-16 and were harvested between P18-66. We differentiated mice into 3 groups: WT, Sham-AAV, and Aβ_{42} -AAV. The WT group did not receive an AAV injection. The Sham-AAV group received an injection of AAV-1/2-hEF1a-EGFP-WPRE-bGHp(A) which causes GFP expression, and the Aβ_{42} -AAV group received an injection of AAV-1-hEF1a-EGFP-Aβ_{1-42}-WPRE-bGHp(A) which produces Aβ_{42} protein. The Sham-AAV and Aβ_{42}-AAV groups received bilateral injections of 0.2 μl of the AAV construct in both hemispheres (0.4 μl total).

**Slice preparation and biocytin fills:** To patch astrocytes we added Biocytin 0.2–0.4% to the intracellular solution. The cells were then patched and filled for 20 min at minimum. The slices were then fixed overnight at 4°C in 4% PFA/PBS, cryo-protected in 30% sucrose PBS, and incubated in 0.1% streptavidin-Alexa Fluor 488 conjugate and 0.1% Triton X-100 for 3 hrs at room
temperature (RT). Then, using Fluoromount-G mounting medium (Cat# 0100-01; SouthernBiotech, Birmingham, AL), the slices were then mounted onto microscope slides.

**Confocal laser scanning microscopy image acquisition:** Astrocyte confocal images were acquired using a Zeiss LSM710 inverted microscope equipped with 488 nm Ar laser. All images were acquired as z-stacks using a 40X/1.4 NA oil-immersion objective. To visualize full cells, we stitched together z-stacks (1024×1024 pixels) collected by averaging four frames for each focal plane (1 μm z-step).

Images of biocytin-filled neurons from WT, Sham-AAV, and Aβ42-AAV mice were obtained using a Zeiss LSM 710 confocal microscope. The images were acquired using a 40X oil objective; λ<sub>ex</sub>: 649 nm; λ<sub>em</sub>: 666 nm; Frame size: 512 x 512; Pixel size: 1.16 px/μm; Pinhole size: 1 Airy Unit; Averaged frames: 2.

**Protein-retention expansion microscopy and two-photon imaging:** The expansion factor is measured using hippocampal slices with 4% PFA/PBS overnight at 4°C, cryoprotected in 30% sucrose PBS and stored in PBS. The hippocampal slices were incubated with DAPI Fluoromount G overnight at 4°C (Cat# 0100-20; SouthernBiotech, Birmingham, AL) before the proExM protocol was followed. Slices containing biocytin filled astrocytes were fixed, cryo-protected, and stored in PBS labelled with streptavidin-Alexa Fluor 488. These were then incubated overnight at room temperature in 200 μl of anchoring solution. After incubating, the slices were gelled and digested with Proteinase again overnight and at room temperature. The following day, the slices were expanded by incubating thrice in distilled water for 15 min each. Those slices stained with DAPI were imaged during each incubation period. This imaging was done using a 2X/0.06 NA air objective on an EVOS FL Cell Imaging System equipped with DAPI filter set (λ<sub>ex</sub>: 357/44 nm, λ<sub>em</sub>: 447/60 nm; ThermoFisher Scientific, Waltham, MA). Imaging occurred at 0.1 fps and was
followed by a manually tracing to determine slice perimeter and surface area using IMOD (https://bio3d.colorado.edu/imod/) These expanded gels were then covered with 2% agarose and submerged in distilled water. Images were taken using a two-photon laser-scanning microscope. This scanning system (Coherent, Santa Clara, CA) tuned to 760 nm and connected to an upright microscope with a 60X/1.0 NA objective (Olympus Corporation, Center Valley, PA). Using a 565 nm dichroic mirror, the green fluorescent signal could be separated from the red. It was then filtered using FITC filter sets (Olympus Corporation, Center Valley, PA). The average amount of frames for each optical section, about 512x512 pixels) was 8 and enough z-stacks were collected for a total distance of about ~200 μm (in 1.2 μm steps).

**Astrocyte Reconstructions:** Images were reconstructed using Bitplane Imaris 9.6. TIFF files from 2-photon laser scanning microscopy were imported into Imaris and the dimension of each voxel was scaled as follows: x: 0.507 μm, y: 0.507 μm, z: 1.2 μm. Next, we applied a gaussian filter to reduce background noise in the image. We created a surface to isolate the astrocyte of interest. Extraneous segments were removed by selecting seed points that only include the astrocyte soma. Furthermore, the threshold was set to ensure we cover the boundaries of the selected astrocyte; this creates the cytoplasmic volume. Next, we created a mask of the cytoplasmic volume and using that mask we created another surface using a threshold value of 1 and surface smoothing to 5 μm, this becomes the bounding volume. Once the surfaces are created, Imaris calculates statistics like total area and volume encompassed by the surface. After the cytoplasmic and bounding volumes were created, we created filaments. The filaments were created using the mask of cytoplasmic volume, the largest diameter for starting points was set to 27.3 μm and the thinnest diameter was set to 1 μm. Next the starting points threshold is altered only to include the soma, and the seed points threshold is set to have coverage of all astrocyte branches. Once the
filaments are created, we assign a color code to them based on the branch level and set the colormap to jet. Additionally, we set the branches to be viewed as cylinders with a diameter of 1 μm. Lastly, we run a MATLAB plug-in for Imaris called Branch Hierarchy which gives us statistics on branch levels.

**Stereotaxic Surgeries:** We performed bilateral stereotaxic injections of AAV-1-hEF1a-EGFP-Aβ1-42-WPRE-bGHp(A) (here referred to as Aβ42-AAV) and AAV-1/2-hEF1a-EGFP-WPRE-bGHp(A) (here referred to as Sham-AAV) in region CA1 of the hippocampus in mice of either sex aged P12-16. We placed mice in an induction chamber and administered 5% isoflurane. We then removed the fur on the top of the head with a hair remover lotion (such as Nair) and the mouse was positioned into the microinjection stereotaxic frame (Neurostar, Tübingen, Germany) on a hand warmer to prevent an unsustainable drop in body temperature. In the frame, the mouse received a continuous dosage of 2-3% isoflurane with a flow rate of 0.8-1.2 liters per minute. Lidocaine was applied to the scalp for two minutes to allow for proper anaesthetization. The scalp was then cleaned with three alternating applications of betadine and 70% ethanol. Artificial tears were applied to the eyes to prevent drying. A tail pinch was used to determine the depth of the anesthesia and if no reaction was observed, we proceeded with the surgery. A midsagittal incision was made in the scalp and bregma, the point where the frontal and the two parietal bones fuse, was located and was used as a point of reference for the injection. The coordinates of the hippocampus are AP: -1.9 mm, ML: ±1.6 mm; DV: -1.4 mm. We used a drill to thin the skull in the area of interest. A Hamilton syringe was used to inject 0.2 μl of the construct at a rate of 0.05 μl/min. After the injection finished, we waited one minute before retracting the needle halfway, and then another 30 s before retracting the needle fully to ensure diffusion of the viral construct. The incision site was closed with surgical glue and lidocaine is applied once again, along with an antibiotic
ointment. We performed a subcutaneous injection of a 10% v/w saline to rehydrate the mouse. The mouse was then placed in the recovery cage on another hand warmer separate from the mother to recover from anesthesia and surgery properly. We monitored body weight, behavior, site of incision, and overall well-being of the mouse over the next 72 hr.

**Dendritic Spine Reconstructions:** Images were reconstructed using Bitplane Imaris 9.6.1. CZI files from confocal microscopy were converted into TIFF files using ImageJ. Next, we created a surface to isolate the dendrite of interest and its associated spines. For more accurate surface creation and to exclude other dendrites and background noise in the image the surface was created using a region of interest fit to our dendrite of interest. We then masked the surface to create a channel including just our dendrite of interest. Next, we used the newly created channel for manual filament creation. For filament creation, we first set the beginning and end points of the dendrite and rebuild the dendrite diameter. Then, we began to create spines by setting the size of seed points. Seed point size was determined by measuring the diameter of the smallest spine head. Seed points must be added manually at the head of each spine. Once seed points were added to each spine head, the spines were reconstructed. To classify spines, we used the Classify Spines XTension which determines differences in spine morphology and classifies them into categories. The categories are differentiated by name, classification rule, and color. Classification rules were determined experimentally (Table 1). Lastly relevant statistical measures recorded by Imaris were exported into Igor Pro 6.37 for analysis. Spine head, neck, and ground statistics were calculated by Imaris with the distinction that the first 25% of the spine is identified as the ground, the next 50% is the neck, and the last 25% is the head.
Table 1 Spine Classification Rules

<table>
<thead>
<tr>
<th>Spine Type</th>
<th>Classification rule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mushroom</td>
<td>$\frac{\text{mean head width}}{\text{mean neck width}} \geq 1.15$</td>
</tr>
<tr>
<td>Thin</td>
<td>$\frac{\text{mean head width}}{\text{mean neck width}} \geq 0.94 \text{ AND } \frac{\text{mean head width}}{\text{mean neck width}} &lt; 1.15 \text{ AND spine length } &lt; 2$</td>
</tr>
<tr>
<td>Stubby</td>
<td>$\frac{\text{mean head width}}{\text{mean neck width}} \geq 0.6 \text{ AND } \frac{\text{mean head width}}{\text{mean neck width}} &lt; 1.4 \text{ AND spine length } \leq 1.0$</td>
</tr>
<tr>
<td>Filopodia</td>
<td>$(\text{mean neck width } &lt; 0.3 \text{ AND } (\text{mean neck width } - 0.1) &lt; \text{mean head width}) &lt; (\text{mean neck width } + 0.1)$</td>
</tr>
</tbody>
</table>

Data Analysis: Data was exported from the Bitplane Imaris software and analyzed with Igor Pro 6.37 (Wavemetrics, Lake Oswego, OR). Data are presented as mean ± SEM unless otherwise stated. All experiments were performed on multiple mice of either sex. Statistical significance was determined by Student’s paired or unpaired t test, as appropriate (IgorPro 6.36). Differences were considered significant at $p < 0.05$ (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Electrophysiology: For IPSC recordings from CA1-PCs, stimulating and recording electrodes were both placed in the CA1 hippocampus ~100 μm away from each other. Each electrical stimulus was 0.1 ms long and was delivered every 10 s. The resistance of the recording electrode was ~1.5 MΩ and was monitored throughout the experiments. Data were discarded if the resistance changed >20% throughout the course of the experiment. We recorded IPSCs in the presence of the GABA receptor antagonist (PTX, 100 μM), at a holding potential of 0 mV. Recordings were performed in acute striatal slices from C57BL/6 WT mice. All recordings were
obtained using a Multiclamp 700B amplifier and filtered at 10 kHz (Molecular Devices), converted with an 18-bit 200 kHz A/D board (HEKA), digitized at 10 kHz, and analyzed offline with custom-made software (A.S.) written in IgorPro 6.36 (Wavemetrics). All recordings were performed at RT.

For NMDA/AMPA ratio recordings from CA1-PCs, we obtained hippocampal slices from C57-BL/6J mice of either sex (P12-16) that were deeply anesthetized with halothane and decapitated in accordance with SUNY Albany Animal Care and Use Committee guidelines. We rapidly removed the brain and placed it in ice-cold slicing solution bubbled with 95% O2-5% CO2, containing (in mM): 119 NaCl, 2.5 KCl, 0.5 CaCl2, 1.3 MgSO4·H2O, 4 MgCl2, 26.2 NaHCO3, 1 NaH2PO4, and 22 glucose; 320 mOsm; pH 7.4. We prepared striatal slices (250 μm thick) using a vibrating blade microtome (VT1200S, Leica Microsystems, Wetzlar, Germany). After sectioning, we stored the slices in this solution in a submersion chamber at 36°C for 30 min and at room temperature for at least 30 min and up to 4 hr. The recording solution contained (in mM): 119 NaCl, 2.5 KCl, 2.5 CaCl2, 1 MgCl2, 26.2 NaHCO3, 1 NaH2PO4, 22 glucose; 300 mOsm; pH 7.4. We obtained whole-cell patch clamp recordings from CA1-PCs and astrocytes that were identified under infrared-differential interference contrast using an upright fixed stage microscope (BX51 WI, Olympus Corporation, Tokyo, Japan). The internal solution that we used to record the CA1-PCs contained (in mM): 120 KCH3SO3, 10 EGTA, 20 HEPES, 2 MgATP, 0.2 NaGTP, 5 QX-314Br, 5 NaCl; 290 mOsm; pH 7.2.
Results

A. Hippocampal astrocytes imaged with confocal microscopy show no circadian changes in morphology

The hippocampus is a subordinate circadian oscillator with a role in learning and memory formation. Astrocytes are star shaped glial cells that have been shown to be subordinate circadian oscillators that regulate synaptic activity and plasticity. Previous research shows that, in the D phase, astrocytes reduce the number of fine processes and synapses (McCauley et al., 2019). Because astrocytes are implicated in glutamate clearance, a reduction in their fine processes would impair this process and potentially lead to excitotoxicity. Here, we image biocytin-filled astrocytes using confocal microscopy to observe morphological changes between astrocytes taken from L and D phases of the circadian rhythm (Fig. 1A).

Using Bitplane Imaris, we reconstruct confocal microscopy images to visualize the cytoplasmic and bounding volumes of astrocytes (Fig. 1B-C). No significant differences were observed in either cytoplasmic or bounding volume of astrocytes between L and D phases (Fig. 1D-G). These results suggest that astrocyte morphology is not affected by circadian rhythms. However, there is a possibility these results are hindered by the limitations of our confocal microscopy. The spatial resolution of confocal microscopy is around 250 nm when using a 488 nm excitation wavelength, while astrocytic fine processes can be smaller than 100 nm. Additionally, confocal microscopy is also prone to photobleaching which restricts the ability to observe fine astrocytic processes.
B. ProExM and the expansion of hippocampal slices

Due to the limitations of confocal microscopy, we decided to explore alternate imaging methods, particularly, protein-retention expansion microscopy (pro-ExM). Pro-ExM allows for the equal expansion of a 100 nm process to 200 nm using two cycles of expansion, and more cycles could lead to more expansion (Fig. 2A). This expansion of small processes helps in the visualization of fine astrocytic processes that would otherwise be lost in confocal microscopy. For our experiments, we conducted three rounds of expansion which expanded our samples nearly 4 times the size of the original. Furthermore, this expansion was done equally in all directions so there was no distortion (Fig. 2B, C). Because we are interested in the circadian oscillations in astrocyte morphology, we ensured pro-ExM affects samples from both the L and D phases equally (Fig. 2D). Following pro-ExM, two-photon laser scanning microscopy (2P-LSM) allowed us to image deep within biological samples and ensure we capture fine processes.

C. ProExM and 2P-LSM reveal circadian dependent changes in gross astrocyte morphology

We reconstructed images generated from proExM and 2P-LSM using Bitplane Imaris to observe morphology changes in astrocytes (Fig. 3A). Using Bitplane Imaris, we reconstructed the cytoplasmic volume, bounding volume, and astrocytic branches (Fig. 3B, C). During our reconstructions, we ensured the reconstruction parameters were similar for astrocytes in L and D phases so as not to have a biased analysis (Fig. 3D). Unlike in confocal microscopy, here we observe differences in both cytoplasmic and bounding volumes of astrocytes between L and D phases. Specifically, in the L phase, both cytoplasmic volume and bounding volume are larger (Fig. 3E). After observing changes in gross astrocyte morphology, we decided to observe and analyze the extent of changes in astrocyte morphology using filament analysis. Astrocytes from
the L and D phase show similar branch levels (Fig. 3E), but more subtle differences are present. In the L phase, there are more filament segments per branch (Fig. 3F).

D. CA1-PCs show an increase in Tonic inhibition in the D-phase

To determine the functional impact of the observed decrease in cytoplasmic and bounding volumes in the D phase, we recorded current from hippocampal area CA1 pyramidal neurons. Initially, current is larger in neurons taken from the D phase. However, both L and D phase currents decrease following the application of picrotoxin (PTX). PTX is a GABA$_A$ antagonist (Fig. 4A). The observed decrease in amplitude for both L and D phase currents following PTX application indicates that these currents are GABA-mediated. To further explore this relationship, we decided to compare the current density (pA/pF) of L and D phase cells. Current density gives us a more complete view of circadian modulation by adjusting for tonic currents that are artificially inflated because of large cell size. When comparing current density, we find an increase in tonic inhibition in the D phase (Fig. 4B).

GABA is an inhibitory neurotransmitter, and our results indicated tonic inhibition in L and D phase CA1-PCs is mediated by GABA. When recording inhibitory postsynaptic current, we find no change in the peak normalized IPSC after a single stimulation between L and D phases (Fig. 4C). Furthermore, there is no change in either rise time or latency of the IPSC (Fig. 4D). This effect holds for a stimulus train of 15 pulses at 50 Hz (Fig. 4E). We see similar values for L and D phase normalized IPSC amplitude and peak (Fig. 4F). Finally, we recorded the normalized cumulative IPSC amplitude (Fig. 4G). The normalized cumulative IPSC amplitude fits a linear model after 100 ms, the y-intercept of this linear model represents the readily releasable pool of vesicles while the slope represents the rate at which vesicles are recycled. Neither the y-intercept nor the slope are statistically significant among L and D phases (Fig. 4H).
E. Aβ_{42}-AAV induces Aβ_{42} accumulation in the mouse hippocampus

To determine if Aβ_{42}-AAV causes buildup of Aβ_{42} we performed stereotaxic injections of Aβ_{42}-AAV and Sham-AAV in the hippocampus (Fig. 5A). Following stereotaxic injections, mice brains revealed Aβ_{42} buildup for mice injected with Aβ_{42}-AAV but not Sham-AAV which indicates the accumulation of Aβ_{42} was not due to the stereotaxic AAV approach but rather our Aβ_{42} virus. This is further confirmed by a radioimmunoprecipitation assay (RIPA) which shows an increased level of insoluble Aβ_{42} (Fig. 5B). Insoluble Aβ_{42} causes the formation of extracellular amyloid plaques present in AD (Bloom, 2014).

F. Aβ_{42}-AAV does not change the gross morphology of CA1-PCs

Following the validation of our AAV approach, we wanted to determine if Aβ_{42} accumulation affected the morphology of CA1-PCs. We performed Sholl analysis on biocytin-filled CA1-PCs identified in acute hippocampal slices of wild type mice (WT) and of mice that received stereotaxic injections of either Sham-AAV or Aβ_{42}-AAV three weeks prior to data collection (Fig. 6A). Sholl analysis allows us to count the number of intersections dendritic branches form within circles of increasing radius, centered on the soma of each cell (O'Neil et al, 2015). Sholl analysis shows similar numbers of total intersections and a similar distribution of intersections as distance from the soma increases for WT, Sham-AAV, and Aβ_{42}-AAV (Fig. 6, B-E). This suggests that Aβ_{42} does not affect the gross morphology of CA1-PCs.

G. Aβ_{42}-AAV increases spine density and changes spine morphology

Because our data suggests Aβ_{42} causes no changes in gross morphology of CA1-PCs, we explored finer morphology changes in dendritic spines. We reconstructed the imaged neurons using Bitplane Imaris to visualize changes in spine morphology. Spine density was calculated manually using ImageJ, spine density analysis showed that Aβ_{42} caused an increase in spine density
(Fig. 7A). Reconstruction in Bitplane Imaris revealed similar distributions of spine types (mushroom, thin, stubby) among WT, Sham-AAV, and Aβ42-AAV (Fig. 7B) Analysis of spine morphology indicated no changes in spine area, head volume, head diameter or neck length (Fig. 7C, D).

H. Aβ42-AAV reduces NMDA/AMPA ratio

Dendritic spines contain NMDA and AMPA receptors which bind glutamate. Glutamate is an excitatory neurotransmitter that evokes excitatory postsynaptic potentials (EPSCs) when bound to NMDA and AMPA receptors. To explore the functional effects of Aβ42 accumulation on CA1-PCs, we recorded EPSCs and determined the AMPA/NMDA ratios. First, we looked solely at AMPA receptors; to do this, PTX was added to block GABA receptors and the cell was held at -70mV (Fig. 8A). No differences were detected in AMPA paired pulse ratio (PPR) between WT, Sham, and Aβ42-AAV groups (Fig. 8B). This indicates the release probability of vesicles is not affected by Aβ42-AAV. There were also no changes in EPSC rise time, amplitude, and decay time (Fig. 8C).

To determine the effect of Aβ42-AAV on NMDA receptors, we used 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline (NBQX) to block AMPA receptors and changed the holding potential to +40 mV to remove the Mg2+ block from NMDA receptors (Fig. 8D). Much like our data for AMPA receptors, we found no changes in NMDA PPR or EPSC rise time, amplitude, and decay time between our three conditions (Fig. 8E, F). There was a significant decrease in the NMDA/AMPA ratio for the Aβ42-AAV condition (Fig. 8H). This suggests a decrease in the expression or activity of either NMDA or AMPA receptors in CA1-PCs.
**Discussion**

Circadian rhythms affect sleep, wakefulness, and hormone release; in humans, the 24-hr circadian cycle governs our daily lives as we follow regular eating, sleeping, and working patterns. Circadian rhythmicity is coordinated by subordinate circadian oscillators, including astrocytes. To determine the effects of circadian rhythmicity on hippocampal astrocyte morphology we reconstructed astrocytes in 3d using Bitplane Imaris. Changes in gross astrocyte morphology were detected following imaging using proExM and 2P-LSM which allowed us to better visualize finer astrocytic processes (Fig. 3A). Our analysis showed a significant decrease in both cytoplasmic and bounding volume of astrocytes in the D-phase (Fig. 3B, C). This suggests a retraction of fine astrocytic processes in the D phase. Previous work by McCauley et al. (2019) shows that LTP is impaired in the D phase. Astrocytes are responsible for removing glutamate from the extracellular space and converting it into glutamine. The loss of astrocytic processes in the D phase indicates glutamate clearance and release is reduced, which consequently impairs LTP. These findings are consistent with previous work done by McCauley et al. (2019).

The SCN is the main circadian master clock; glutamate is needed for molecular timekeeping in the SCN. Astrocytes control glutamate release and uptake from the extracellular space. Glutamate is necessary for molecular timekeeping in the SCN, circadian oscillations of extracellular glutamate are anti-phasic to neuronal intracellular calcium but in phase with astrocyte intracellular calcium. Astrocytes exercise control over circadian oscillations of extracellular glutamate. Research by Brancaccio et al. (2017) shows that D phase SCN astrocytes release high levels of glutamate into the extracellular space which increases GABAergic inhibitory tone across the SCN circuit. Due to the combination of higher glutamate release and reduced uptake in the D-phase, we can assume a decrease in the number of fine astrocytic processes. This is supported by our experimental data showing decreases in cytoplasmic and bounding volume in the D-phase.
GABA plays a key role in the SCN as well. In the absence of GABA, the SCN displays burst firing leading to increased levels of intracellular calcium. However, this effect is abolished following application of GABA. While the SCN is the main circadian clock, the hippocampus is a subordinate circadian oscillator. When studying astrocytes in the hippocampus, we see an increase in GABA-mediated tonic inhibition in the D phase (Fig. 4B). Experiments by (McCauley et al., 2019) found that a decrease in the number of astrocytic processes also created an increase in the distance between post-synaptic density and the nearest astrocyte process. Our experimental data supports this as the number of filament segments per branch level is lower in the D-phase indicating that there is a decrease in the number of astrocytic processes. This impairs the ability of astrocytes to uptake GABA from inhibitory synapses and causes the increase in tonic inhibition.

Previous research by Scimemi et al. (2013) has shown that Aβ_{42} reduces the clearance of glutamate from the extracellular space by reducing GLT-1, a glutamate transporter, surface expression. Changes in the synaptic environment brought on by Aβ_{42} can also affect the shape, size and turnover of dendritic spines as both astrocytic processes and dendritic spines localize near synapses (McCauley et al., 2019; Haber et al., 2006). To explore changes in dendritic morphology in response to Aβ_{42}, we analyzed CA1-PCs from mice injected with Aβ_{42}-AAV. Our experiments indicated that gross dendritic morphology does not change following Aβ_{42}-AAV application (Fig. 6). Furthermore, we observe a significant increase in spine density in the Aβ_{42}-AAV condition (Fig. 7A). These results are surprising as previous research by Montero-Crespo et al. (2021) suggests that Aβ_{42} accumulation can cause neuronal degeneration and synaptic deterioration. This would be signified by a decrease in spine density and number of intersections for CA1-PCs treated with Aβ42. One possibility is that these results are because our Aβ_{42}-AAV had 3 weeks for
transfection before slices were prepared for imaging unlike AD where patients are affected for multiple years.

Our analysis of dendritic spines showed similar distributions of spine types and sizes among WT, Sham-AAV, and Aβ42-AAV (Fig. 7B-D). Spines also have distinct characteristics depending on spine type. Mushroom spines are thought to be sites of long-term memory storage. Thin spines are more dynamic and responsible for forming new memories when changes in synaptic plasticity occur. Stubby spines are found in small amounts in adult brains and filopodia are very rare (Bourne & Harris, 2007). Although our results show no changes in spine type for the Aβ42-AAV condition, this may be due to our imaging approach, which does not capture subtle changes in fine dendritic morphology.

Dendritic spines contain excitatory synapses, NMDA, and AMPA receptors and thus changes in spine morphology should be accompanied by changes in stimulus evoked EPSCs (Boros et al., 2017). Specifically, a decrease in spine density or the overall surface area of spines would correspond to a reduction in EPSC amplitude as fewer receptors would be available to bind glutamate. We recorded EPSCs from NMDA and AMPA receptors to determine and found no significant changes in EPSC kinetics (Fig. 8 C, F). However, we did observe a significant decrease in the NMDA/AMPA paired pulse ratio (PPR) (Fig. 8H). This indicates that Aβ42 causes a reduction in the NMDA/AMPA PPR, meaning that in response to the activation of AMPA receptors, the probability of EPSCs occurring at NMDA receptors is reduced. This phenomenon could be due to a reduction in the number of receptors, which is supported by current literature.
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Figure 1. Structural differences in astrocytes imaged using confocal microscopy

(A) Confocal microscopy image of a hippocampal slice prepared in the L-phase. (B) The reconstructed cytoplasmic volume of astrocytes in the light and dark phase. (C) The isolated astrocyte of interest, in white, surrounded by the reconstructed bounding volume, in yellow. (D) Summary of analytical criteria used to create the cytoplasmic volume. (E) Summary of analytical criteria used to create the bounding volume. (F) Summary chart showing no significant change in cytoplasmic volume between light and dark phases. (G) Summary chart showing no significant change in bounding volume between light and dark phases.
Figure 2. Expansion of an astrocyte using pro-ExM

(A) Left, the original slice imaged. Middle, fluorescence microscopy image of the same hippocampal slice after expansion. Right, the expanded image scaled down to the pre-expansion slice’s size (B) An increase is shown in the hippocampal slice area over time, in slices prepared during the L-phase. (C) As in (B), in slices prepared during the D-phase. (D) Summary of the surface area increase in hippocampal slices. (E) An increase is shown in the hippocampal slice perimeter over time, in slices prepared during the L-phase. (F) As in (E), in slices prepared during the D-phase. (G) Summary of the perimeter increase in hippocampal slices.
Figure 3. Structural differences between biocytin-filled astrocytes in acute hippocampal slices prepared during the light and dark cycle

(A) The astrocyte of interest is isolated in white. (B) The cytoplasmic volume in pink, surrounded by the bounding volume in yellow. (C) Filaments reconstructed and color-coded based on branch level. (D) Summary graph of the analytical criteria used to perform the structural analysis of astrocytes using Bitplane Imaris. (E) Changes in cytoplasmic volume and bounding volume were observed across light and dark cells. (F) Changes in the number of filament segments per branch level but not the total number of branch levels were observed across astrocytes taken from the L and D phase.
Figure 4. IPSC recording in CA 1 Pyramidal Neurons

(A) Time course of holding currents from CA1-PCs in the L/D-phases (black and blue, respectively) before and after the application of the GABA$_A$ receptor antagonist PTX (100 µM). (B) Left, Summary graph showing the magnitude of the PTX-sensitive holding current. Right, As in B, after in-cell normalization by the cell capacitance. (C) Peak normalized IPSC recorded from CA1-PCs in slices prepared during the L/D-phases. (D) Summary graph of the IPSC latency, rise time, and decay time. (E) Train of IPSCs normalized by the peak of the first IPSC, evoked by a stimulus train of 15 pulses at 50 Hz. (F) Left, Summary graph of the decay time of the IPSC train. Middle, normalized IPSC amplitude over 300 ms. Right, normalized IPSC peak over 300 ms (G) Normalized cumulative IPSC amplitude during the train. The lines represent a linear fit of the last 5 points, used to calculate the values of $y_0$ and slope. (H) Summary graphs showing that the $y$-intercept and slope of the linear fit, which provide information on the size of the RRP and the vesicle recycling rate, are similar during the L/D-phases. The release probability also does not change.
Figure 5. Stereotaxic injections of Aβ₄₂-AAV cause Aβ₄₂ accumulation in CA1 hippocampus

(A) Schematic depicting stereotaxic injections of Aβ₄₂-AAV and Sham-AAV into the hippocampus. (B) Left, bar graph depicting accumulation of Aβ₄₂ in the hippocampus. Right, bar graph depicting the amount of insoluble Aβ₄₂ in the hippocampus. Data represent mean ± S.E.M.
Figure 6. Aβ42-AAV does not alter the gross morphology of CA1-PCs

(A) Representative confocal images of biocytin filled CA1-PCs imaged using confocal microscopy. (B) Dendrite sholl analysis of WT neurons showing the number of intersections as distance from the soma increases. (C) As in (B) but for Sham. (D) As in B but for neurons transfected with Aβ42-AAV. (E) Total number of intersections derived from Sholl analysis of biocytin-filled CA1-PCs in WT, Sham, and Aβ42 treated mice. Data represent mean ± S.E.M.
Figure 7. Spine morphology changes in CA1 pyramidal neurons

(A) Left, representative images of CA1-PCs in WT, Sham, and Aβ42 treated mice. Right, bar graph depicting spine density statistics for CA1-PCs in WT, Sham, and Aβ42. (B) Proportions of spine types (mushroom, thin, and stubby) for CA1-PCs in WT, Sham, and Aβ42 treated mice. (C) Left, bar graph depicting spine area for CA1-PCs in WT, Sham, and Aβ42 treated mice separated by type of spine. Right, as in C, but depicting statistics for spine head volume. (D) Left, bar graph showing spine head diameter separated by spine type for CA1-PCs in WT, Sham, and Aβ42. Right, As in D, for spine neck length. Data represent mean ± S.E.M.
Figure 8. Aβ42-AAV reduces the NMDA/AMPA ratio in hippocampal CA1-PCs

(A) Paired AMPA EPSCs recorded from CA1-PCs in response to Schaffer collateral stimulation. Each CA1-PC was voltage clamped at -70 mV. Each trace represents the average of ... consecutive sweeps. (B) Summary graph showing the quantification of the paired pulse ratio (PPR) for AMPA EPSCs. (C) Summary graphs of the amplitude and kinetics of AMPA EPSCs recorded from CA1-PCs in WT (white bar), Sham (green bar) and Aβ42-AAV-treated mice (green). (D-F) As in A-C, for NMDA EPSCs recording in the same cells at a holding potential of +40 mV. (G) Example of AMPA and NMDA EPSCs recorded from CA1-PCs in each one of the three groups mentioned above. (H) Summary graph showing NMDA/AMPA ratio. Data represent mean ± S.E.M.