Effects of sleep deprivation and aging on long-term and remote memory in mice

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Sleep deprivation (SD) following hippocampus-dependent learning in young mice impairs memory when tested the following day. Here, we examined the effects of SD on remote memory in both young and aged mice. In young mice, we found that memory is still impaired 1 mo after training. SD also impaired memory in aged mice 1 d after training, but, by a month after training, sleep-deprived and control aged animals performed similarly, primarily due to remote memory decay in the control aged animals. Gene expression analysis supported the finding that SD has similar effects on the hippocampus in young and aged mice.

Two major concerns in developed countries are the aging population and the prevalence of sleep deprivation (SD) (Luyster et al. 2012). Both aging and SD have clear negative impacts on cognitive function, including specific forms of memory. In particular, aging is associated with deficits in hippocampus-dependent episodic memories with associative, contextual, or spatial components (Moscovitch et al. 1986; Spencer and Raz 1995; Eichenbaum 1999; Old and Naveh-Benjamin 2008). Rodent studies show that hippocampus-dependent memory formation and synaptic plasticity are impaired by both SD (Graves et al. 2003; Guan et al. 2004; Ruskin et al. 2004; Vecsey et al. 2009) and aging (Barnes and McNaughton 1985; Tanila et al. 1997; Burke and Barnes 2006; Robitsek et al. 2008; Foster et al. 2012). Further, aging causes abnormalities in the amount, quality, and timing of sleep (Bonnet and Arand 1989; Stone 1989; Wimmer et al. 2013; Pace-Schott and Spencer 2014), particularly nonrapid eye movement (NREM) sleep (Cajochen et al. 2006). Thus, it is possible that aging induces a form of SD that could mediate some of the effects of aging on memory (Hornung et al. 2005). A goal of this study was therefore to assess how aging and SD interact to affect memory.

Studies of post-training SD have shown clear memory impairments in hippocampus-dependent tasks such as contextual fear conditioning (CFC), but memory is typically not abolished completely (Graves et al. 2003; Ruskin et al. 2004; Vecsey et al. 2009). Therefore, we were interested to determine what happens to the remaining memory after longer post-training intervals. Memory has several stages (Abel and Lattal 2001), including acquisition, consolidation, retrieval, reconsolidation, and systems consolidation, in which memory is transformed such that its storage and recall become dependent on different brain systems (Winocur et al. 2010). Systems consolidation may involve transfer of the memory from one brain area to another for storage (Frankland and Bontempi 2005), or at least reorganization of the memory traces such that recall involves a new set of brain regions (Winocur et al. 2010; Sutherland and Lehmann 2011). In the case of CFC, the hippocampus appears to be required for all of the stages of memory occurring during the first few weeks following learning. When memory is assessed at more "remote" time points, the hippocampus is less strongly required for retrieval, indicating that systems consolidation has taken place (Scoville and Milner 1957; Kim and Fanselow 1992; Anagnostaras et al. 1999; Bontempi et al.

1999). However, recent evidence indicates that the hippocampus is likely to continue to play a role in remote memory storage and recall (Moscovitch et al. 2005; Goshen et al. 2011). Using CFC, we studied the effects of SD and aging on recent and remote memory to determine how these two factors interacted. We used CFC for a number of reasons. First, because SD had been shown to cause deficits in the memory consolidation for this task, as described above. Second, because the process of systems consolidation was best characterized for this task. And third, because this learning task has the advantage of inducing very long-lasting memory with a single training trial, it allowed us to carry out SD following training and to assess memory at different intervals following learning. This meant that we could target the memory consolidation stage with SD while leaving acquisition and systems consolidation unaffected. Last, we compared the molecular effects of aging and SD by assessing gene expression in the hippocampus.

Young adult (2-mo old) and aged (22- to 23-mo old) male C57BL/6 mice from the National Institute of Aging (NIA) were used for behavioral experiments testing memory and for gene expression analysis. Mice were individually housed in a temperature-controlled environment, which was on a 12 h/12 h light/dark schedule, and had ad libitum access to food and water. All experiments were conducted according to National Institutes of Health Guidelines for Animal Care and Use and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

All behavioral experiments were carried out during the first half of the light period, as previously described (Vecsey et al. 2009). Mice were trained in one of four chambers (Med Associates), two of which had circular floors and two of which had square floors. The chambers had walls made of clear Plexiglas and shock grid floors. Blue and white striped paper was placed behind two of the four walls of the square chambers and around half of the circular chambers. There were also X's of varying size made with red tape on the same walls covered with paper (two per wall for square chambers, three for circular chamber). The chambers were located in a windowless, dimly lit room. Each

mouse was handled for 2–3 min daily for 5–6 d prior to training, at approximately the same time that they were trained. On the training day, each mouse was carried to the testing room in its cage and was placed in one of the four chambers for 2 min and 28 sec. For the next 2 sec, a 1.5-mA foot shock was given. The mice remained in the chambers for another 30 sec following the shock and were then returned to their home cages. Four mice were trained simultaneously using the four chambers. Between each training or testing set, the chambers were cleaned with 70% ethanol.

Half of the mice were tested after 24 h (Recent test). They were placed in the same chambers they were trained in for 5 min. The fear response was measured at intervals of 5 sec by recording levels of freezing, which is the absence of all movement except for respiration (Blanchard and Blanchard 1969; Fanselow 1980). The same mice were retested in an altered context 24 h after testing. For the altered chamber, smooth, black panels covered the floor, laminated cardboard served as the top, and a metal sheet was placed in the chamber to divide it in half (diagonally for the square shape). Mice were placed in a chamber with the opposite shape than the one in which they were trained and initially tested. The chambers were cleaned with a 15% Lemon Joy (Procter & Gamble)/water mixture. The other half of the mice were tested in the trained context 30 d after training (Remote test), in the same manner as the mice tested after 24 h, followed by altered context testing the following day.

In behavioral experiments, SD began immediately following behavioral training. In gene expression analysis experiments, SD in handled but untrained animals began 2 h after lights-on. Sleep-deprived mice were kept awake for 5 h by gentle handling (Vecsey et al. 2013), which consisted of tapping of the cage, lifting of the cage lids, and uncovering of the cages for short periods of time. The young and aged nonsleep-deprived mice were left undisturbed in their home cages and allowed to rest. Although quantitative measurements were not made, no qualitative differences were observed in the degree of manipulation required to keep young and old mice awake.

Freezing levels were scored by an observer blind to condition, by tallying the number of times the mice were freezing, at 5-sec intervals, during the 5-min tests. The percent freezing was calculated by dividing the freezing levels by the number of 5-sec intervals during testing (Abel et al. 1997). Freezing levels and percent freezing were calculated for both sets of mice for the original and altered context. A "delta" score was also calculated by subtracting the percent freezing in the altered context from the percent freezing in the original context for each animal (Paylor et al. 1994). This score reflects freezing that is specific to the original context (Frankland et al. 1998). For behavioral experiments, young mice were trained and tested in separate experimental cohorts from aged mice. We performed two-way ANOVAs with treatment (FC alone versus FCSD) and testing time (Recent versus Remote) as factors, run separately for each age. We also performed two-way ANOVAs with age (Young versus Old) and treatment (FC alone versus FCSD) as factors, run separately for each of the two test times. All behavioral data ANOVAs showing a significant effect were followed by post hoc Tukey tests, and were carried out using JMP8 and JMP10 (SAS Institute).

For gene expression analysis, RNA preparation, cDNA synthesis, and qPCR analysis was performed as previously described (Vecsey et al. 2012). Quantitative real-time RT-PCR (qPCR) reactions were carried out in the ABI Prism 7000 with an initial activation at 50°C for 2 min followed by incubation at 95°C for 15 min and 40 subsequent cycles of 95°C for 15 sec, 56°C for 30 sec, and 72°C for 30 sec. Primer sequences, data analysis, and relative quantification of gene expression have been previously described (Vecsey et al. 2012). Hippocampal samples from young and aged

mice were tested simultaneously. A two-way ANOVA was performed to compare fold change values for each gene, with age (young versus aged) and treatment (sleep-deprived versus nonsleep-deprived), followed by post hoc Tukey tests (Statistica 7, Statsoft, Inc.).

Sleep deprivation following contextual fear conditioning impairs memory when tested 1 or 30 d after training in young mice

In young mice, SD for 5 h immediately following CFC impaired memory during both the Recent and Remote tests, as seen in Figure 1B for freezing in the trained context ($F_{(1,79)} = 15.8$, $P = 0.0002$) and in 1D for memory specificity (delta score) ($F_{(1,79)} = 11.8$, $P = 0.0009$). In contrast, freezing in the altered context (indicative of fear memory that is not specific for the trained context) was not affected by SD ($F_{(1,79)} = 0.45$, $P = 0.51$), but instead showed a significant overall decrement with time after training ($F_{(1,79)} = 22.43$, $P = <0.0001$), with no interaction between SD and test time ($F_{(1,79)} = 1,31$ $P = 0.26$). There was no interaction between the effects of SD and test time on either freezing in the trained context ($F_{(1,79)} = 0.12$, $P = 0.74$) or on the delta score ($F_{(1,79)} = 0.49$, $P = 0.49$), indicating that SD caused comparable deficits at either time of testing. The deficits in 30-d remote memory support the idea that SD after learning impairs memory consolidation and does not simply disrupt recall during the test the following day.

This experiment also demonstrated that memory in the trained context tends to improve over time in young mice ($F_{(1,79)} = 4.72$, $P = 0.033$). This, combined with the decrease in freezing in the altered context, led to a pronounced improvement in memory specificity, as evident in the delta score in Figure 1D ($F_{(1,79)} = 42.7$, $P < 0.0001$). Although memory was highly unspecific in young fear-conditioned and sleep-deprived (FCSD) animals during the Recent memory tests, this did improve over time, suggesting that systems consolidation in the absence of continuing SD is able to improve the quality and accuracy of memory (compare darker gray bars in Fig. 1B). This effect has been observed previously, and has been termed memory "incubation" (Oler and Markus 1998; Houston et al. 1999; Pickens et al. 2009), Despite this improvement, FCSD animals still showed a memory deficit compared with the FC alone group, suggesting that the long-term quality of the memory was still dependent on the initial consolidation event, which had been impaired by the SD occurring immediately post-training.

Sleep deprivation in aged animals causes memory impairments at 1 d, but not at 30 d

When the same experiment was carried out in aged mice, SD significantly impaired memory during the Recent test. This was true both for freezing in the trained context alone, and most strikingly in the delta score, where memory specificity was essentially null in sleep-deprived mice of both ages. However, in contrast to what was observed in young mice, SD did not impact freezing levels during the Remote test in aged mice. As in young mice, freezing in the altered context decreased significantly with testing time ($F_{(1,83)} = 11.64$, $P = 0.001$), but showed no significant effect of SD ($F_{(1,83)} = 0.34$, $P = 0.56$), and no interaction between SD and testing time ($F_{(1,83)} = 0.21$, $P = 0.65$). For the delta score, there was a significant overall effect of SD ($F_{(1,83)} = 7.0$, $P = 0.01$). There was also a trend toward an interaction between SD and testing time ($F_{(1,83)} = 3.8$, $P = 0.05$), with a stronger effect of SD at the
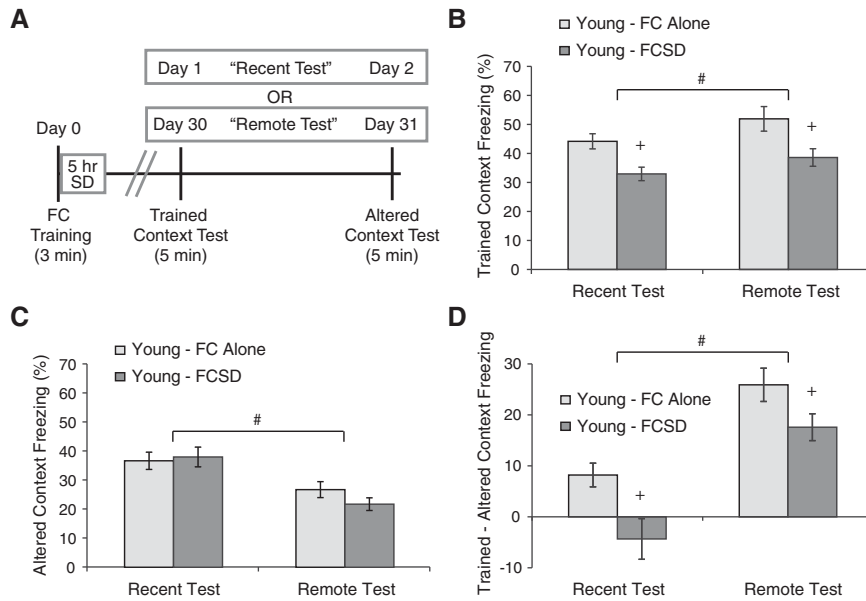
Figure 1. Sleep deprivation after training in young mice impairs memory tested 1 or 30 d after training. (*A*) A timeline of experimental procedures used for both young and old mice. C57BL/6 NIA mice (either 2-mo old (young) or 22- to 23-mo old (aged)) received single-trial contextual fear conditioning and were immediately either sleep-deprived for 5 h by gentle handling (FCSD) or left undisturbed in their home cages (FC Alone). Half of the mice from each group were then tested for retention, as assessed by percent time spent freezing, in the same environment (trained context) 1 d later, followed by testing in a different environment (altered context) the following day (Recent tests). The other half of the mice were tested in the trained context 30 d following training, again followed by testing in the altered context the next day (Remote tests). The specificity of memory was calculated by subtracting the amount of freezing in the altered context from the freezing in the trained context. (*B*) In young mice, SD significantly impaired memory for the trained context during the Recent and Remote tests. (*C*) SD did not have a significant effect on freezing in the altered context, which instead lessened over time in both the FC Alone and FCSD groups. (*D*) SD impaired the specificity of memory for the trained context during the Recent and Remote tests. Notably, young mice showed an improvement in the specificity of memory from the Recent to the Remote test. Data are graphed as the mean ± SEM. (#) A significant overall effect of testing time, and (+) a significant overall effect of SD ($P \leq 0.05$ was considered significant). Number of animals: FC Alone Recent = 20, FCSD Recent = 20, FC Alone Remote = 21, FCSD Remote = 22.

the Recent tests (Fig. 2A,C), there was ample room for improvement by the time of the Remote tests.
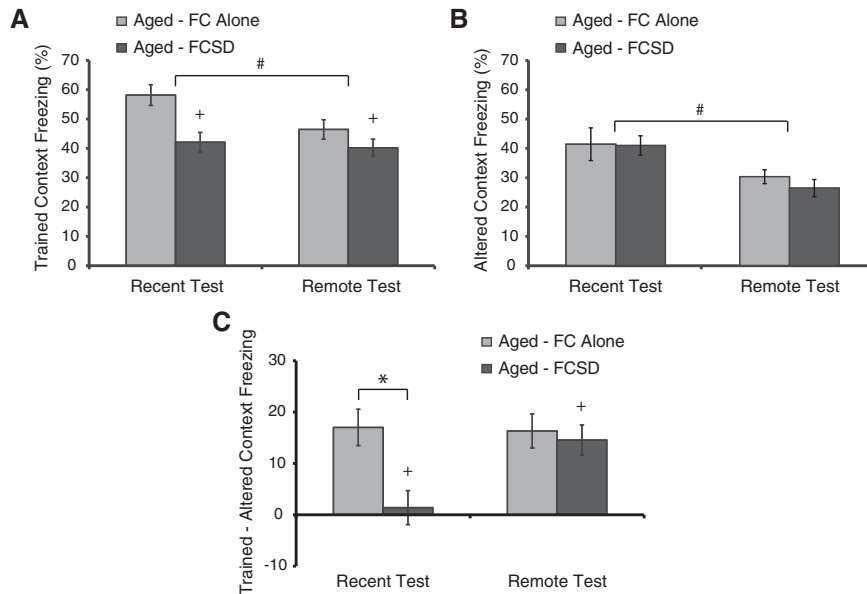
Sleep deprivation has equivalent effects on hippocampal gene expression in young and aged mice

We had previously shown that SD in young mice causes widespread changes in hippocampal gene expression (Vecsey et al. 2012). Therefore, in this study we examined whether aging altered the effects of SD on the expression of a subset of those previously identified genes, chosen to represent many of the functional clusters of SD target genes (Vecsey et al. 2012). Across young and aged mice, SD significantly increased the expression levels of *Prkab2* (energy metabolism and regulation of sleep Carling 2005; Chikahisa et al. 2009; Dworak et al. 2010), *Arc* (synaptic plasticity Tzingounis and Nicoll 2006), *Tsc22d3, Hspa5, and Hspb1,* (stress response Gething 1999; Gusev et al. 2002; Naidoo et al. 2005, 2007; Ayroldi et al. 2007) (Fig. 3A, two-way ANOVA for each, $P < 0.05$). SD also significantly decreased expression levels of *Rbm3* and *Hnrpdl* (mRNA metabolism and trafficking (Kawamura et al. 2002; Smart et al. 2007)) and *Usp2* (deubiquitination and control of circadian rhythms (Metzig et al. 2011; Scoma et al. 2011; Yang et al. 2012)) (Fig. 3B; two-way ANOVA for each, $P < 0.05$).

In contrast to the widespread effects of SD, the only significant effect of aging was a slight increase in the expression of *Rbm3* and *Usp2* (two-way ANOVA, $P < 0.05$). For both of these genes, SD caused a similar decrease in their expression, even with increased baseline expression in aged animals. An interaction between aging and SD was found only for *Hspb1* (two-way ANOVA, $P = 0.007$). Thus, across the eight genes tested, the effects of SD on gene expression were similar in both young and aged mice, and age itself had only minor effects. For example, *Hspb1*, *Rbm3*, and *Usp2* had small, but significant, increases in hippocampal expression due to aging, but all experienced similar responses to SD. Combined with the behavioral findings in Figures 1, 2 showing that SD impaired memory at the 1-d test in both young and aged animals, these findings support the conclusion that SD has a similar impact on hippocampal function in young and aged animals. It is likely that the SD-induced impairment in remote memory in aged animals is mediated by a different set of genes and/or by a different brain area other than the hippocampus.

One of the brain areas thought to be key for systems memory consolidation is the anterior cingulate cortex (ACC) (Frankland et al. 2004, 2006; Teixeira et al. 2006; Goshen et al. 2011). It is possible that aging negatively affects plasticity mechanisms within this area, or limits the ability of the hippocampus to pass information to the ACC. One study (Miller et al. 2010) found that expression of *calcineurin*, a negative regulator of plasticity and memory

Recent test than at the Remote test. Post hoc tests found a significant deficit in memory specificity due to SD during the 1-d test, but no deficit at the Remote test.

Unlike young mice, in which overall freezing and specificity for the trained context increased over time following learning ($F_{(1,79)} = 4.7, P = 0.03$), aged mice did not show an improvement over time. In fact, freezing in the trained context showed a trend toward a decline in aged mice from 1 to 30 d ($F_{(1,83)} = 3.9, P = 0.05$), reflective of an age-dependent impairment in "systems" memory consolidation (compare light gray bars in Fig. 2A). This is reminiscent of previously published reports of age-related deficits in memory tests performed at remote time points, but not soon after training (Moscovitch et al. 1986; Winocur 1988; Oler and Markus 1998; Houston et al. 1999). Interestingly, in aged mice, the specificity of memory shows a significant improvement over time in the FCSD group, but not in the FC Alone group (Fig. 2B). It should be noted that the aged, FC Alone mice showed evidence for a strong memory that was highly specific for the trained context during the 1-d tests (Fig. 2A,C). The lack of a further enhancement in memory specificity in the aged mice may indicate a lower saturation point in aged animals than in young animals, such that no further improvement in the specificity of memory was possible in the FC Alone group. In contrast, in the FCSD group, because SD initially impaired memory specificity during

A

B

C

Figure 2. Sleep deprivation after training in aged mice impairs memory at 1 d, but not 30 d, after training. Training, SD, and testing procedures for aged (22- to 23-mo old) C57BL/6 NIA mice were the same as shown in Figure 1A. (*A*) In aged mice, SD significantly reduced freezing in the trained context during the 1-d test, but had no significant effect on freezing during the Remote test. (*B*) SD did not have a significant effect on freezing in the altered context, which lessened over time in both the FC Alone and FCSD groups, similar to what was observed in young mice. (*C*) SD impaired the specificity of memory for the trained context during the Recent memory test, but had no effect during the Remote test. Aged FCSD mice showed a significant improvement in the specificity of memory over time, whereas aged FC Alone mice did not. Data are graphed as the mean ± SEM. (#) A significant overall effect of testing time, (+) a significant overall effect of SD, and (*) a significant post hoc comparison following a significant interaction between SD and testing time ($P \leq 0.05$ was considered significant). Number of animals: FC Alone Recent = 20, FCSD Recent = 19, FC Alone Remote = 23, FCSD Remote = 24.

(Mansuy 2003), was reduced in the dorsomedial prefrontal cortex (a region that includes the ACC) 30 d after CFC training, an effect that was correlated with hypermethylation of the gene locus. There are discrete time windows following learning when gene expression and protein synthesis events occur that mediate the initial phase of memory consolidation (Bourtchouladze et al. 1998; Igaz et al. 2002), but this is not the case for systems memory consolidation, which seems to happen piecemeal over the course of weeks. Screens for mutants with selective deficits in systems memory consolidation will be necessary to identify the genes involved in this process. It will also be important to identify the subset of these genes that may be affected by aging. In fact, no broad assessment of aging on ACC gene expression has been performed. Thus, it would be of interest to perform an experiment in which FC is performed in young and aged mice, and gene expression is broadly analyzed 30 d following training in the ACC versus hippocampus.

Studies in humans have observed several sleep disturbances with healthy aging, including reduced slow wave and REM sleep density as well as alterations in circadian rhythms (Pace-Schott and Spencer 2014). This is very similar to

the effects of age on sleep in rodents (Welsh et al. 1986; Stone 1989; Hasan et al. 2012; Wimmer et al. 2013). It has been suggested that these changes in sleep contribute to age-related cognitive decline, including deficits in the consolidation of episodic, hippocampus-dependent memories (Pace-Schott and Spencer 2014).

The data presented here show that aged mice do not have impaired acquisition or initial consolidation of long-term contextual fear memory, demonstrating robust memory when tested a day after training. This suggests that ongoing age-related sleep abnormalities do not significantly impair memory consolidation in this task. In contrast, young and old mice both showed clear deficits in memory due to acute post-training SD. Although old mice tend to have reductions in sleep compared with young mice, they still obtain substantial amounts of sleep, whereas gentle handling SD has been shown to be highly effective at eliminating all sleep during the SD period (Hasan et al. 2012). Consistent with this view, a study in humans found that episodic memory in both young and old subjects was better preserved across a period of sleep than a period of wakefulness, demonstrating that the sleep older people obtain is still beneficial for memory (Aly and Moscovitch 2010). One might have expected that young mice, presumably achieving greater amounts of more consolidated sleep than aged mice, would have demonstrated better initial memory consolidation, but they did not, either in terms of total freezing or the specificity of memory for the trained context. A possible explanation is that there may be a threshold quality of sleep that is satisfactory for normal initial

A

B
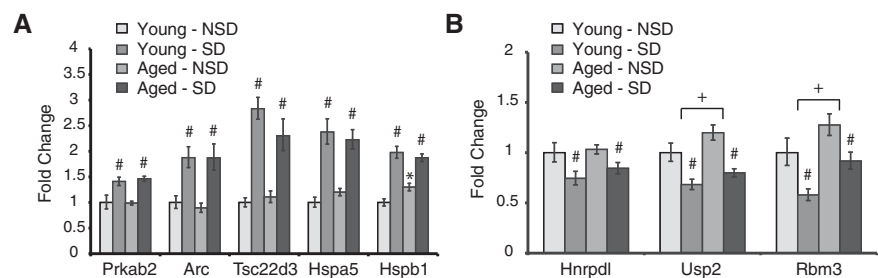
Figure 3. Sleep deprivation causes similar changes in hippocampal gene expression in young and aged mice. Young (2 mo) and aged (22–23 mo) C57BL/6 NIA mice were sleep-deprived for 5 h by gentle handling. Immediately afterward, hippocampal tissue was dissected and analyzed by quantitative real-time RT-PCR for the expression of several genes that had previously been shown to be up- or down-regulated by SD in the hippocampus of young mice (Vecsey et al. 2012). (*A*) Up-regulated genes. There was an overall effect of SD across both age groups on each gene. No significant effects of age were observed. The exception was Hspb1, for which there was a significant interaction between age and SD, indicating that SD caused a smaller up-regulation in aged animals than in young animals. (*B*) Down-regulated genes. There was an overall effect of SD across both age groups in each gene. For *Usp2* and *Rbm3*, aged animals had significantly higher levels of gene expression, although there was no interaction between SD and aging. For a given gene, (#) a significant overall effect of SD relative to NSD, (+) a significant overall effect of age, and (*) a significant post hoc comparison following a significant interaction between SD and age ($P \leq 0.05$ was considered significant). Data are graphed as the mean ± SEM, and $n = 8$ for each group.

consolidation, but which is not enough to support gradual systems consolidation.

Not only did aged mice not show a deficit in memory during the Recent memory test, they showed significantly better memory specificity than young mice at the Recent test ($F_{(1,76)} = 4.04$, $P = 0.048$), with no significant interaction between age and whether the mice were sleep-deprived ($F_{(1,76)} = 0.18$, $P = 0.67$). In contrast, the trend had completely reversed by the Remote test, with younger mice showing greater memory specificity ($F_{(1,86)} = 5.35$, $P = 0.023$), again with no interaction between age and SD treatment ($F_{(1,86)} = 1.09$, $P = 0.30$). The finding that aged mice performed better than young mice during the Recent test was unexpected. However, this may be a consequence of a limitation of our study, which was that young mice were trained and tested in separate experimental cohorts from aged mice. Thus, it is possible that environmental conditions were not identical between age groups, which could have created an initial difference in the overall freezing levels between the two age groups. In future studies, it would be ideal to run all age groups intermixed within each cohort to prevent issues of batch effects. The fact that memory in aged mice became worse than in young mice by the Remote test, on the other hand, was not surprising. Our data showed that memory in young mice improves over a month-long interval, whereas it decays in aged mice, consistent with previous reports (Oler and Markus 1998; Houston et al. 1999; Pickens et al. 2009). This deficit in long-term retention in aged animals may be a result of issues with memory storage in the hippocampus over the time-course of weeks, or because of problems with transferring information to cortical regions such as the ACC (Frankland et al. 2004; Frankland and Bontempi 2005). Although SD after training impairs initial memory consolidation, our findings suggest that the portion of the memory that *is* formed can be maintained effectively and eventually transferred into permanent storage. This indicates that changes in memory over prolonged delays after learning may be independent of the influence of SD occurring during initial memory consolidation.

In the current study, altered context tests were always run 24 h after the trained context test. Thus, reactivation of the fear memory during the trained context test could have had an impact on the fear response during the subsequent altered context test through extinction or a reconsolidation effect (Debiec et al. 2002). However, the design of the experiment allowed us to analyze freezing in the trained context alone (see Figs. 1B, 2A), which is free of confounds of prior reactivation. The patterns of the effects of age and SD on memory in the trained context were very similar to those for memory specificity, in which freezing in the altered context was subtracted from freezing in the trained context. Thus, we do not believe that the order of testing affected our conclusions.

In general, our findings suggest that aging and acute SD disrupt episodic memory via different mechanisms, and that acute SD has similar effects on initial, hippocampus-dependent memory consolidation, and hippocampal gene expression in young and aged animals. It will be of future interest to explore the cellular and molecular nature of the deficits in remote memory seen in aged animals, and to determine if aging-related sleep abnormalities do in fact contribute to worsening in other areas of cognitive function or general health.

Acknowledgments

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