Behavioral and Molecular Changes Following Acute Sleep Deprivation in Juvenile Wild-Type and *Shank3*$^{WT/\Delta C}$ Mice

By

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Abstract

Sleep is a critical to health and wellbeing, and lack of sleep can have numerous detrimental effects on human health. These effects can be overrepresented or exasperated in certain populations, such as those with autism spectrum disorders (ASD). Mutations in the *Shank3*ΔC gene have been used as a mouse model of autism, and these mice have been shown to have a distinct sleep phenotype. A popular measure of sleep deprivation effects is Novel Object Recognition (NOR), as sleep-deprived (SD) mice show disruptions to learning and memory. Sleep is hypothesized to play a key role in memory consolidation through homeostatic synaptic downscaling. Some key proteins involved in or mediating this process are Arc and mGluR5. The current study will analyze behavioral and molecular changes following SD in juvenile mice by administration of NOR to wild-type (WT) mice and Western Blotting of forebrains of WT and *Shank3*$^{WT/\Delta C}$ mice for protein quantification. Due to their vulnerability, I hypothesized lack of novel object identification in NOR following both two-hour and four-hour sleep deprivation. I also expected increase of Arc, mGluR5, and SHANK3, with *Shank3*$^{WT/\Delta C}$ animals having more intense responses. The study found juvenile WT mice to successfully learn only following a two-hour sleep deprivation, indicating a limited tolerance. Arc showed significant increase post sleep deprivation in both genotypes, while SHANK3 effects were only observed in *Shank3*$^{WT/\Delta C}$ mice. Contrary to the hypothesis, mGluR5 did not show variability among groups. Further study is suggested with specified procedural modifications and larger data samples to include sex as a variable of analysis.

Introduction

Sleep is a vital function with overarching impacts on both the body and the brain. Conversely, lack of sufficient sleep can cause serious harm, especially in terms of alertness, sensory perception, emotional processing, and degradation of learning and memory (Killgore, 2010). Around 20% of Americans experience adverse health consequences due to chronic sleep disorders (Colten *et al*., 2006), affecting people of all age groups (Colten *et al.*, 2006; Chattu *et al*., 2018). Certain populations experience sleep problems and/or sleep deprivation at a higher rate than average. One of these cases is people with autism spectrum disorders (ASD), where prevalence of sleep disorders is significantly higher than in the average population, notably in children (Chen *et al*., 2021; Galli *et al*., 2022). Implications of sleep disorders in this population include developmental and cognitive delays and emotional and behavioral difficulties (Galli *et al*., 2022), thus further highlighting the significance of development of therapeutic or pharmacological intervention for lasting symptom mitigation.

SH3 and multiple ankyrin repeat domains 3 (SHANK3) is a synaptic scaffolding protein, insufficiency and mutations of which are associated with Phelan-McDermid syndrome and ASD (Durand *et al*, 2007; Uchino & Waga, 2012). Its deficiency is also known to produce an ASD-like phenotype in mice (Uchino & Waga, 2012; Delling & Boeckers, 2021), with notable sleep differences (Ingiosi *et al*., 2019). *Shank3* knock-out mice (*Shank3*$^{\Delta C/\Delta C}$; C-terminal truncation through deletion of exon 21) are also characterized by varying sleep patterns from wild-type (WT) mice: they sleep less but demonstrate higher levels of REM sleep early in life (Medina *et al.*, 2022). Additionally, heterozygous *Shank3*$^{WT/\Delta C}$ mice have been shown to be more vulnerable to sleep deprivation: with sociability, sensory processing, and locomotion variation in males and risk aversion reduction in females (Lord *et al*., 2022). Therefore, SHANK3 mice serve as a

mouse model of ASD and also demonstrate significant differences in sleep phenotypes from WT mice, with both age and sex being key factors (Medina *et al.*, 2022; Lord *et al*., 2022).

Degradation of memory and learning is one of the common consequences of sleep deprivation (Killgore, 2010). A popular of memory measurement in mice is the novel object recognition (NOR) task, which consists of a training phase with familiar objects and testing with an introduction to an unfamiliar one. As mice tend to explore novel objects in their environment, they are expected to spend more time with the unfamiliar object in the testing phase to demonstrate learning and memory (Cohen & Stackman, 2015). Specifically, sleep-deprived mice have been shown to have decreased object recognition in this task (Huang *et al*., 2022; Palchykova *et al*., 2006), thus allowing it to apply to gradual measurements of sleep deprivation.

Though behavioral consequences of sleep deprivation have been widely studied, its molecular basis is largely unknown. A key area of sleep research, also being pertinent to learning and memory, is synaptic plasticity, an ability of synaptic transmission strength to change (Diering, 2022). A large component of this learning and memory models is based on long-term potentiation (LTP) and depression (LTD) in an input-dependent manner, also known as Hebbian plasticity (Abbott & Nelson, 2000). There is also homeostatic plasticity, which is input-independent and allows for modification of all synapses in a neuron (Turrigiano, 2008). Combining the two, the synaptic homeostasis hypothesis of sleep states that sleep allows for global synapse scaling-down to account for synapse strengthening (LTP) that occurs through activity during wake (Tononi & Cirelli, 2014; Diering, 2022).

Activity regulated cytoskeleton associated protein (Arc) is an immediate early gene that is heavily involved in synaptic plasticity and memory. It is induced in neurons after learning behavior or other activities that require Hebbian plasticity (Saha & Dudek, 2013; Diering, 2022).

Arc regulates AMPA-type glutamate receptors, which in turn allows for global, but selective, synaptic downscaling (Shepherd *et al.,* 2006; Chowdhury *et al.,* 2006; Diering, 2022). In certain hippocampal neurons, Arc local translation was shown to be mediated through metabotropic glutamate receptor 5 (mGluR5) activation (Wilkerson *et al*., 2014). Both Arc and mGluR5 have implications in sleep. Arc expression is increased in response to sleep deprivation, and it was found to be heavily involved in induction of numerous genes that are typically induced by sleep deprivation (Suzuki, Yanagisawa, & Greene, 2020). Without mGluR5, mice presented with a distinct sleep phenotype: they had decreased wake time and bout length, while increasing NREM sleep (Aguilar *et al*., 2020). With its relevance to both memory and sleep, Arc and mGluR5 are crucial candidate of study to further characterize molecular basis of sleep.

Due to the multi-faceted nature of sleep and consequences of its deprivation, this study will consist of behavioral and molecular companion experiments, focusing on the various changes produced by sleep deprivation. A unique focus of the study will be exploration of juvenile mice rather than adults, because postanal day 23-29 has been identified as a key point of development for sleep in mice (Medina et al. 2022).

Sleep deprivation has been shown to degrade memory, especially in the hippocampus (Guan, Peng, & Fang, 2004). Thus, because of the interconnected nature of sleep need, memory consolidation, and synaptic plasticity, the first experiment will utilize NOR to compare behavioral translations of gradually increasing amounts of sleep deprivation (zero, two, and four-hour sleep deprivation). As sleep deprivation effects were demonstrated to be time-dependent at protein-level (Wang *et al.*, 2018), behaviorally, I am expecting a range of phenotypes that will diverge when undergoing increasing amounts of acute sleep deprivation. In adult mice, this is mirrored through a lack of identification of a novel object after a six-hour sleep deprivation but

not four (Gay *et al.*, 2023). So, for juveniles, their increased sensitivity could allow them to have differences even earlier than the typical four-hour sleep deprivation.

For the molecular experiment, I will compare effects of a four-hour sleep deprivation on *Shank3*$^{WT/\Delta C}$ and WT mice. As *Shank3* has already been identified as a promising candidate of therapeutic intervention (Uchino & Waga, 2012; Delling & Boeckers, 2021), better understanding of the molecular expression differences in response to sleep deprivation in *Shank3*$^{WT/\Delta C}$ mice can further advance such efforts. The study will focus on proteins since the proteosome has been observed to be influenced by sleep need rather than circadian rhythms (Brüning *et al.*, 2019; Noya *et al.*, 2019). The tissue of the cortex and the hippocampus of these mice post sleep deprivation will undergo Western Blot analysis to quantify changes in key proteins: Arc, mGluR5, and SHANK3. Expression of all three proteins is expected to increase due to their direct or indirect involvement in synaptic downscaling that is hypothesized to occur during sleep. *Shank3*$^{WT/\Delta C}$ mice are hypothesized to have display a more intense increase due to their vulnerability to acute stress.

Methods

Behavioral: Novel Object Recognition

Mice

Male and female C57BI/6J mice (N = 30) were bred in-house. At p23, they were weaned into cages with littermates and housed in Techniplast vivarium cages in a 12:12 light cycle. Prior to Novel Object Recognition training, each mouse was brought to the experiment room and handled for ten minutes per day for three days to acclimatize. All procedures were approved by UNC Institutional Animal Care and Use Committee (IACUC).

Novel Object Recognition

The experimental set-up was an arrangement of four Kydex-lined cubes on a table, with light sources above each chamber. The cubes were 20 cm by 20 cm in size, and they each had an identical piece of paper on one of the walls to act as visual cues for spatial orientation. AKASO EK7000 Pro cameras were set up above each chamber. The objects used were a glass saltshaker or a plastic canister, both around six inches in height and three inches in diameter. They were filled with marbles to prevent movement and/or tipping.

Training: Each chamber had two identical objects (shaker or canister, assigned at random), and placement of each was measured to be consistent across chambers and experiments. The mice were brought to the experimental room at ZT1. Each mouse was placed in a chamber individually and allowed to explore for ten minutes. Up to four mice were placed simultaneously, in different chambers, based on cohort size. The entire interactions were recorded. The chambers and the objects were wiped down with ethanol between use for different animals, as well as after the end of the experimental run.

Sleep Deprivation: After training, the mice were returned to their cages. The control animals (SD0, N = 11) were taken back to the vivarium. The rest of the mice were sleep-deprived for either two (SD2, N = 12) or four hours (SD4, N = 6) by gentle handling. This method entails tapping the cage or moving around the bedding to prevent sleep. The mice were returned to the vivarium afterward.

Testing: Testing took place the day after training, and the mice were brought to the experiment room at ZT2. The set-up and procedure were identical to that of training, except for the use of one shaker and one canister in each chamber. The mice were returned to the vivarium after the testing concluded.

Scoring

The author developed a scoring function using Oracle OpenJDK 17.0.1. It was used in the IntelliJ IDEA 2021.3.1 IDE by scorers to record beginning and end points (used to calculate interaction length), in milliseconds, of an interaction with each object, while viewing the video recording of the testing sessions. An interaction was defined ongoing as long as all the following condition were being met: 1) the mouse was facing the object, 2) the mouse was within one head-length of the object, and 3) the mouse was not on top of the object. The scorers were blind to mouse sleep condition and familiar/novel object status during the time of scoring. The function also recorded all beginning points of interactions, as well as the beginning and end time points of the scoring process/video. The results were exported in a CSV file using the openCSV package 5.7.1.

Analysis

Interaction times with each object (novel or familiar), discrimination index, and the ratio of interaction time with novel object vs familiar were analyzed in GraphPad Prism 9.5.1 (all measures are taken per mouse). The calculation of the discrimination index (DI) is presented below:

$$DI = \frac{time\ interacting\ with\ novel\ object}{time\ interacting\ with\ novel + familiar\ object}$$

Paired t-tests were used for total interaction time data to compare interaction with novel vs the familiar objects, paired by mouse. An ordinary one-way ANOVA was run to compare discrimination index of ZT4, SD2, and SD4 mice. Turkey's HSD tests were performed post-hoc, when applicable. Unpaired t-tests were used to compare ratios of interaction when canister vs shaker was the novel object. Prior to further analysis, outliers were identified (ROUT method at Q = 1.0%) and removed from analysis (N = 1).

Molecular: Protein Quantification via Western Blotting

Mice

Male and female C57BI/6J mice were crossed with Shank3$^{WT/\Delta C}$ heterozygous mice in-house to produce two litters. Shank3ΔC mice were generously donated by Dr. Paul Worley at Johns Hopkins University. At p23, they were weaned into cages with littermates and housed in Techniplast vivarium cages in a 12:12 light cycle. At postnatal day 28 (p28), one litter of mice was taken from the vivarium at Zeitgeber Time 0 (ZT0; lights on) for a four-hour sleep deprivation (SD4) by gentle handling. Both control (ZT4; N = 6) and sleep-deprived mice (SD4; N = 8) were sacked at ZT4. Their forebrains were frozen and ear samples were collected to be outsourced for automated genotyping by Transnetyx. Both litters had a mix of wild-type (WT; N = 2 (ZT4); N = 4 (SD4)) and Shank3$^{WT/\Delta C}$ heterozygous (HET; N = 4 (ZT4); N = 4 (SD4)) mice. All procedures were approved by UNC IACUC.

Sample Preparation

Each forebrain was put in chilled glass homogenizers with 5 ml of homogenization buffer (320mM sucrose, 10mM HEPES pH 7.4, 1mM EDTA, 5mM Na pyrophosphate, 1mM Na_3VO_4, 200nM okadaic acid, protease inhibitor (Roche)), and 12 strokes were applied to homogenize. The brain samples were then put in the centrifuge at 1000 x g for 10 minutes at 4°C. For each brain sample, 0.5 mL was used to prepare whole cortical lysate (WCL) and 1.5 mL for P2 fraction. For WCL samples, the supernatant (S1) was put in ¼ volume of 5x RIPA buffer (125 mM Tris base pH 7.6, 50mM NaPPi, 0.5% SDS, 5% NP40, 2.5% sodium deoxycholate). For the P2 samples, the nuclear pellet (P1) was discarded and the remaining S1 was centrifuged at 16,000 x g for 20 minutes at 4°C to isolate the 'crude' synaptosome (Wirths, 2017). The P2 pellet was reconstituted in 1x RIPA buffer. The Bradford assay was used to quantify proteins in each sample and adjust loading amounts for Western Blotting as appropriate.

Western Blotting

Tris glycine gels (10% with 5% stacking gel) were prepared and 2-3µL of samples were loaded in each well. The gels were run at 180V for 1 hour The proteins were transferred to nitrocellulose membranes and ran at 100V for 1 hour. The membranes were placed in blocking buffer (5% BSA, 0.02% Na_3N, PBS) on orbital shakers overnight at 4°C. After blocking, they underwent three 5-minute washes on an orbital shaker with TBST prior to addition of the primary antibody in probing buffer (3% BSA, 0.02% Na_3N, TBST). The primary antibody solution was kept overnight at 4°C. The membranes were then put in three 10-minute washes with TBST. They were placed in the secondary antibody solution for 1 hour. They were put in three 30-minute TBST washes prior to imaging with LI-COR Odyssey. The following primary antibodies were used: mGluR5 (1:5000, Abcam AB76316), Shank3 (1:1000, Cell Signaling Technology 64555),

Arc/Arg3.1 (1:1000, Synaptic Systems 156-111), HSC70 (1:1000, Millipore MABE1120). The secondary antibodies were IRDYE 680RD goat anti-mouse (1:10,000, LI-COR 926-68070) and IRDYE 800 CW goat anti-rabbit (1:10000, LI-COR 926-68070).

Analysis

The images were quantified using Image Studio Version 5.2.5 and were exported to GraphPad Prism 9.5.1. A two-way ordinary ANOVA was used to analyze differences of signal intensity between condition (ZT4, SD4) and genotype (WT, HET) for each protein. Uncorrected Fisher's Least Significant Difference tests were performed post-hoc, when applicable

Results

Novel Object Recognition

To look at differences between the three groups of gradual sleep deprivation (SD), the novel object recognition (NOR) results were initially compared for Interaction Time and Discrimination Index (DI) for all three cohorts. Interaction Time was a sum of total time in milliseconds that a mouse spent interacting with the novel or the familiar object throughout the entire duration of the testing session. Both ZT4 (no SD) and SD2 mice spent significantly more time interacting with the novel object rather than the familiar ($P = .0007$, $P < .0001$), whereas SD4 mice had no major difference in the amount of time they spent with the object (Fig. 1A). As there is variability to how much time each mouse spent interacting with any object, DI was also calculated and compared between the three groups of mice. DI measures the fraction of time spent interacting with the novel object, and in this analysis for all cohorts, mice in all three conditions performed similarly, with no significant differences between any groups (Fig. 1B). All means were above 0.50, meaning that all mice had some level of preference for the novel object,

regardless of condition (M = 0.634 +/- 0.085, M = 0.616 +/- 0.061, M = 0.602 +/- 0.137). Due to observations during the course of the initial two cohorts, the objects themselves were compared to check for inherent preferences. To do this, ratio of novel to familiar object interaction was compared for mice that were presented with the shaker vs the canister as the novel object (Fig. 1C). There was a statistically significant preference for the shaker (P = .0006).

As the preference for the shaker was identified after the first two cohorts and new shakers and canisters (identical to previous but never previously used in experiments) were used for the third, the data was split in two groups (Cohorts 1-2, Cohort 3) to account for the change. Both object preference and DI analysis for cohorts 1-2 were similar to that of the combined data presented in Figure 1. There was a strong preference for the shaker (P = .0012), and DI did not significantly differ between different conditions (Fig. 2 A-B). Conversely, cohort 3 did not demonstrate a significant preference for either object (Fig. 2C), and their DI showed a gradual decrease with increasing sleep deprivation (Fig. 2D), with ZT4 and SD4 showing a significant difference (F (2,8) = 7.266, P = .0159; Turkey's HSD P = .0127).

Protein Quantification

Western blotting was used to quantify and compare protein amounts in mice varying in both condition and genotype. HSC70 was intended to be used as a loading control to normalize signal across the samples, but the protein showed significant differences between WT and the two conditions of HET animals (F (1,10) = 15.25, P = .0029; Fisher's LSD P = .0007), as well as between both genotypes under the SD4 condition (F (1,10) = 5.085, P = .0478; Fisher's LSD P = .0033) . Therefore, HSC70 expression values were not used for normalization (Fig. S1A).

Arc protein was run in samples of both whole cortical lysate (WCL) and the P2 fraction. There was a significant increase of Arc following sleep deprivation in both the WCL ($F(1, 10) = 66.62$, $P < 0.0001$) and P2 fraction samples ($F(1, 10) = 155.1$, $P < 0.0001$) regardless of condition (WCL: Fisher's LSD $P = .001$ for WT, $P < .0001$ for HET; P2: Fisher's LSD $P < .0001$ for both) (Fig. 3B-C).

All remaining proteins were only quantified in the P2 fraction to maintain a focus on the synaptosome. SHANK3 was also found to have significant differences in two groups ($F(1, 9) = 18.3$, $P = .002$). One of the samples had to be excluded due to blot staining that interfered with quantification. The protein was found to be significantly highly expressed in HET SD4 mice, both when compared to mice in their condition (Fisher's LSD $P = .218$) and genotype (Fisher's LSD $P = .0013$). Notably, the difference in SHANK3 between WT and HET was not significant at ZT4 (Fig. 4B). Finally, mGluR5 was not found to have significant differences amongst any of the groups tested (Fig. 4C).

Discussion

Juvenile mice were able to learn following sleep deprivation under all conditions but may have been influenced by object bias.

The combined cohort data showed that both ZT4 and SD2 mice are successfully able to identify the novel object, with the SD4 group having more mixed results as interaction time varied but not the discrimination index (Fig. 1). The results deviate from the expectations of a gradually increasing differences in phenotypic effects of sleep deprivation as all groups of mice were able to successfully learn which object was novel in their environment. Though the discrimination in the SD2 group could be indicative of a level of tolerance to sleep deprivation in juveniles, the lack of significant difference between control and SD4 group is notable, because it

is inconsistent with prior findings, where juveniles were the only age group that was unable to discriminate between the objects after a four-hour sleep deprivation (Gay *et al.*, 2023). To investigate possible causes of the discrepancy, factors other than learning that could influence object preference were identified. Figure 1C shows that in instances that the shaker was the novel object, the ratio of interaction was significantly higher than when the canister was introduced. This is indicative of a preference for shaker, possibly due to its multiple holes and ridges that may make it more difficult to fully remove all previous scents from the object. As this observation was made prior to finalized analysis, cohort 3 mice were given unused objects to minimize problems associated with cleaning them between mice.

In cohort with no significant object preference, juvenile mice were not able to identify the novel object after a four-hour sleep deprivation.

Mice from cohort 3 showed predicted behavior: discrimination index decreased as sleep deprivation length increased (Fig. 2D). The control mice (ZT4) and SD4 significantly varied, which is consistent both with prior experiments and expectations based on literature. As adult mice typically are not hindered in this task after only a four-hour sleep deprivation, the significant difference in cohort 3 could be indicative of the increased vulnerability of the younger mice. Though there was a trend to a decrease of the discrimination index for cohort 3 (Fig, 2D), a two-hour sleep deprivation may not be sufficient to show behavioral differences. This demonstrates that even younger mice have a degree of tolerance to sleep deprivation, and two hours is not long enough for it to impair memory consolidation. Due to the object preference interference in the first two cohorts, the experiment should be repeated with modified objects to further explore the gradual trend of discrimination index decrease that was observed in cohort 3.

Arc is induced following sleep deprivation in juvenile mice.

In both WT and *Shank3*$^{WT/\Delta C}$ mice, Arc expression was significantly elevated after a four-hour sleep deprivation (Fig. 3). This difference could be seen in the whole cortical lysate, but also when looking only at the P2 fraction to focus on the synapses. This finding is consistent with previous studies that have quantified Arc expression (Suzuki, Yanagisawa, & Greene, 2020), and a stark increase was thus hypothesized. Within the context of the homeostatic sleep hypothesis, Arc is explored as to have a crucial role in selectively regulating downscaling of the synapses during sleep (Suzuki, Yanagisawa, & Greene, 2020; Diering, 2022). Though precise mechanisms of Arc are not explored in this study, its accumulation, especially specifically in the P2 fraction (Fig. 3C), could support that the protein is needed during sleep to perform unique synaptic functions from wake. It is of note that previous research is primarily focused on adult animals, while this study looks at juvenile mice that are still in the developmental window where sleep habits and mechanisms change. Evidence of a similar response at a younger age point could be indicative that certain sleep mechanisms are already development or relatively stable by p21. Alternatively, it could also indicate far-reaching impacts of Arc as it has numerous functions across neurons.

SHANK3 differences are revealed post sleep deprivation.

Even in animals that vary in genotype and are heterozygous for *Shank3*, there was not a significant difference in protein expression until the sleep deprivation was applied. Additionally, HET mice had a significant increase in SHANK3 expression post-SD, while the WT mice did not (Fig. 4B). As hypothesized, the results underscore the vulnerability of HET animals. The results also mirror behavioral results that have been observed in *Shank3*$^{WT/\Delta C}$ mice following acute stress: even with the absence of a varying phenotype without interference, after undergoing

stress, HET animals demonstrate different behavior from WT animals (Lin *et al.*, 2021). Both suggest that there may be a similar 'baseline', but even short-term stress is sufficient to unmask differences between the genotypes. A limitation of SHANK3 samples, in particular, is the low blotting signal, which could be comparable to background. For further analysis, more blotting or use of a different antibody should be considered. An additional consideration would be quantification within a more-enriched PSD fraction sample rather than P2 for stronger signal acquisition.

mGluR5 did not have significant condition or genotype effects.

Unlike the other proteins in the study, mGluR5 remained relatively consistent among all four groups of mice (Fig. 4C). This finding is contrary to the hypothesis, which, similarly to Arc, expected an increase in mGluR5 expression following a sleep deprivation as Arc translation can go through mGluR5 (Wilkerson *et al.*, 2014). There was also literature that identified a cerebral increase in mGluR5 after sleep deprivation in humans (Holst *et al.*, 2017), but the discrepancies could also be due to the large variation of the study paradigm and techniques. This protein is also an instance where lack of normalization could be strongly affecting quantification analysis. Though HSC70 was not successful as a loading control, when it was used for normalization, mGluR5 showed a significant increase in HET SD4 mice (Fig. S1B). This observation is not appropriate or sufficient to draw conclusions, but it could provide basis for further study of the protein, especially with proper loading controls.

Limitations and Future Directions

Both behaviorally and on a molecular level, there is evidence that sleep deprivation affects males and females differently (Lord *et al.*, 2022; Shi *et al*., 2024). Though the current study included mice of both sexes, in approximately an even ratio, sex was not considered as a

variable for project analysis in either the behavioral or the molecular experiments. Due to the time constraints that prevented expansion of the number of animals, there was not sufficient statistical power for consistent analysis of sex across all groups. In future experiments, it is imperative to treat sex as a statistical variable to explore differences that arise, especially at an earlier developmental point, to better understand when and how the two may diverge.

As mentioned previously, there are procedural modification that should be considered, such as use of new objects for NOR and a new loading control for normalization of protein quantification for further exploration of trends identified in this study. In addition, I suggest expansion of the experiments to bridge the behavioral and molecular components of the study. This could include quantification of proteins of SD2 mice to find out time-dependence of their accumulation and inclusion of HET mice in NOR to explore whether their increased vulnerability translates to behavior with a shorter sleep deprivation than WT mice.

Conclusion

The novel object recognition task showed that juvenile WT mice are able to discriminate between a novel and a familiar object in control conditions and following a two-hour SD, but this ability is blunted after a longer, four-hour SD. Further study is needed utilizing objects that are not inherently preffered by the mice. Both WT and *Shank3*$^{WT/\Delta C}$ mice showed a significant accumulation of Arc following a four-hour SD. The heterozygous mice did not display a significant increase of SHANK3 at baseline, but displayed differences after undergoing SD. Though mGluR5 did not vary in its expression, experiments are suggested with proper loading controls to quantify all proteins more accurately. The responses to SD demonstrate the pertinence of the study focusing both on juvenile and autism model mice, as both groups show a distinct and more vulnerable response to sleep deprivation. Understanding mechanisms that may make these

populations more sensitive is crucial in exploration of treatment. Especially for conditions that are life-long, problems can begin early and have lasting effects. Identification of critical developmental points can mitigate these effects into adulthood and lead to better outcomes.

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Figures

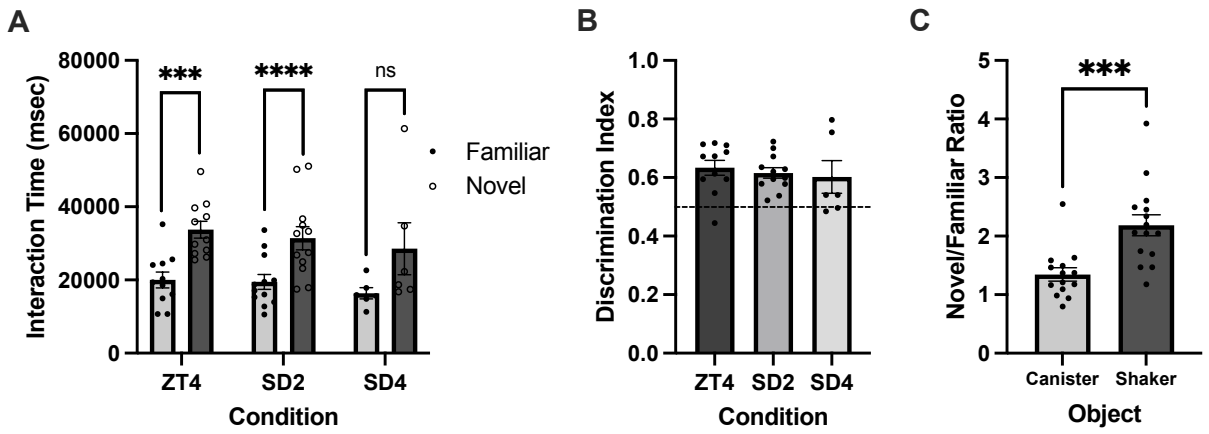


Figure 1. Combined Interaction Time, Discrimination Index and Object Preference. NOR data from all three cohorts were used for calculations and visualization. (A) Total interaction time with each object in milliseconds, grouped by sleep condition. (B) Discrimination Index grouped by sleep condition. Dashed line at DI = 0.50 to clearly display preference. (C) Ratio of time spent with novel vs familiar object while canister or shaker was the novel object. Data from all sleep conditions. ***P ≤ .001, ****P ≤ .0001, ns: not significant. Individual values plotted, showing mean and SEM with error bars.

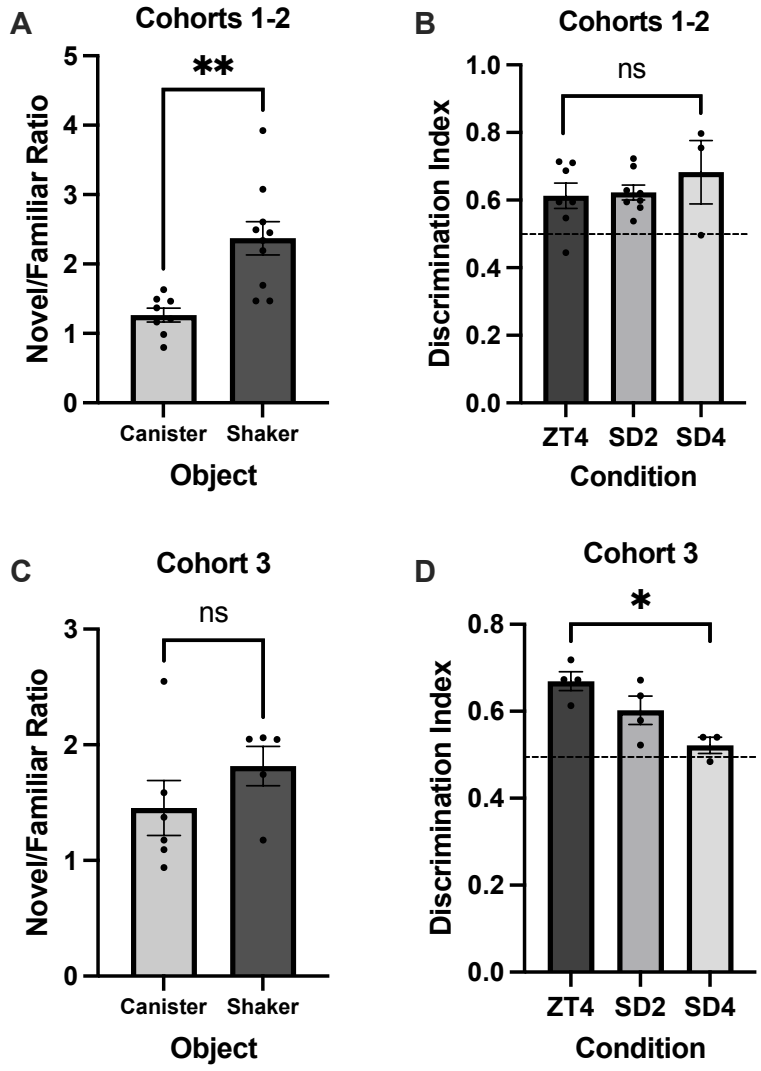


Figure 2. Object Preference May Affect Post-SD Discrimination. NOR data split to only include cohorts 1-2 or cohort 3. **(A)** Ratio of time spent with novel vs familiar object while canister or shaker was the novel object. Data from all sleep conditions in cohorts 1 and 2. (B) Discrimination Index grouped by sleep condition in cohorts 1 and 2. Dashed line at DI = 0.50 to clearly display preference. (C) and (D) represent the same measures as (A) and (B), respectively, but with cohort 3 mice only. **P ≤ .01, *P ≤ .05, ns: not significant. Individual values plotted, showing mean and SEM with error bars.

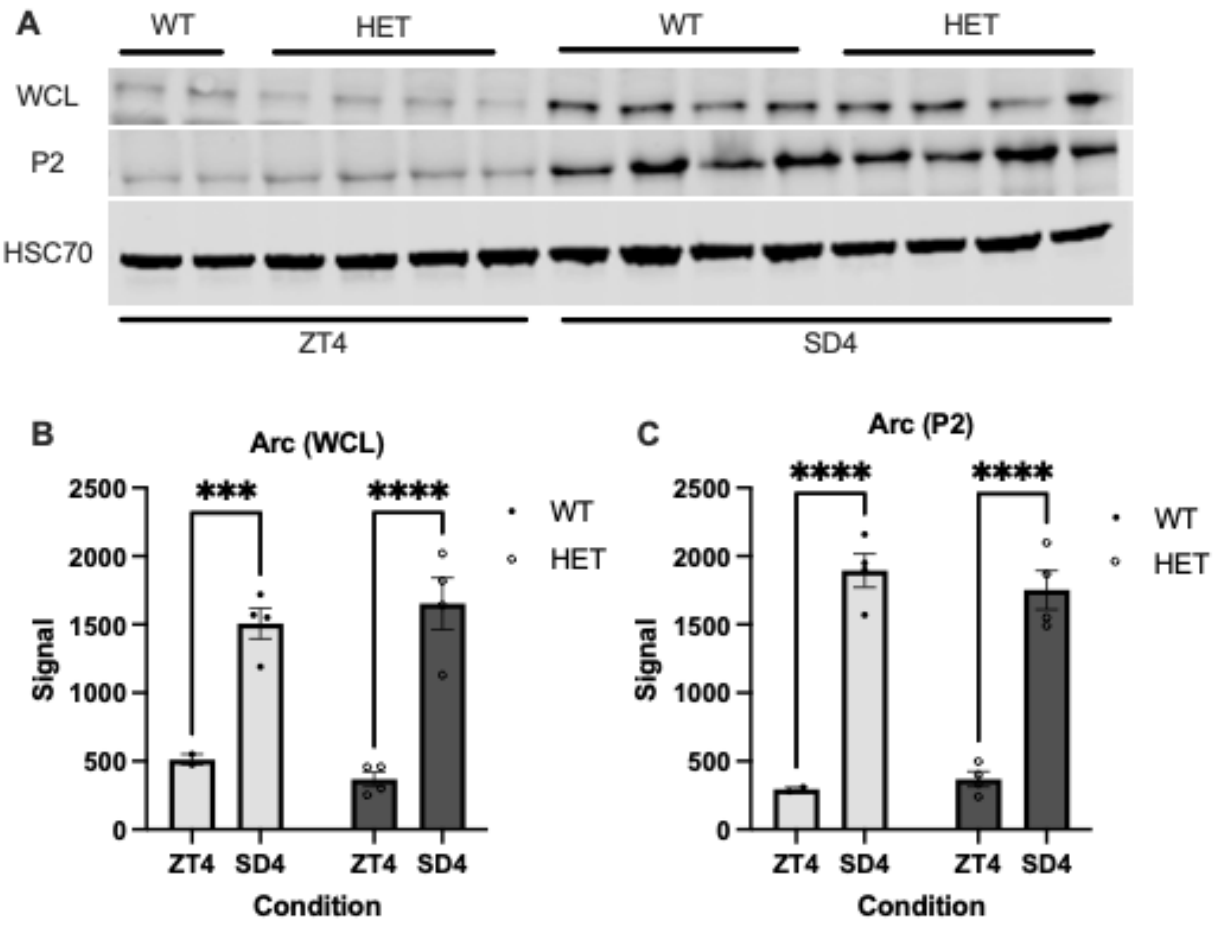


Figure 3. Arc Protein Levels Increase Following SD. WB quantification of Arc in WT and HET mice at ZT4 or following SD4. (A) Representative blot used for WB quantification and analysis of the Arc protein in WCL and P2. HSC70 presented for comparison. (B) and (C) show protein quantification and visualization of Arc in WCL and P2 fraction, respectively. The values are not normalized. ***P ≤ .001, ****P ≤ .0001. Individual values plotted, showing mean and SEM with error bars.

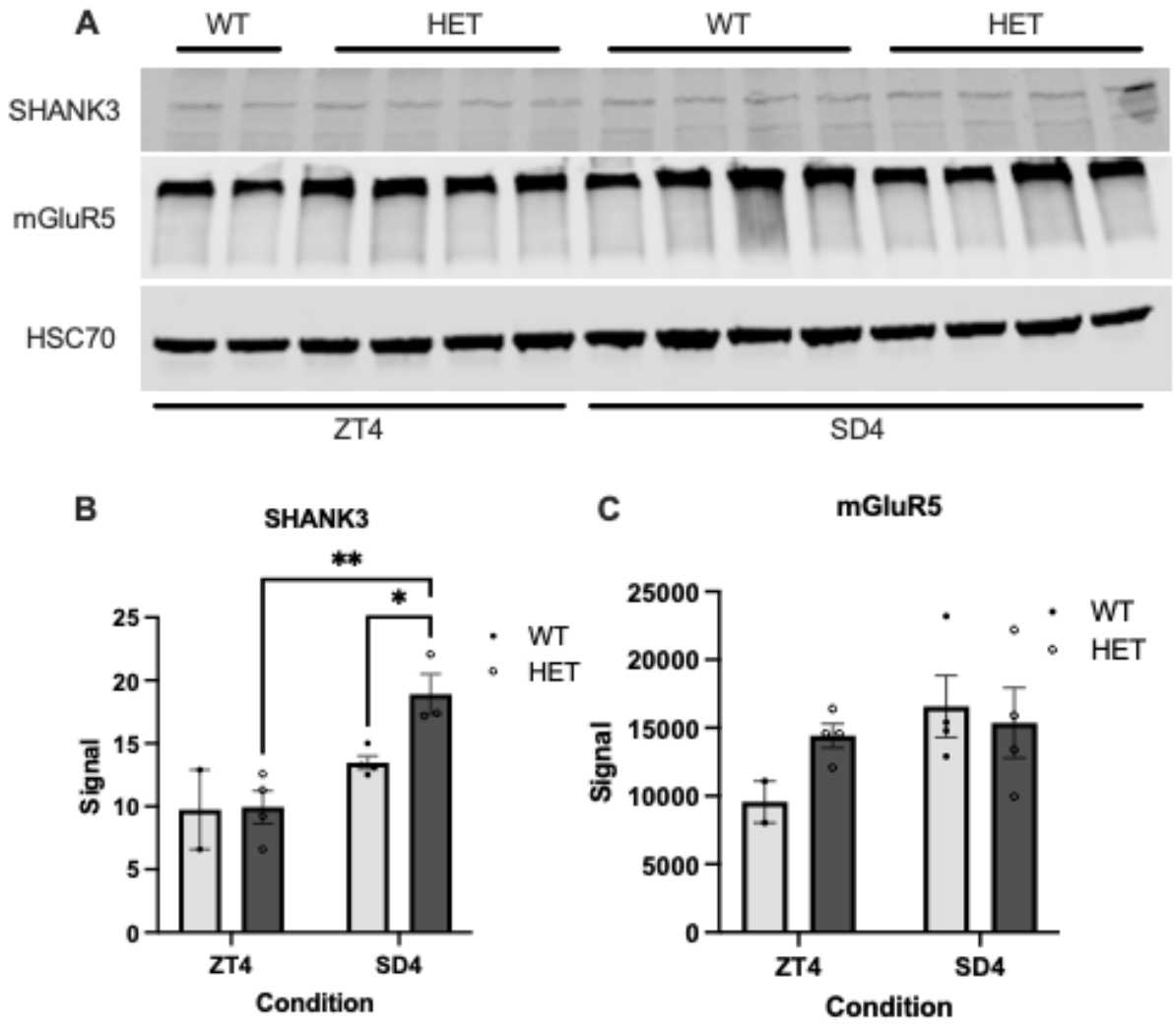
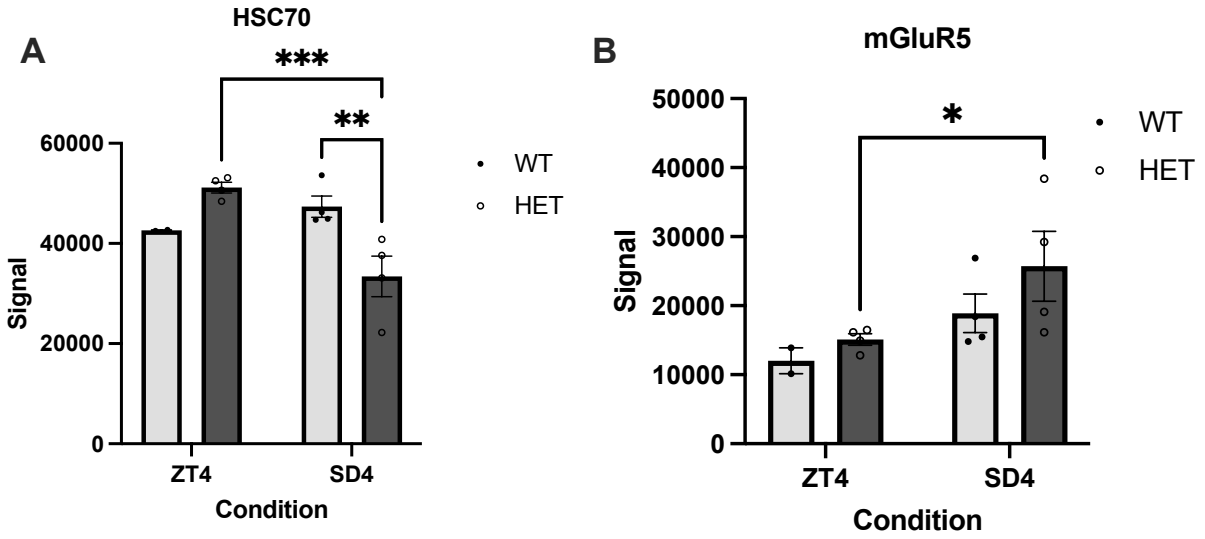


Figure 4. SHANK3 Shows Variation Post-SD, and mGluR5 Remains Consistent Across Groups. WB quantification of SHANK3 and mGluR5, both in P2 fraction. (A) Representative blot used for WB quantification and analysis of the SHANK3 and mGluR5 proteins. HSC70 presented for comparison. (B) and (C) show protein quantification and visualization of SHANK3 and mGluR5, respectively. The values are not normalized. **P ≤ .01, *P ≤ .05. Individual values plotted, showing mean and SEM with error bars.



Supplemental Figure S1. HSC70 shows a significant decrease in HET SD4 mice. WB quantification of HSC70 and mGluR5, both in P2 fraction. (A) Protein quantification and visualization of HSC70 to test whether it can serve as a loading control. (B) Protein quantification and visualization of mGluR5 with normalization applied from HSC70. ***P ≤ .001, **P ≤ .01, *P ≤ .05. Individual values plotted, showing mean and SEM with error bars.

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