Title Page

Research Article

Title: Sleep deprivation selectively reactivates hippocampal CA1 pyramidal neurons.

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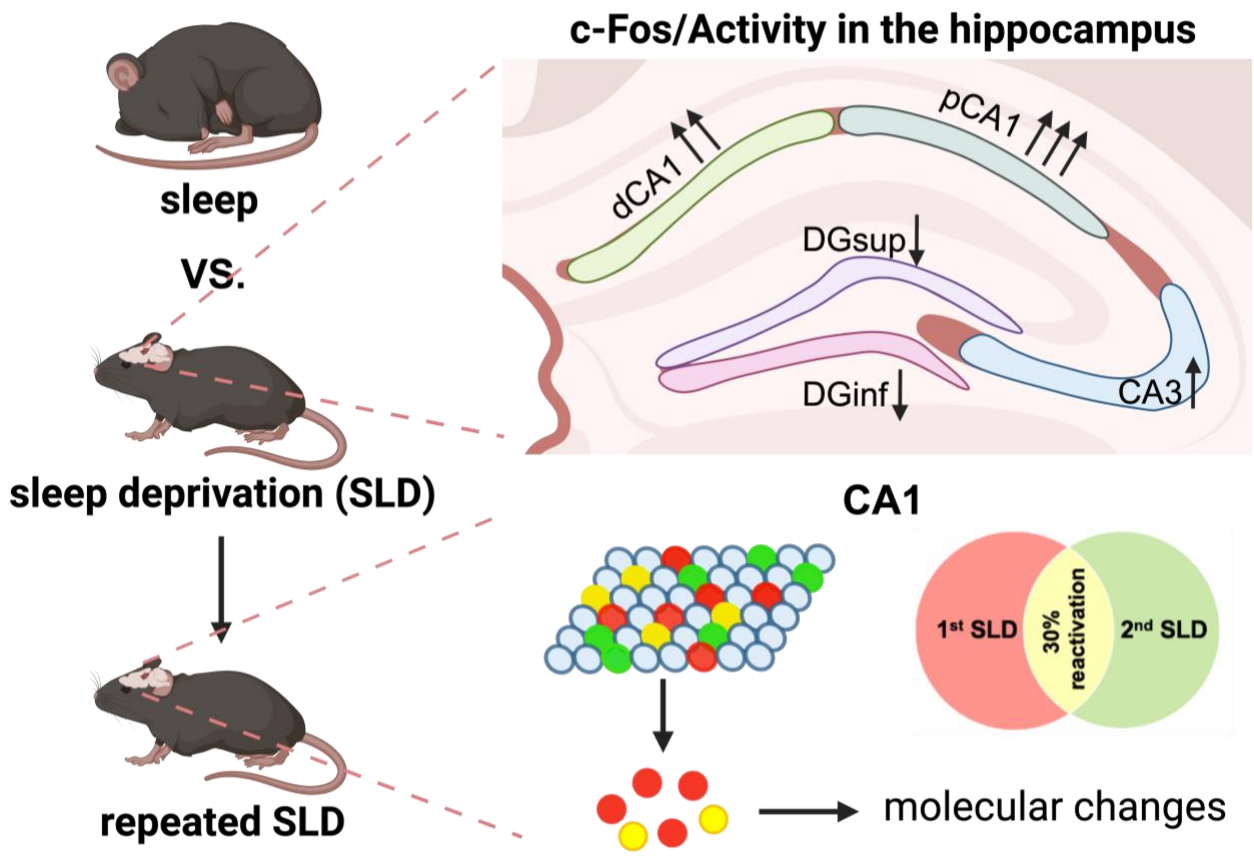
Abstract:

Sleep supports a variety of physiological processes, ranging from metabolic to immune system homeostasis, and plays a critical role in cognition and memory. A brief period of sleep loss impairs memory, particularly hippocampus-dependent memories, and alters molecular signaling and synaptic plasticity in the hippocampus. Studies have shown that sleep deprivation (SLD) alters neuronal activation as indicated by broad changes in gene expression signatures and by the altered expression of c-Fos, an immediate early gene (IEG) that functions as a molecular marker of neuronal activity. In the present study, we examined hippocampal subregion-specific c-Fos induction patterns. We find that CA1 pyramidal neurons exhibit the most robust c-Fos induction after SLD. Within CA1, the proximal region, where spatial information is processed and encoded, shows higher c-Fos levels after SLD as compared to the distal region. Using an activity-driven ribosomal tagging system and a repeated SLD model, we identify a specific population of excitatory neurons in area CA1 that are reactivated by repeated SLD. This approach also enables analysis of individual gene expression from c-Fos+ neurons to identify specific molecular signatures of neurons sensitive to repeated sleep loss. In summary, our study provides a detailed view of the activation of hippocampal neurons after SLD, revealing a subset of CA1 pyramidal neurons having higher sensitivity to the effect of sleep loss and laying the groundwork for further research investigating molecular changes in neurons specifically impacted by repeated sleep loss.

Keywords: sleep deprivation; hippocampus; CA1; pyramidal neurons; c-Fos; RiboTag

Graphical Abstract



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Statement of Significance:

Sleep loss is a prevalent problem in modern society and even brief periods of sleep loss impair hippocampal functions. Here, we map c-Fos expression via immunohistochemical staining in hippocampal subregions after sleep deprivation (SLD) and find distinct patterns of neuronal activation in each hippocampal subregion. We combined an activity-driven neuron labeling system with repeated SLD and for the first time report that a subset of CA1 pyramidal neurons is reactivated by repeated SLD. Our work uncovers a population of CA1 pyramidal neurons that are sensitive to repeated sleep loss and sheds light on a possible link between acute and chronic sleep loss at the cellular level.

Introduction

Sleep facilitates numerous physiological processes including cognition, learning, and memory consolidation[1]. Over 30% of adults in the United States routinely experience insufficient sleep, leading to subsequent health problems[2,3]. Sleep loss has pronounced impacts on cognitive function, with hippocampus-dependent memories being especially vulnerable[4]. The hippocampus plays a critical role in consolidating spatial, episodic, and contextual memories in rodents[5] and declarative memory in humans[6]. It is comprised of subfields with specialized roles in memory: the Dentate Gyrus (DG) enables pattern separation[7], Cornu Ammonis 3 (CA3) integrates separation and completion[8], and Cornu Ammonis 1 (CA1) recodes CA3 outputs while linking to the neocortex for memory retrieval[9]. Brief periods of sleep deprivation (SLD) impact hippocampal function via impairments in the cAMP signaling and related long-lasting forms of synaptic plasticity[10-12], suppressed protein synthesis [13], and altered synaptic connectivity[14-16].

c-Fos, an immediate early gene (IEG), is widely recognized as a marker of neuronal activity. The c-Fos protein is rapidly activated during synaptic plasticity associated with learning and memory[17], and its downstream effector genes, such as *Timp1* and *Mmp9*, regulate the neuronal structural and functional plasticity required for memory formation[17]. c-Fos expression has been used as a marker to locate and identify the neuronal populations activated by sleep and extended wakefulness[18-21]. While sleep is typically linked to low c-Fos expression and neuronal activity across most brain regions[18,19], several hours of SLD significantly increases c-Fos expression, particularly in the cortex, medial preoptic area, posterior hypothalamus, and the hippocampus[18-21]. This brain-wide activation resembles the increased c-Fos expression pattern during spontaneous wakefulness. In humans, sleep loss can often span several nights or even weeks. Work using rodents to model chronic sleep restriction has demonstrated impairment of hippocampal synaptic plasticity and severe spatial memory deficits that are resistant to recovery sleep[22,23]. However, whether repeated bouts of sleep loss impact the same neuronal population

is not known. Given the anatomical and functional complexity of the hippocampus, this study aims to precisely map c-Fos expression patterns within hippocampal subregions after 5h of SLD and after repeated SLD exposures.

Our results suggest that neuronal activation measured using c-Fos expression varies across the hippocampus following both single and repeated SLD. The CA1 subregion, particularly proximal CA1 (pCA1), showed the most pronounced increase, while DG exhibited a decrease. Applying the Target Recombination in Active Populations (fosTRAP) method in conjunction with the ribosome tagging strategy (RiboTag)[24,25], we devised an active neuron labeling system that was amenable to analysis of individual gene expression. Through this c-Fos-RiboTag approach, we confirmed that a substantial proportion of CA1 pyramidal neurons are reactivated by repeated bouts of SLD. As prior genomic studies identified translation-level changes in brain regions exhibiting increased c-Fos expression after SLD, including the hippocampus[26-29], the c-Fos-RiboTag approach also provides access for mapping activity-dependent alterations in translation of plasticity-related genes.

Methods & Materials

Animals

All experimental animals were 3- to 5-month-old male C57BL/6J mice obtained from the Jackson Laboratories (Catalog# 000664, Bar Harbor, ME). Mice were group housed (5 per cage) in standard ventilated cages with access to food (NIH-31 irradiated modified mouse diet #7913) and water *ad libitum.* Animals were maintained in the animal care facility at the University of Iowa on a 12h light/dark cycle, in a temperature- and humidity-controlled environment (21-22°C and 60-70%, respectively). All experiments were conducted in accordance with the standards established by the US National Institutes of Health Guidelines for Animal Care and use was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Iowa.

Adeno-associated virus (AAV) constructs and stereotactic surgeries

Surgeries were performed under isoflurane anesthesia (5% induction, 2% maintenance) following administration of Meloxicam (5mg/kg subcutaneous, s.c.). Animal health was monitored for 5 days following surgery along with administration of Meloxicam (5mg/kg, s.c.) for the first 2 days post-surgery. To label the SLD activated hippocampal neurons, 10-12 week old mice were injected intrahippocampally with a cocktail of AAV8-c-Fos-ERT2-Cre-ERT2-PEST-WPRE (titer- 1.57E+13 GC/mL, generated and packaged by Stanford University Gene Vector, catalog# GVVC-AAV-139) and AAV9-EF1a.DIO.HA-mRpl22.IRES2.eYFP.WPRE.hGH (titer- 1.14E+13 GC/mL, generated and packaged by the University of Pennsylvania Viral Vector Core). For labeling cells activated by SLD throughout the hippocampal subregions, the AAV cocktail was injected into the dorsal hippocampus bilaterally (1000nL at a rate of 200nL/min) using the following coordinates relative to bregma: anteroposterior (AP) -1.9mm, mediolateral (ML) ±1.5mm, dorsoventral (DV) -1.5mm from the surface of the brain. For labeling cells activated by SLD specifically in area CA1, the AAV cocktail was injected into dorsal CA1 bilaterally (300nL at a rate of 50nL/min), the following

coordinates were used upon validation: AP -1.8mm, ML ±1.45mm, DV -1.65 from the surface of the skull. Experiments were performed after 3 to 4 weeks to allow recovery from surgery and for spread of the viral particles in the targeted brain regions.

Drug preparation

4-hydroxytamoxifen (4-OHT) solution was prepared fresh on the day of experiment as previously described[30]. 4-OHT (Catalog #H6278, Millipore Sigma, MO, USA) was dissolved in 100% ethanol (EtOH) at 37°C for 15min with constant rotation to prepare a 20mg/mL stock solution. Stock solution was then diluted to 10mg/mL in 100% corn oil followed by another 15min incubation at 37°C with constant rotation. The solution was vacuum centrifuged for 15min to evaporate the EtOH, yielding an injectable solution of 10mg/mL 4-OHT in corn oil, and was administered intraperitoneally (i.p.) at a dose of 50mg/kg body weight.

Sleep deprivation

Total SLD was performed acutely, for 5h beginning at ZT0 using the gentle handling method, which involved lightly tapping and shaking the cage to keep the animal awake[12]. Mice were single housed in cages with corncob bedding and a handful of soft bedding for nest building for 7 days prior to SLD. Mice had *ad libitum* access to food and water in these housing conditions and during the sleep deprivation experiments. Mice were habituated to the experimenter and gentle handling stimuli, which was done by the experimenter holding each mouse in the palm for 2min and cage tapping for 2min for 4 consecutive days prior to the 1st sleep deprivation bout. Injection habituation occurred for 2 consecutive days prior to the first sleep deprivation experiment in which mice received an i.p. injection of saline after the 2min of handling and before being placed back in the cage for 2min of light cage tapping. All mice received an i.p. injection of 15mg/kg or 50mg/kg 4-OHT at ZT2 on the day of first SLD/NSD. After 5 hours, mice were returned to the original standard housing room where NSD mice were kept undisturbed throughout the experimental time window

(ZT0-ZT5). The second SLD or NSD bout was performed a week later using the same gentle handling method and timeline. After the second bout of SLD/NSD, mice went through perfusion fixation for immunohistochemistry validation of c-Fos/RiboTag expression or cervical dislocation to collect tissue for RNA extraction from the hippocampal CA1 (see Supplemental Materials for details).

Immunohistochemistry (IHC)

Perfusion fixed brains (4% v/v PFA in 1X PBS, Catalog #15710, Electron Microscopy Sciences, PA, USA) were equilibrated in sucrose solution (30%w/v in 1X PBS) for 48h. The brains were then sliced into 30µm sections using a Leica cryostat (Leica CM3050S, IL, USA) at -20°C and stored in cryoprotectant solution (30% w/v sucrose, 30% v/v ethylene glycol, and 0.01% Sodium Azide in 1X PBS). Sections containing the dorsal hippocampus were rinsed three times for 5min each in 1X PBS , followed by a 1h incubation in blocking solution (3% normal Donkey/Goat serum in 0.4% triton-X100/PBS buffer) and an overnight incubation at room temperature (RT) with primary antibodies: rabbit anti-c-Fos (1:3000, #226008, Synaptic Systems, Gortingen, Germany), guinea pig anti-c-Fos (1:3000, #226308, Synaptic Systems), rabbit anti-HAtag (1:2000, #3724, Cell Signaling, MA, USA), mouse anti-NeuN (1:1500, #ab104224, Abcam, MA, USA), rabbit anti-Sox9 (1:1500, #ab185966, Abcam). Following overnight incubation with primary antibodies, slices were rinsed three times for 5 min in 1X PBS and incubated with secondary antibodies: Alexa Fluor 488 goat anti-rabbit (1:1000, #A11008, Invitrogen, CA, USA), Alexa Fluor 647 donkey anti-rabbit (1:1000, #A32795, Invitrogen), Alexa Fluor 555 goat anti-guinea pig (1:1000, #150186, Abcam), Alexa Fluor 488 donkey anti-mouse (1:1000, #A21202, Invitrogen) for 2h at RT followed by three rinses in 1X PBS for 5min each. Slices were mounted onto Superfrost Plus (Fisherbrand) slides with Prolonged Diamond Antifade mountant with DAPI (P36962, Thermo Fisher Scientific, MA, USA) to stain cell nuclei.

Confocal Microscopy and Image Analysis

Following IHC, images of the dorsal hippocampus were acquired using a Leica SPE Confocal microscope equipped with lasers at 405nm, 488nm, 561nm, and 635nm. All images (8-bit) were obtained with identical settings for laser power, detector gain, and pinhole diameter (1.0AU) using a 20X oil immersion objective at 1024 × 1024-pixel resolution and 1.5X optical zoom.

The Adult Mouse Allen Brain Reference Atlas was used as a reference for the hippocampus and hippocampal subregions. Images were processed using the ImageJ software. Background fluorescent signals generated from AAV infusion surgery was subtracted from c-Fos and HAtag channels using the Image Calculator function in ImageJ. Images were converted to 16-bit for c-Fos+ cell quantification. The c-Fos+ cells within the cell body layers of each subregion were then plotted and counted automatically using the Analyze Particle function in ImageJ. The HAtag+ cells, DAPI+ nuclei, and double-labeled with HAtag/NeuN/Sox9 cells were counted manually.

Statistical analysis

All statistical analyses were performed using GraphPad Prism v10 with $\alpha = 0.05$ for analyses. Imaging data comparing c-Fos and RiboTag expression and neuronal reactivation were analyzed using unpaired two-tailed t-tests to compare the NSD and SLD groups (Figure 1, 3C, 4, 5, 6). Two-way ANOVA comparisons were used to determine the interaction between c-Fos activation and number of sleep deprivation epochs (Figure 3D). Data is presented as Mean ± SEM and *n* represents the number of animals in all experiments, IHC data is presented as an average of 3-4 sections per animal per condition. In figures, ns refers to non-significant, * refers to a p value < 0.05, ** refers to $p < 0.01$, *** refers to $p<0.001$, and **** refers to $p<0.0001$.

Results

Sleep deprivation causes non-uniform activation of hippocampal subregions as measured by c-Fos expression

Induction of IEGs, such as *Fos, Arc, and Egr1*, mark recent neuronal activation, and previous studies have investigated the impact of sleep and sleep loss on neuronal activity across rodent brains by measuring mRNA or protein levels of IEGs[18-21,26,28,31]. To investigate the impact of acute SLD on neuronal activation within hippocampal subregions, we sleep deprived mice for 5h by the gentle handling method. This method was chosen to avoid confounds with methods that may cause novelty-induced hippocampal activation. Following our 5h SLD we mapped c-Fos protein expression, a well characterized marker of neuronal activity, within the hippocampus as an indicator of neuronal activation. Quantification of c-Fos protein abundance by immunohistochemistry in SLD and NSD mice found increased c-Fos in SLD samples suggesting an overall increase in hippocampal activity (Fig. 1A-B) after 5h of SLD. As the hippocampal subregions have distinct functions, we quantified c-Fos expression within each hippocampal subregion finding that sleep deprivation for 5h induced c-Fos expression in pyramidal cell layers in CA1 and CA3 subfields. However, we observed decreased c-Fos expression in the superior (DGsup) and inferior blades (DGinf) of the DG (Fig. 1B). Furthermore, although CA1 and CA3 both showed an increase in c-Fos expression after 5h of SLD, the induction of c-Fos expression was more prominent in CA1 (8-fold induction compared to NSD) compared to CA3 (1.6-fold induction compared to NSD).

We then asked whether a specific cell type within each subregion is selectively activated by sleep deprivation. To answer that question, we performed co-staining with c-Fos and cell-type specific markers (NeuN for neurons and Sox9 for astrocytes). Our results suggest that neurons (NeuN+) represent the majority of activated cells in all the subregions while a minor fraction of those c-Fos+ cells in each subregion are astrocytes (Sox9+) (Fig. 2). In addition, depending on the

subregion, a variable fraction of activated cells (c-Fos+) were classified as other cell types (e.g. microglia, oligodendrocytes) because they were negative for NeuN and Sox9.

Repeated SLD has similar effects on c-Fos expression

In humans, SLD is not usually a singular event, but frequently occurs on multiple nights or a weekly basis. However, how hippocampal neuronal activity is affected by repeated sleep loss remains poorly understood. To address this gap and to determine whether the prominent c-Fos induction in CA1 was due to stochastic neuronal activity or an inherent bias that makes a subset of neurons susceptible to sleep loss, we subjected animals to a repeated SLD paradigm (Fig. 3A). In this paradigm animals were subject to SLD twice with a 1 week-long recovery between the first and the second SLD. This allowed us to determine how repeated SLD alters c-Fos expression across hippocampal subregions. A second SLD period revealed a similar pattern of c-Fos expression in the hippocampus as a single SLD with induction in CA1 and CA3, and a reduction trend in DG inferior blade (Fig. 3 B,C). The repeated protocol itself does not have a significant effect on c-Fos abundance within subregions. We observed similar c-Fos expression levels between single and repeated NSD and SLD groups (1xNSD vs 2xNSD, and 1xSLD vs 2xSLD) within subregions, although the DGsup region showed decreased c-Fos after repeated NSD (Fig. 3D). These results indicate consistent levels of neuronal activation after each individual SLD session. More importantly, the c-Fos induction patterns evident with a single SLD were conserved after the second SLD: high c-Fos induction in CA1 (7-fold) and a smaller induction in CA3 (3.5-fold). Together, the results suggest that SLD consistently induces region-specific changes in c-Fos expression and neuronal activation within the hippocampus, with the CA1 and CA3 regions showing increased c-Fos and the DG consistently showing decreased c-Fos expression.

c-Fos driven RiboTag labels hippocampal neurons activated by sleep deprivation

Although there is a significant induction of c-Fos expression in CA1 pyramidal neurons after both single and repeated SLD compared to NSD, the number of c-Fos+ neurons represent a small portion (about 12.5%) of the total number of CA1 pyramidal neurons (data not shown). While the results from the repeated SLD paradigm suggest that CA1 neurons are activated by SLD, it remains unclear if the neurons within CA1 that show c-Fos induction are randomly recruited by individual SLD sessions or whether neurons activated by an initial SLD period are then more likely to be activated by additional periods of SLD. To further characterize the neurons activated by repeated bouts of SLD, we took advantage of the fosTRAP system, which can label neurons activated by defined stimuli[24].

We employed an AAV-based neuron labeling system in which animals were injected intrahippocampally with a cocktail of a c-Fos driven Tamoxifen-dependent Cre vector (AAV8-Fos-ERT2-Cre-ERT2) and a Cre-dependent RiboTag vector (AAV9-EF1a-DIO-HA-mRpl22-IRES-eYFP). Administration of 4-OHT facilitates expression of the tamoxifen-inducible c-Fos-driven Cre recombinase (Cre^{ERT2}) upon neuronal activation, which subsequently drives expression of mRpl22-HA (RiboTag)[24,25] (Fig. 4A) to label CA1 pyramidal neurons. Using this strategy, we first tested CA1 neuronal labeling efficacy, indicated by RiboTag (HAtag) expression, using different labeling durations, 4-OHT doses, and AAV infusion methods (Fig. S1 and Fig. 4).

Unlike c-Fos protein itself, which is expressed rapidly following neuronal activation, expression of the c-Fos activation-promoted RiboTag depends on Cre^{ERT2} mediated recombination of loxP sites and consequent expression of mRpl22-HA (RiboTag), which likely takes a longer period of time. We first assessed the amount of time needed to get robust expression of HA following 4-OHT delivery and SLD or NSD. In a 5h labeling test (Fig. S1A-C), where 4-OHT was administered at ZT0 and tissue was collected after SLD or NSD (ZT0-5), we observed only weak expression of the RiboTag, and there were no differences between the SLD and NSD groups, confirming that the RiboTag marker takes longer to develop. To provide a longer expression window, we waited

one week based on previous results showing that repeated sleep deprivation leads to increased c-Fos positive cells in area CA1 and area CA3 that are comparable to a single SLD (Fig. 3). Consequently, this approach requires the use of c-Fos-driven RiboTag labeling for neurons activated by an initial period of SLD and subsequent c-Fos immunohistochemistry to identify neurons activated by a second period of sleep deprivation. We divided mice into repeated sleep deprivation (SLD-SLD) or repeated sleep (NSD-NSD) groups. All mice received an injection of 4-OHT at ZT2 of the 1st SLD/NSD[30] to drive RiboTag expression in neurons activated during the 5h of SLD/NSD. We also tested two concentrations of tamoxifen and found that a higher dose of 4-OHT (50mg/kg) was necessary to reflect the proportion of cells activated by SLD (Fig. S1D-F).

To further optimize RiboTag expression, we targeted the AAV cocktail infusion to hippocampal area CA1 bilaterally. Specific CA1 expression revealed a 3-fold induction of RiboTag expression after SLD compared to the NSD group (Fig. 4). The expression level of RiboTag using this CA1 targeting strategy was higher than the normalized number of cells showing c-Fos expression that we observed in both NSD and SLD for the single or repeated SLD groups (Fig. 1 and 3), particularly for the NSD groups. This could be due to the short half-life (~2h) of c-Fos[32]. Further, the c-Fos protein that was induced in the early stages of the light cycle may not be reliably detectable at ZT5 using IHC in both the NSD and SLD conditions, whereas the RiboTag will persist upon neuronal activation. Another factor that is likely to increase RiboTag expression in the NSD group is the unavoidable wakefulness and subsequent c-Fos expression induced by i.p. injection of 4-OHT. Together, these optimization strategies established a method for activity-driven RiboTagging that successfully labels CA1 pyramidal neurons that are activated by SLD.

CA1 pyramidal neurons are reactivated by repeated SLD

To determine if subsets of neurons reactivate during repeated SLD in the CA1 or whether hippocampal CA1 pyramidal neurons are randomly activated by repeated sleep loss, we performed RiboTagging combined with c-Fos immunolabeling. Immunofluorescence was used to

identify the cells positive for RiboTag (the neurons that were activated during first SLD or NSD) and c-Fos (the neurons that were activate during the second SLD or NSD). Cells that are positive for both c-Fos and the HA tag are neurons that undergo reactivation during the second SLD period (Fig. 5A). Our analysis showed that about 30% of CA1 neurons activated by the first SLD (HAtag positive) were also activated by the second SLD (c-Fos positive), and 40% of CA1 neurons active during the second SLD were previously activated by the first SLD (Fig. 5B, C). Overlap was minimal in the NSD-NSD group: about 5% of RiboTagged cells during 1st NSD were activated by the 2nd NSD, and double-labeled cells make up approximately 15% of the limited population of c-Fos+ cells after the second NSD. This overlap is likely due to background RiboTag expression (Fig. S1G). This neuronal reactivation confirms that repeated SLD stimulates a similar population of pyramidal neurons in hippocampal CA1 rather than a random activation, which suggests that a subset of neurons are more sensitive to repeated bouts of SLD.

Sleep loss preferentially activates proximal CA1

Within the CA1, differences exist between the inputs and functions of the distal (dCA1) and proximal (pCA1) sections. The dCA1 receives major inputs from the lateral entorhinal cortex (LEC) and the pCA1 from the medial entorhinal cortex (MEC) via direct and tri-synaptic pathways[33], which projects back to LEC and MEC via the subiculum. dCA1-LEC and pCA1-MEC circuits are suggested to underlie the separate processing of objective and spatial information [33,34]. Consequently, we analyzed the subregional CA1 immunohistochemistry labeling for HA and c-Fos (Fig. 6A) to determine potential differences within CA1. Our comparison between dCA1 and pCA1 revealed a higher activation rate in pCA1 during the first SLD and after the second SLD (Fig. 6) shown as higher co-labeling of HA and c-Fos expression in pCA1. To further confirm whether the higher co-labeling of HA and c-Fos expression is due to a higher number of cells within pCA1, we compared the DAPI signal and observed the same nuclei density between dCA1

and pCA1. In addition, overlap ratio between c-Fos and HAtag shows no difference in neuronal reactivation rate between dCA1 and pCA1 (Fig. 6C).

Discussion

The hippocampus is a structurally intricate and functionally diverse brain region, comprised of interconnected subregions that contribute uniquely to memory encoding processes[7-9]. To better understand how sleep deprivation affects hippocampal function, we investigated c-Fos expression patterns across different hippocampal subregions. Previous studies utilizing varying methods and durations of SLD have yielded inconsistent results regarding c-Fos expression within the hippocampus[20,28,31]. In this study, we sleep-deprived animals from ZT0-ZT5 by gentle handling, a widely accepted approach that minimizes stress[35], while avoiding the confounding effects of spatial or object novelty on neuronal activation in the hippocampus. In the present study, we examined hippocampal subregional c-Fos expression after single and repeated SLD. Results indicated that c-Fos expression is altered disparately among hippocampal subregions: CA1, particularly proximal CA1, shows the most robust c-Fos induction; CA3 shows a moderate activity increase; and the DG shows reduced c-Fos expression. (Fig. 1-3). This differential pattern of neuronal activation is consistent with previous spatial transcriptomic studies, which identified unique gene expression changes and enriched molecular functions specific to hippocampal subregions after SLD[36,37].

Utilizing a viral c-Fos-RiboTag active neuron tagging approach, we successfully captured the robust induction of CA1 neuronal activation (Fig. 4) and labeled a substantial number of activated neurons compared to a previous study that reported minimal labeling of CA1 neurons following sleep deprivation using a TRAP transgenic mouse model[38]. Our results also demonstrate over 30% overlap between c-Fos+ and RiboTag-labeled neurons between sleep deprivation events (Fig. 5). This represents a substantial overlap of neuronal activation after repeated SLD compared to the hippocampal engram reactivation rate observed using immediate early gene (IEG)-dependent tagging methods (e.g. TRAP2 and Tet-tag) during memory consolidation and retrieval, which typically ranges from 5-20% [37,39-41]. This prominent overlap provides compelling evidence

supporting the hypothesis that a specific subset of CA1 pyramidal neurons have distinct molecular or electrophysiological characteristics that confer heightened sensitivity to repeated and chronic sleep loss[42,43]. Based on our active neuron tagging strategy, further experiments investigating the differences between CA1 neurons that are sensitive (c-Fos+) and insensitive (c-Fos−) to SLD may elucidate key factors underlying neuronal susceptibility to chronic sleep loss. Understanding these molecular determinants could offer critical insights into the mechanisms of hippocampal dysfunction associated with chronic sleep restriction, with broader implications for memory and cognitive decline.

Comparing the c-Fos and RiboTag expression within CA1, we found that sleep loss preferentially activates pCA1 compared to dCA1. pCA1 and dCA1 are connected to distinct entorhinal cortex subregions and are involved in processing different information[33,34]. Entorhinal cortex (EC) mediates the dialogue between hippocampus and neocortex and plays crucial roles in memory formation[44,45]. However, unlike the hippocampus and cortex, the EC has been less extensively studied following sleep loss. The limited research available suggests that sleep deprivation affects gene expression and synaptic plasticity in the EC. Early brain-wide *Fos* mapping showed that sleep loss slightly increases *Fos* mRNA levels in EC in rats[20]. A recent study performing 4h of total sleep deprivation in rats revealed that sleep loss differently affects the surface expression of ionotropic glutamate receptors (AMPARs and NMDARs) subunits in EC: GluA2 and GluA3 subunits of AMPARs are upregulated, whereas GluA1 of AMPAR and GluN1 and GluN2B of NMDARs are downregulated[46]. The disrupted AMPARs and NMDARs subunit expression might consequently alter excitatory synaptic transmission and plasticity in EC and limit information transmission within the neocortex-EC-hippocampus circuitry[46]. Because medial and lateral EC send excitatory projections to pCA1 and dCA1 and processes allocentric spatial and egocentric information respectively[33,34], our finding of higher activity within pCA1 might indicate MEC and

LEC being differently affected by sleep loss, leading to circuitry-specific effects on memory-related processes.

c-Fos expression has broadly been used as indicator of engram activation and reactivation in hippocampus-dependent memory tasks. The replay of neuronal sequences during hippocampal sharp-wave ripples (SWR) provides one means of reactivation[47,48] and it's been shown that disruption of SWR, within which the sequential neuronal reactivation happens, disrupts memory consolidation[47,48]. Recent evidence demonstrates that c-Fos-expressing CA1 neurons exhibit higher place field preference, higher place field activity, higher stability of spatial maps, and more precise spatial information decoding in spatial learning tasks[49]. c-Fos itself plays important roles in shaping place codes as neurons with disrupted c-Fos function present less reliable activity, decreased spatial mapping, and lower across-day place fields stability[49]. However, several hours of sleep deprivation immediately following learning induces significant c-Fos expression in CA1[37,50]—a similar pattern observed in our SLD model without learning, yet impairs memory consolidation. Comparing the c-Fos-expressing neuron populations after learning and during SLD might help elucidate the mechanism of SLD-induced memory impairment. It is possible that SLD may activate a neuronal population distinct from those involved in engram formation, and thereby disrupting the network replay of memory-relevant engrams[51]. Alternatively, SLD may activate similar neuronal ensembles induced by learning, but interfere with gene expression and protein synthesis, and thus interrupt the sequential replay required for memory consolidation.

In addition to tagging activated neurons, the RiboTag approach we applied in this study also allows analyzing gene expression and mRNA translation in specific cell types, in our case, the c-Fos+ neurons[25,27]. We pooled actively translating mRNA from CA1 RiboTag-labeled neurons using immunoprecipitation of the HAtag from NSD and SLD mice (Fig. S2A) in which all mice were sleep deprived to label the sleep loss-sensitive neurons, and then divided into NSD and SLD groups to induce changes in activity and gene expression. Consistent with prior genomic studies[27-29,52,53],

our preliminary qRT-PCR assay comparing plasticity-related genes showed trends for increased translation of immediate early genes (*Fos, Arc*) and transcription factors (*Egr1, Nr4a1*), and trends for decreased translation of *Rbm3*, *Cirbp, and Actb* (Fig. S2B). Along with dysregulated transcription of actin binding/related genes, increased cofilin activity[14,53], and the involvement of *β-actin* in dendritic spine regulation[54-56], our preliminary data here contributes to the explanation of decreased spine density and neuronal connectivity in CA1 observed after SLD[14]. In future studies, use of the repeated sleep paradigm and subsequent Translating Ribosome Affinity Purification sequencing (TRAP-seq) technique[27] will facilitate identification of genome wide changes induced by SLD in sleep loss sensitive (RiboTag labeled) CA1 neurons.

In the present study, we observed distinct patterns of neuronal activation among hippocampal subregions caused by sleep deprivation, but we didn't further investigate mechanisms underlying this subregion-specific effect. Activation of hippocampal neurons is regulated through complicated interactions among different circuits and neurotransmitters (NT) including glutamate, norepinephrine (NE), serotonin (5-HT), and GABA. Differences in NT receptors expression and activity within subregions might help explain the different pattern of c-Fos expression and neuronal activation. With sleep deprivation, expression level of transcripts involved in GABAergic synapse pathway in CA1 and glutamatergic synapse encoding transcripts in DG are down-regulated, whereas 5-HT receptor-encoding transcripts (*Htr5b, Htr1a, Htr1b*) in CA1 are upregulated[37] along with elevated extracellular 5-HT levels[57]. In addition, the NE released from locus coeruleus (LC), which induces c-Fos expression in hippocampal and cortical areas[58] and specifically enhances CA1 pyramidal spike output via activating β-adrenergic receptors[59,60], is also upregulated during sleep deprivation[58]. Collectively, the disparate activity changes we observed across hippocampal subregions may result from complex NT interactions, where distinct NT play a more prominent modulatory role in different hippocampal subregions.

A limitation to address of this study is the labeling accuracy of the c-Fos-RiboTag approach. A critical factor influencing the labeling accuracy of different IEG-tagging approaches is the exact onset and duration of the tagging window. Although the fosTRAP system used in this study offers improved performance compared to the TetTagging system, which relies on dietary administration of doxycycline, the precise tagging window of the fosTRAP method remains unclear. This partly arises from the limited knowledge about the half-life of 4-OHT and the duration of activated Cre^{ERT2} recombinase in mouse neurons in vivo. To maximize labeling efficacy for neurons activated throughout the 5h SLD and to minimize labeling outside, especially the active phase before, the SLD window, we administered 4-OHT at ZT2, approximately the midpoint of the 5h manipulation. Future research should aim to precisely characterize the onset and duration of the fosTRAP tagging window to enhance labeling accuracy.

In conclusion, this study reveals the subregional impact of sleep deprivation (SLD) on hippocampal neuronal activation, which highlights the importance of spatially segregated analysis to understand the response to sleep loss. The reactivation of CA1 pyramidal neurons after repeated SLD supports the premise that certain excitatory neuron populations are particularly susceptible to repeated sleep loss. Our research provides a detailed insight into hippocampal neurons responsive to SLD and establishes a foundation for future molecular investigations of neuron subsets activated by sleep loss. Identifying these subsets will help inform strategies to mitigate cognitive deficits associated with SLD.

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Author Contributions:

Y.W. and T.A. designed the study with input from E.N.W., J.R., and L.C.L.. Y.W. and E.N.W. performed sleep deprivation and tissue collection. Y.W. performed protein and mRNA expression experiments and data analysis. Y.W. wrote the manuscript with input from all other authors.

Disclosure Statement:

All the authors declare that there are no conflicts of interest.

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Figure Legends

Figure 1. c-Fos expression is differently altered within hippocampal subregions after acute sleep deprivation. A) Immunofluorescent images representing c-Fos expression in NSD and SLD hippocampus. **B**) Comparison of area normalized c-Fos expression between NSD (n=5 mice) and SLD (n=6 mice) groups from the entire hippocampus (**1**) and within different subregions (**2**): CA1, CA3, and superior and inferior blades of Dentate Gyrus (DGsup and DGinf). Unpaired two-tailed t-test for $\mathbf{B_1}$: NSD=180.64±11.53, SLD=408.39±14.27, t(9)=12.05, p<0.0001. Multiple unpaired t-tests with Bonferroni-Dunn correction for $\mathbf{B_2}$: CA1: NSD=161.50±17.15, SLD=1072.02±26.98, t(9)=27.10 p<0.000001; CA3: NSD=142.53±6.97, SLD=218.99±14.11, t(9)=4.54, p=0.0056; DGsup: NSD=176.18±11.26, SLD=131.76±6.58, t(9)=3.56, p=0.024; DGinf: NSD=289.11±51.80, SLD=99.07±6.38, t(9)=4.02, p=0.012.

Figure 2. Neurons are selectively activated by SLD. A) Representative images of c-Fos double labeling with NeuN and Sox9 after SLD. **B**) Average percentage of c-Fos+NeuN+ and c-Fos+Sox9+ double labeled cells to the total number of c-Fos+ cells within each subregion in NSD (n=3 mice) and SLD (n=3 mice) groups. c-Fos+NeuN+/c-Fos+ (%): NSD group: CA1=90.8±3.07, CA3=90.2±1.38, DGsup=78.5±2.44, DGinf=79.3±4.14; SLD group: CA1=96.7±1.03, CA3=87.2±1.39, DGsup=70.5±0.92, DGinf=68.7±10.30. c-Fos+Sox+/c-Fos+ (%): NSD group: CA1=6.8±0.39, CA3=4.6±0.86, DGsup=5.6±0.89, DGinf=5.1±3.00; SLD group: CA1=1.2±3.35, CA3=0.9±1.30, DGsup=2.7±1.69, DGinf=3.0±3.40.

Figure 3. Repeated sleep deprivation induces similar pattern of c-Fos expression in the hippocampus. A) Schematic of repeated sleep deprivation. **B**) Immunofluorescent images of c-Fos expression after repeated NSD (NSD-NSD) and repeated SLD (SLD-SLD) in the hippocampus. **C**) Comparison of area normalized c-Fos expression between NSD-NSD (n=6 mice) and SLD-SLD (n=6 mice) within whole hippocampus (1) and subregions (2). C1) Unpaired two-tailed t-test comparing NSD-NSD (113.13±15.84) and SLD-SLD (336.38±36.42), t(10)=5.62,

p=0.0002. C2) Multiple unpaired t-tests with Bonferroni-Dunn correction were run to compare NSD-NSD and SLD-SLD within each subregion. CA1 NSD-NSD (151.68±38.14) and SLD-SLD (1029.62±126.03), t(10)=6.67, *p*=0.0002. CA3 NSD-NSD (60.47±9.98) and SLD-SLD (223.66±37.98), t(10)=4.16, *p*=0.0078. DGsup NSD-NSD (94.06±20.43) and SLD-SLD (112.86±10.19), t(10)=0.82, *p*>0.9999. DGinf NSD-NSD (169.60±24.32) and SLD-SLD (105.76±24.04), t(10)=1.87, *p*=0.3659. **D**) Comparison of area normalized c-Fos expression between single (1x NSD n=5 mice, 1x SLD n=6 mice) and repeated sleep deprived (2x NSD n=6, 2x SLD n=6) within whole hippocampus (1) and hippocampal subregions (2). Two-way ANOVA with Bonferroni post-hoc tests were performed for whole hippocampus (1) and each subregion (2) comparison in D. D1) There is a significant effect of sleep deprivation (F(1,19)=75.69, *p*<0.0001) and a significant effect of repeat manipulation (F(1,19)=7.24, *p*=0.0063), but no significant effect of interaction (F(1,19)=0.01, *p*=0.9224). Post hoc analyses show no significant difference in c-Fos measured between the first and second NSD (1xNSD vs 2xNSD *p*=0.3251) and between the second and first SLD (1xSLD vs 2xSLD *p*=0.1996). D2) CA1: no significant effect of repeated manipulation (F(1,19)=0.1, *p*=0.7283, post hoc 1xNSD vs 2xNSD *p*>0.9999, 1xSLD vs 2xSLD *p*>0.9999). CA3: no significant effect of repeated SLD (F(1,19)=3.0, *p*=0.0966, post hoc 1xNSD vs 2xNSD *p*=0.1147, 1xSLD vs 2xSLD *p*>0.9999). DGsup: there is a significant effect of repeated manipulation (F(1,19)=14.23, *p*=0.0013, post hoc 1xNSD vs 2xNSD *p*=0.0027, 1xSLD vs 2xSLD *p*>0.9999). DGinf: no significant effect of repeated SLD (F(1,19)=3.8, *p*=0.0649, post hoc 1xNSD vs 2xNSD *p*=0.0590, 1xSLD vs 2xSLD *p*>0.9999). See supplemental materials Table S2 for full analysis results.

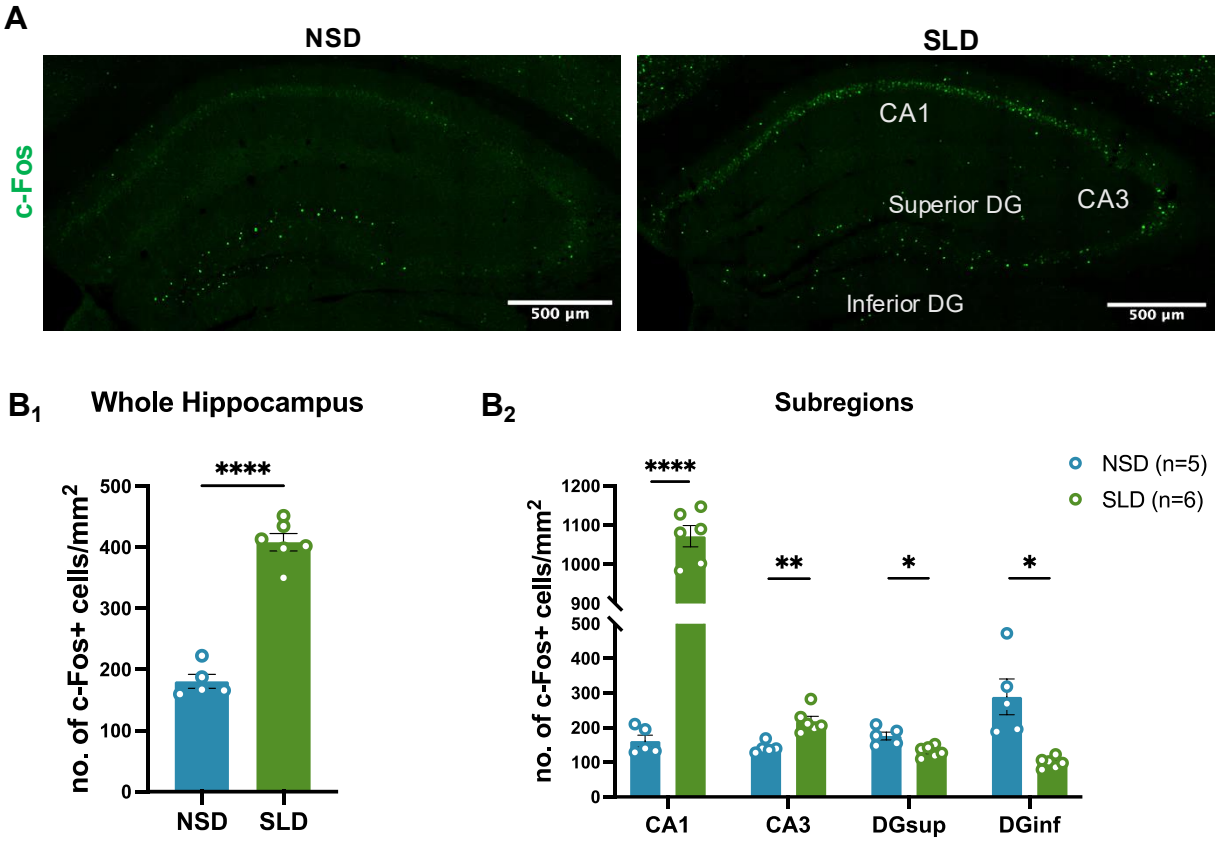
Figure 4. c-Fos-RiboTag strategy to label activated cells in area CA1. A) Diagram of the activity (c-Fos)-driven ribosomal tagging (RiboTag) strategy**. B**) Diagram of CA1 neuron labeling using c-Fos-RiboTag strategy with repeat sleep deprivation. **C**) Representative images of neuron labeling shown as RiboTag (HAtag) expression in the NSD-NSD and SLD-SLD hippocampus. **D**)

Neuronal labeling efficacy in hippocampal CA1: area normalized HAtag+ cells in NSD-NSD (n=5) and SLD-SLD (n=4) groups. Unpaired two-tailed t-test performed, NSD-NSD=476.91±02.01, SLD-SLD=1408.06±186.79, t(7)=4.79, *p*=0.0020.

Figure 5. CA1 pyramidal neurons are reactivated by repeated SLD. A) Immunofluorescence images of c-Fos and HAtag double labeling in area CA1 in NSD-NSD (n=5) and SLD-SLD (n=4) groups. **B**) Neuronal reactivation: percentage (%) of HAtag+ neurons that are double labeled in CA1 in NSD-NSD (3.58±1.08) and SLD-SLD (30.97±5.56) are significantly different (Unpaired two-tail t(7)=5.44, *p*=0.0010). **C**) The percent of c-Fos+ cells that are double labeled in CA1 in NSD-NSD (14.88±4.54) is significantly less than that in SLD-SLD group (38.96±6.03) (Unpaired two-tailed t(7)=3.26, *p*=0.0138).

Figure 6. Proximal CA1 shows greater c-Fos expression than distal CA1 after SLD. A) Representative image of DAPI, c-Fos+, HAtag+, and double labeled cells in distal vs proximal CA1 in SLD-SLD group (n=6). All comparisons used unpaired t-tests. **B)** Comparison of area normalized DAPI (1), c-Fos+ (2), HAtag+ (3), and c-Fos+HAtag+ cells (4) between dCA1 and pCA1. B1) dCA1=7827.39±190.20, pCA1=7732.98±227.45, t(10)=0.32, *p*=0.7576. B2) dCA1= 776.04±154.53, pCA1=1243.15±140.26, t(10)=2.24, *p*=0.0491. B3) dCA1=1287.53±65.68, pCA1=1993.05±142.29, t(10)=4.50, *p*=0.0011. B4) dCA1=305.80±78.63, pCA1=452.91±41.19, t(10)=1.66, *p*=0.1285. **C**) Comparison of neuronal reactivation rate between distal vs proximal CA1 in SLD-SLD mice. **C1)** percentage (%) of HAtag+ cells that are double labeled in dCA1 (21.97±3.71) and pCA1 (23.99±1.46) is not different (t(10)=0.51, p=0.6230). **C2)** percentage (%) of c-Fos+ neurons that are double labeled in dCA1 (38.43±5.24) and pCA1 (39.62±3.73) is also not significantly different (t(10)=0.18, *p*=0.8572).

Figure 1


A
NSD
SLD
c-Fos
CA1
Superior DG
CA3
Inferior DG
500 µm
500 µm
B1
Whole Hippocampus
no. of c-Fos+ cells/mm2
****
NSD
SLD
B2
Subregions
NSD (n=5)
SLD (n=6)
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*
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CA1
CA3
DGsup
DGinf

Figure 2

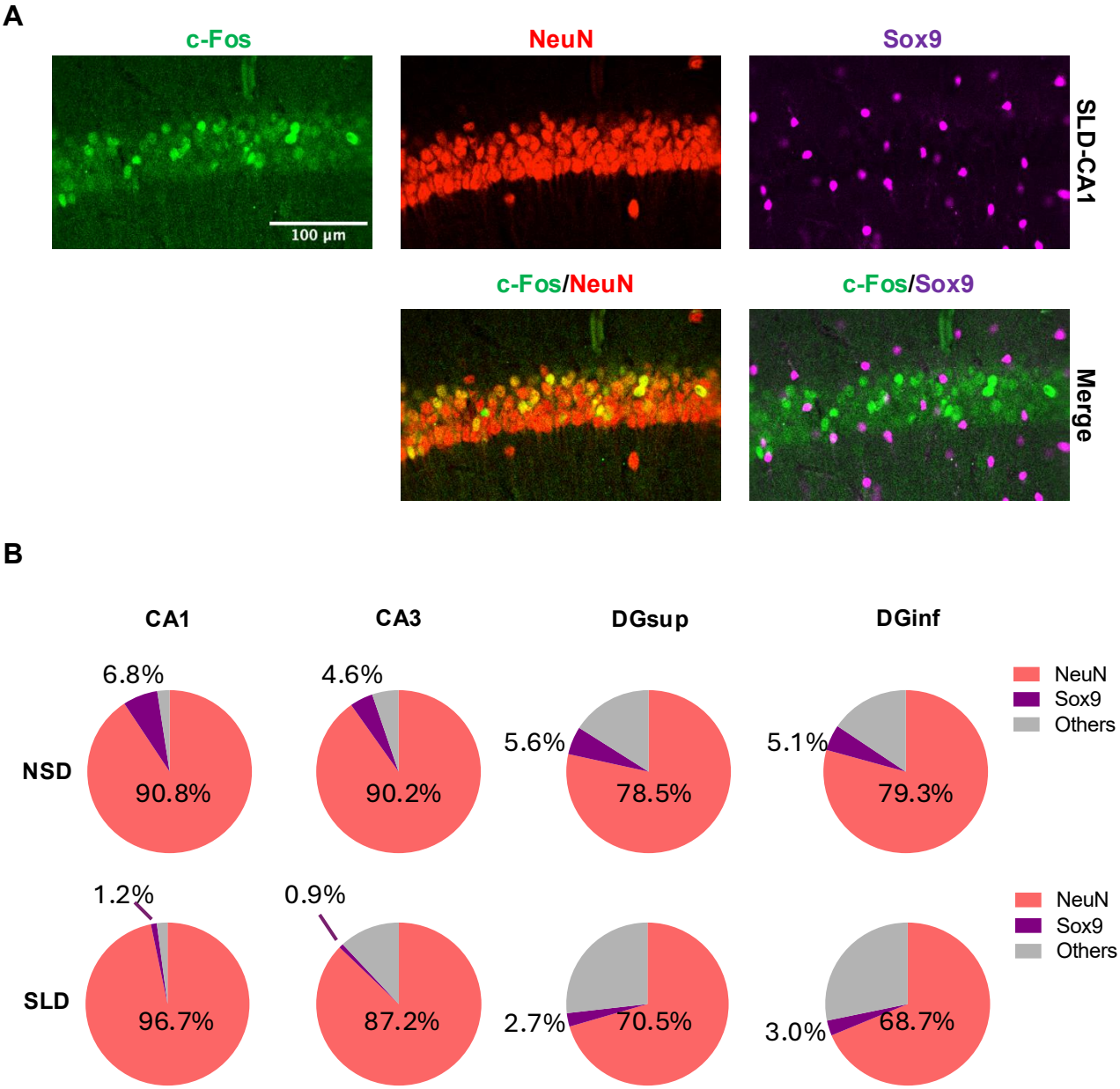

A
c-Fos
NeuN
Sox9
SLD-CA1
100 µm
c-Fos/NeuN
c-Fos/Sox9
Merge
B
CA1
CA3
DGsup
DGinf
NSD
SLD
6.8%
90.8%
4.6%
90.2%
5.6%
78.5%
5.1%
79.3%
1.2%
96.7%
0.9%
87.2%
2.7%
70.5%
3.0%
68.7%
NeuN
Sox9
Others

Figure 3

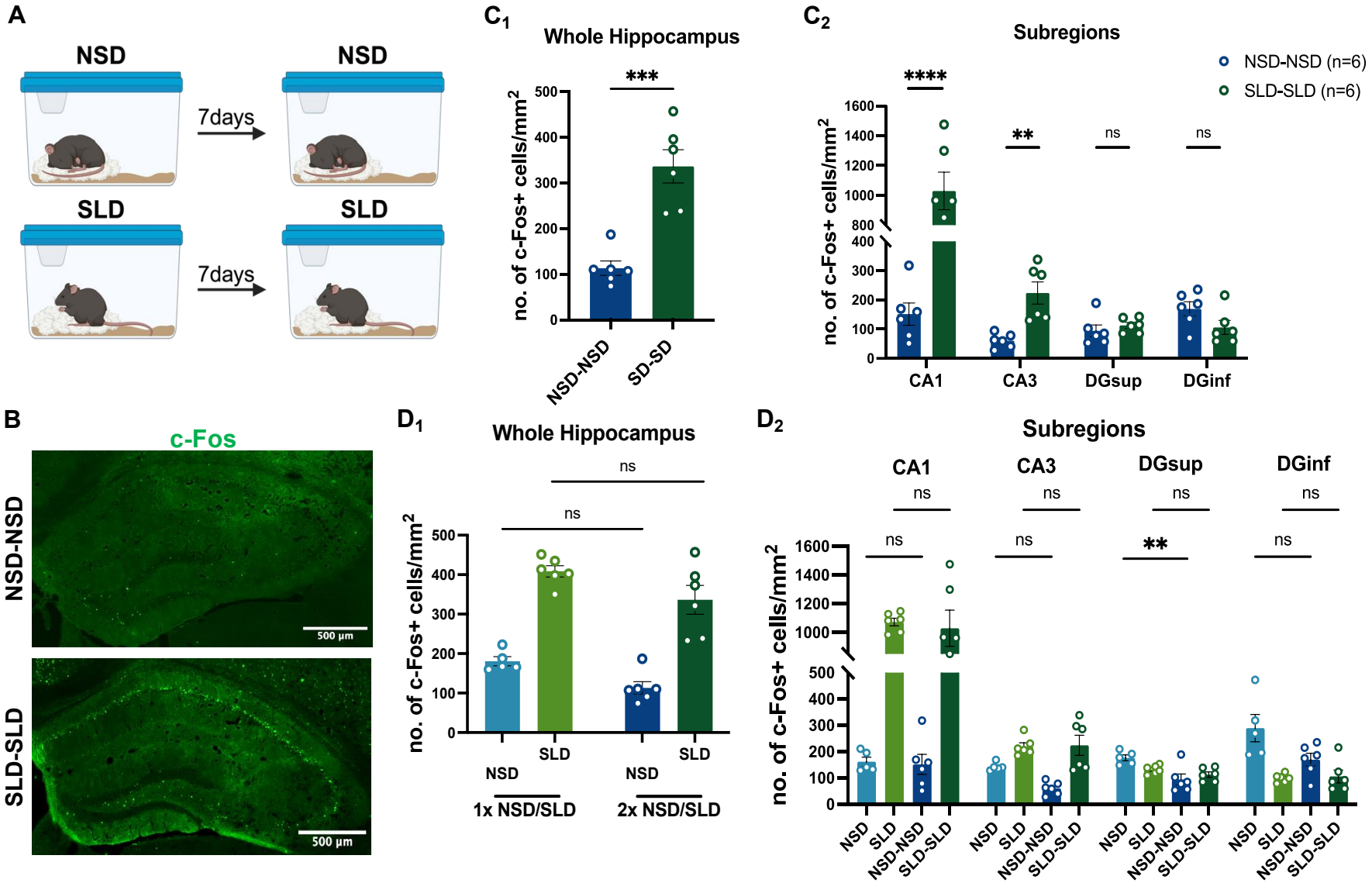

A
NSD
NSD
7days
SLD
SLD
7days
B
c-Fos
NSD-NSD
SLD-SLD
500 µm
500 µm
C1
Whole Hippocampus
***
no. of c-Fos+ cells/mm2
NSD-NSD
SD-SD
C2
Subregions
NSD-NSD (n=6)
SLD-SLD (n=6)
****
**
ns
ns
CA1
CA3
DGsup
DGinf
D1
Whole Hippocampus
ns
ns
NSD
SLD
NSD
SLD
1x NSD/SLD
2x NSD/SLD
D2
Subregions
CA1
CA3
DGsup
DGinf
ns
**
NSD
SLD
NSD-NSD
SLD-SLD

Figure 4

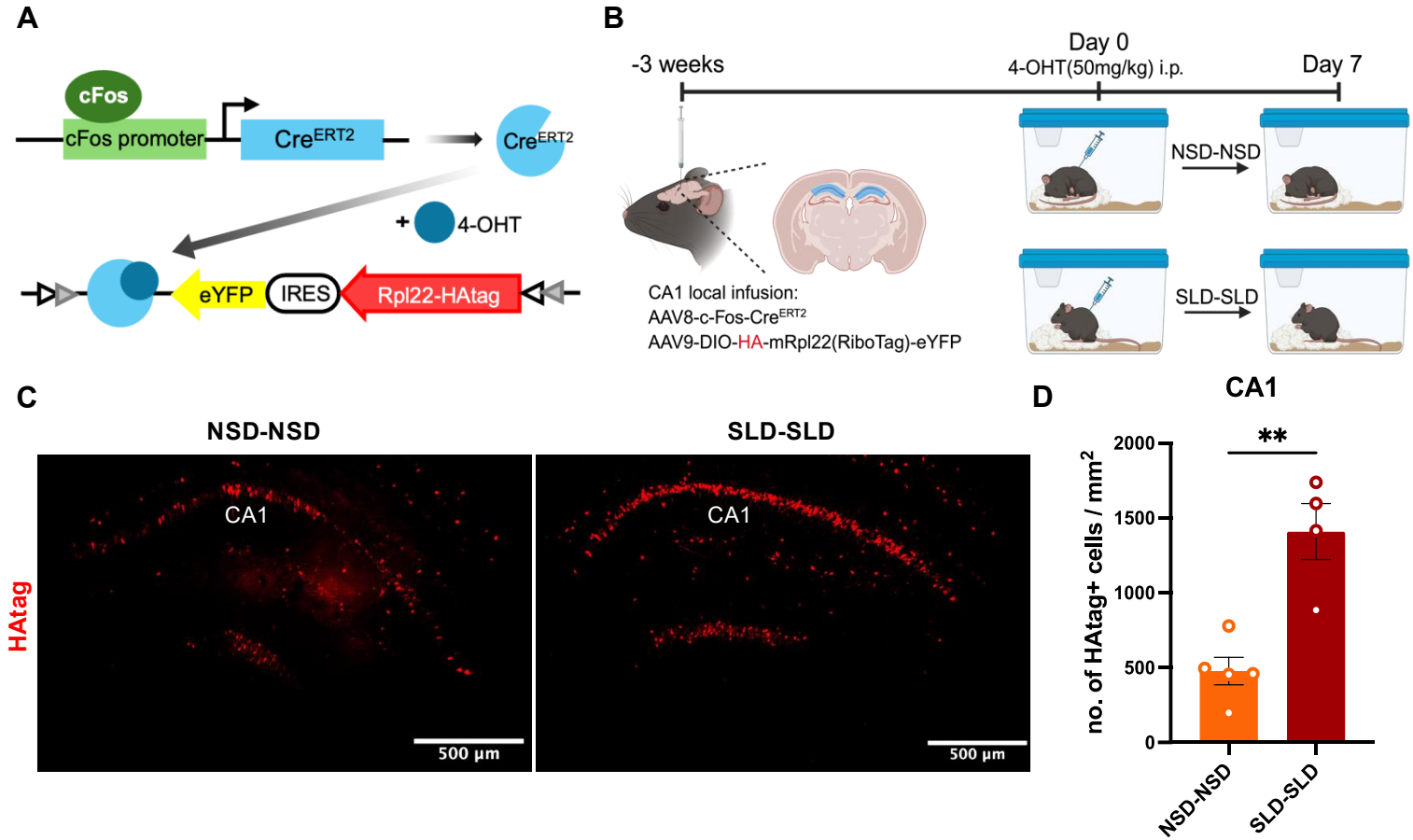

A
cFos
cFos promoter
CreERT2
CreERT2
+ 4-OHT
eYFP
IRES
Rpl22-HAtag
B
-3 weeks
Day 0
4-OHT(50mg/kg) i.p.
Day 7
NSD-NSD
SLD-SLD
CA1 local infusion:
AAV8-c-Fos-CreERT2
AAV9-DIO-HA-mRpl22(RiboTag)-eYFP
C
NSD-NSD
SLD-SLD
HAtag
CA1
CA1
500 µm
500 µm
D
CA1
**
no. of HAtag+ cells / mm2
2000
1500
1000
500
0
NSD-NSD
SLD-SLD

Figure 5

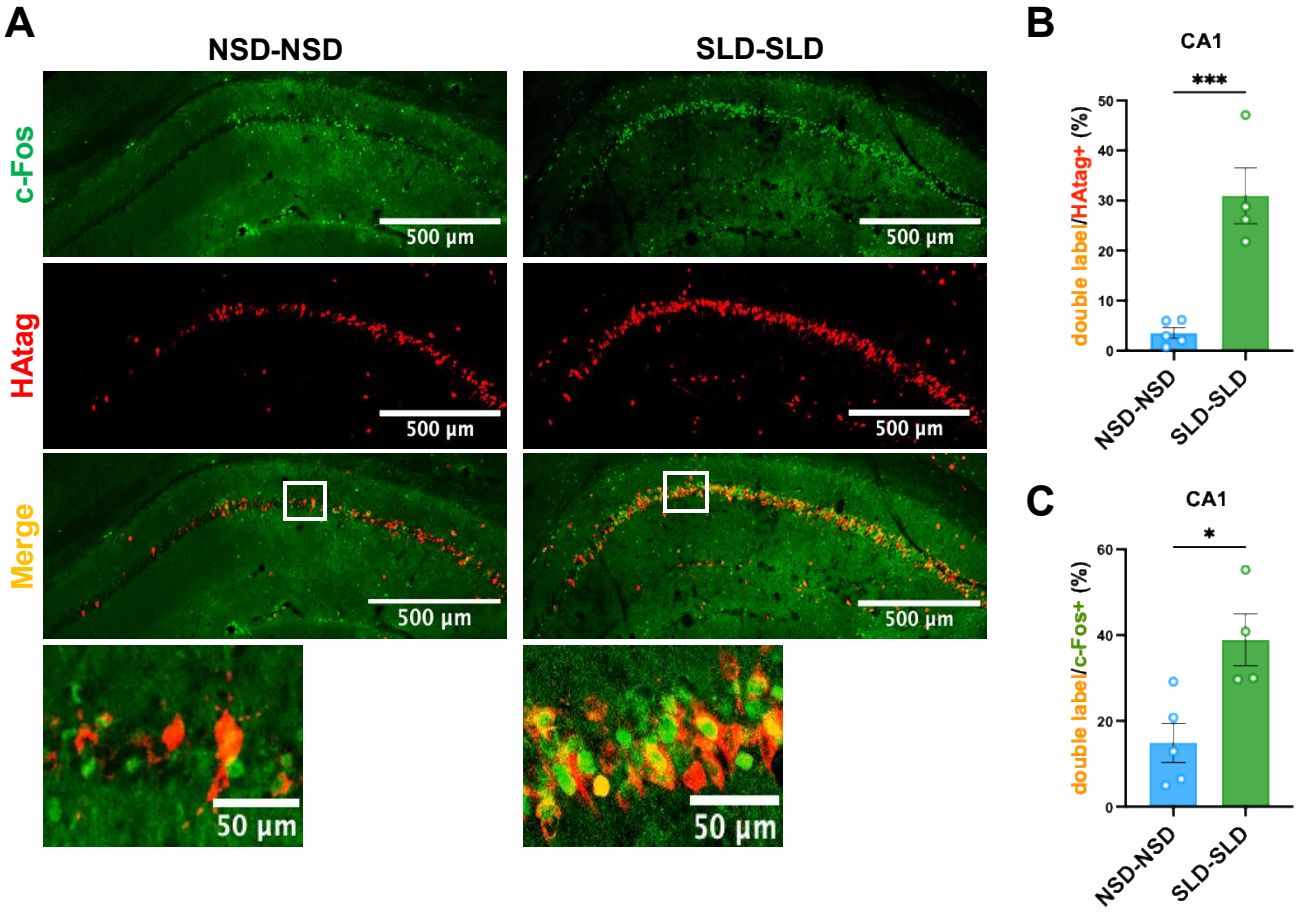
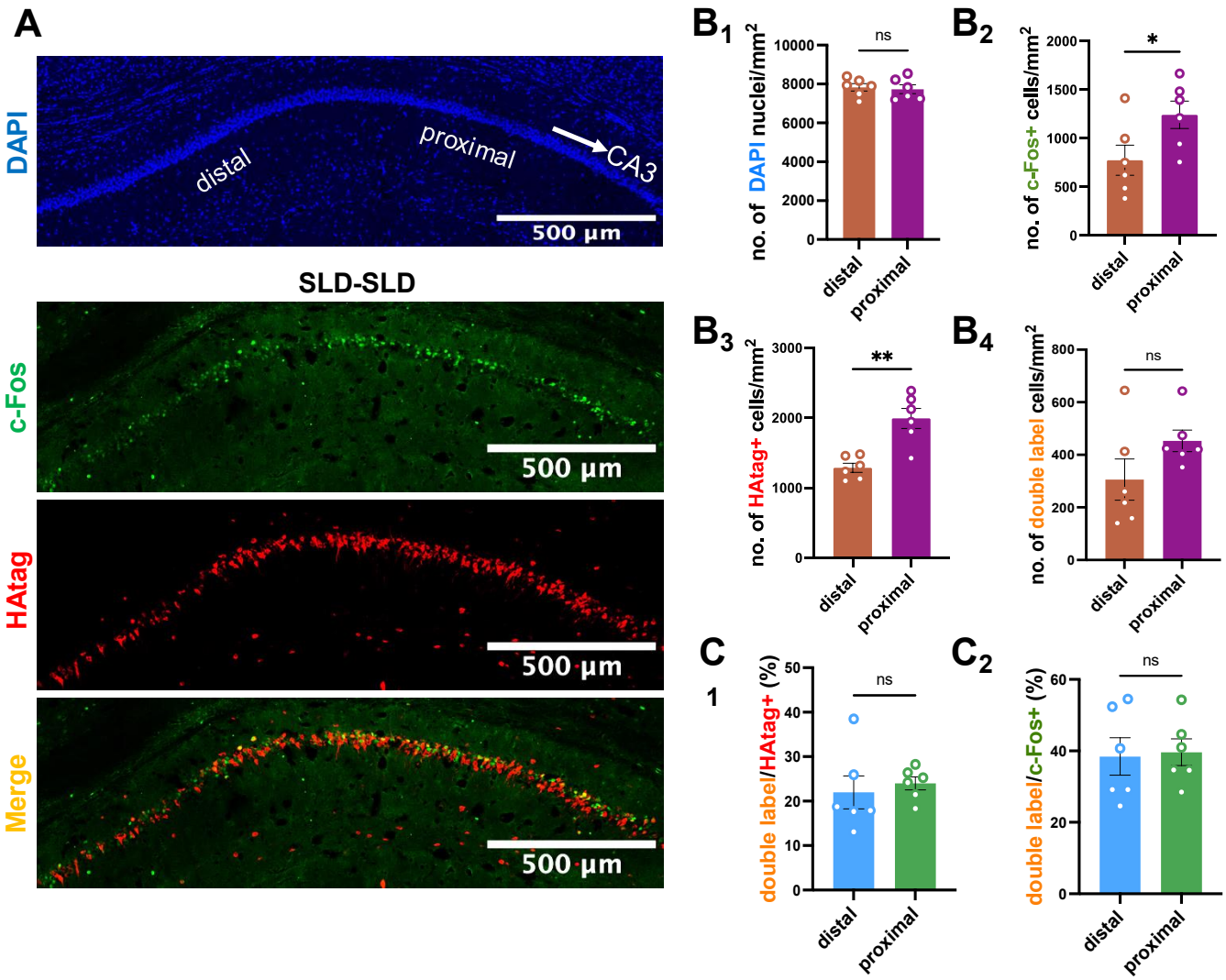

A
NSD-NSD
SLD-SLD
c-Fos
HAtag
Merge
500 µm
50 µm
B
CA1
***
double label/HAtag+ (%)
0
10
20
30
40
50
NSD-NSD
SLD-SLD
C
CA1
*
double label/c-Fos+ (%)
0
20
40
60
NSD-NSD
SLD-SLD

Figure 6


A
DAPI
distal
proximal
CA3
500 µm
SLD-SLD
c-Fos
500 µm
HAtag
500 µm
Merge
500 µm
B1
no. of DAPI nuclei/mm2
ns
distal
proximal
B2
no. of c-Fos+ cells/mm2
*
distal
proximal
B3
no. of HAtag+ cells/mm2
**
distal
proximal
B4
no. of double label cells/mm2
ns
distal
proximal
C1
double label/HAtag+ (%)
ns
distal
proximal
C2
double label/c-Fos+ (%)
ns
distal
proximal