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Sleep deprivation directly following eyeblink-conditioning impairs memory consolidation

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ABSTRACT

The relation between sleep and different forms of memory formation continues to be a relevant topic in our daily life. Sleep has been found to affect cerebellum-dependent procedural memory formation, but it remains to be elucidated to what extent the level of sleep deprivation directly after motor training also influences our ability to store and retrieve memories. Here, we studied the effect of disturbed sleep in mice during two different time-windows, one covering the first four hours following eyeblink conditioning (EBC) and another window following the next period of four hours. Compared to control mice with sleep *ad libitum*, the percentage of conditioned responses and their amplitude were impaired when mice were deprived of sleep directly after conditioning. This impairment was still significant when the learned EBC responses were extinguished and later reacquired. However, consolidation of eyeblink responses was not affected when mice were deprived later than four hours after acquisition, not even when tested during a different day-night cycle for control. Moreover, mice that slept longer directly following EBC showed a tendency for more conditioned responses. Our data indicate that consolidation of motor memories can benefit from sleep directly following memory formation.

1. Introduction

Adequate sleep is important for our everyday performance and sleep disorders constitute a fast-growing problem, often contributing to health problems and mental disorders (Banks & Dinges, 2007; Killgore, 2010; Reis et al., 2018; Spiegel, Leproult, & Van Cauter, 1999). A vast body of evidence, emerging from both human and animal studies, indicates that sleep plays a major role in the formation and consolidation of memories (Diekelmann, 2014; Diekelmann & Born, 2010; Maquet et al., 2000; Marshall & Born, 2007; Ohno et al., 2002; Plihal & Born, 1997; Schabus et al., 2004; Smith, 2001; van Schalkwijk et al., 2019; Walker, Stickgold, Alsop, Gaab, & Schlaug, 2005; Walker & Stickgold, 2006). For example, sleep deprivation affects both the formation and retrieval of hippocampus-dependent declarative memories (Gais & Born, 2004; McDermott et al., 2003; Tartar et al., 2006; Yoo, Hu, Gujar, Jolesz, & Walker, 2007; Oudiette & Paller, 2013; Smith, 2011; Stickgold & Walker, 2013), whereas artificially boosting sleep can improve the retrieval of such memories (Marshall, Helgadóttir, Mölle, & Born, 2006). Interestingly, learning of declarative tasks in turn can also influence the architecture of subsequent sleep (Cox, Hofman, & Talamini, 2012; De Koninck, Lorrain, Christ, Proulx, & Coulombe, 1989; Fogel et al., 2017; Gais, Mölle, Helms, & Born, 2002), suggesting that sleep following hippocampal learning is also relevant for consolidation of memories. This possibility is corroborated by the findings that sleep insufficiencies following declarative learning can negatively affect consolidation of hippocampus-dependent memories, and vice versa that individual declarative memories can be strengthened by reactivating cues that can be associated with them during sleep (Cousins, El-Deredy, Parkes, Hennies, & Lewis, 2016; Cousins, Sasmita, & Chee, 2018; Lowe, Safati, & Hall, 2017; Rasch, Buchel, Gais, & Born, 2007; Rudoy, Voss, Westerberg, & Paller, 2009; van Dongen & Takashima, Barth, Zapp, Schad, Paller, & Fernandez, 2012).

To what extent sleep deprivation also affects cerebellum-dependent consolidation and retention of procedural memories remains to be elucidated. We know that consolidation of procedural memory formation occurs at least partly in the cerebellum (Galliano et al., 2013; Gao, van Beugen, & De Zeeuw, 2012), while learning-dependent timing and spatiotemporal predictions of motor actions are facilitated by sleep (Barakat et al., 2011; Barakat et al., 2013; Grube, Cooper, Chinnery, & Griffiths, 2010; Stoodley, 2012; Verweij, Onuki, Van Someren, & Van der Werf, 2016; Walker, Brakefield, Morgan, Hobson, & Stickgold, 2002). Moreover, new procedural associative reflexes can be acquired in infants while sleeping (Fifer et al., 2010; Tarullo et al., 2016). To further study the impact of sleep deprivation on cerebellum-dependent

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memory consolidation we subjected mice to different sleep regimes after they were subjected to delay eyeblink conditioning (EBC; see e.g., Freeman & Steinmetz, 2011; Nicholson & Freeman, 2000). EBC is a valuable model system to study the relation between sleep and memory consolidation for several reasons. The behavioral paradigm allows for identification of different parts of the learning process, enabling us to separate consolidation from acquisition, extinction, reacquisition as well as retention (Inda, María Delgado-García, Ngel, & Carrión, 2005). Likewise, the neuro-anatomical pathways as well as the electro-physiological correlates that underlie EBC have been largely elucidated

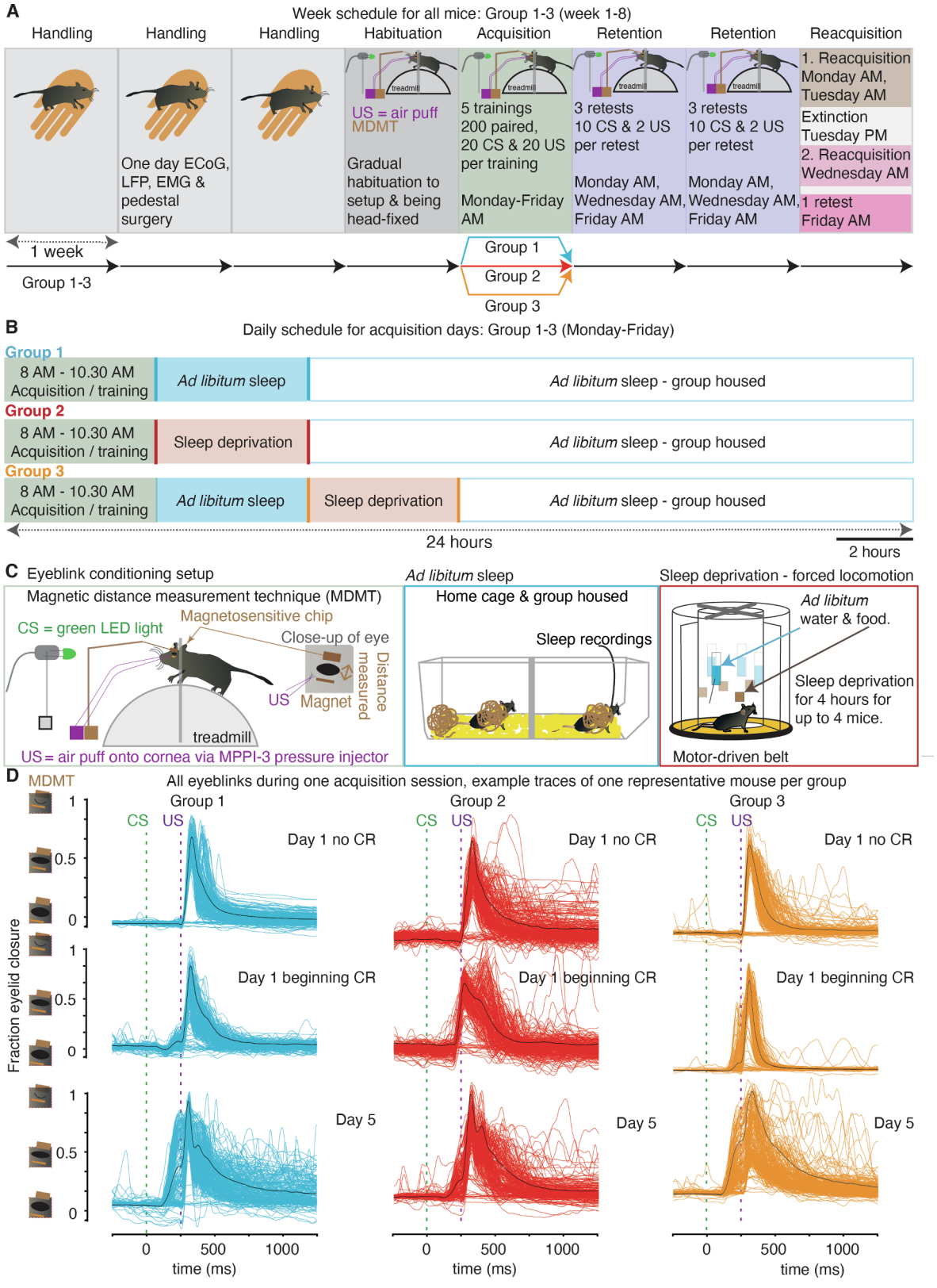

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Fig. 1. Outline of the experimental setup. (A) The week schedule for all mice: Group 1–3. After weaning, mice were handled intensively for 3 weeks. During the 2nd handling week, a surgery was performed to place a pedestal, electrocardiographic (EcoG), local field potential (LFP), and electromyographic (EMG) electrodes. After handling (gray background), mice were gradually habituated to the setup and being head-fixed (dark grey background). Mice had *ad libitum* food and water, except for the time when they were trained in the eyeblink setup. During the acquisition week, we trained mice in the morning (AM) for 5 subsequent days (Day 1–5 (D1-D5)), one acquisition session a day (200 paired trials, 20 conditioned stimuli (CS) and 20 unconditioned stimuli (US)). Mice are nocturnal and the acquisition sessions started between one and two hours after lights were switched on. (B) Group 1 (blue) mice were allowed to have *ad libitum* sleep after every acquisition session without further handling. Group 2 mice (red) were sleep-deprived for four hours after acquisition and group 3 mice (orange) were sleep-deprived for 4 h starting 4 h after finishing the training. Eventually all mice were allowed to have *ad-libitum* sleep for the remaining night and day. (A) After the acquisition week and during retention, all mice were retested Monday, Wednesday and Friday mornings (AM) for 2 weeks (purple background). Every retest session consisted of 1 × US, 10 × CS, and 1 × US trial. In the mornings of day (D) 22 and 23 (brown background), the reacquisition week, mice were presented a reacquisition protocol to test for the rate of re-learning (savings) compared to the rate of original learning. One reacquisition session consisted of 200 paired trials, 20 CS and 20 US trials. The reacquisition on D23 was followed by a prolonged extinguishing (grey background) of the learned memory. The day after, (pink background) mice were presented a reacquisition protocol to test for savings after prolonged extinction and finally a last retest was performed in the morning of the Fridays of the acquisition week (dark pink background). (C) The delayed eyeblink (EBC) setup to the left, in the middle a representation of the home cage, where mice are housed in groups with a possibility to record activity of the brain and muscles. If mice were recorded, mice were separated by a Plexiglas wall with holes to allow smelling each other. To the right, the sleep deprivation device. A rotating drum (∅ 39 cm, height 37 cm), divided into 4 semicircular compartments by stationary central walls was developed at the University of Grenoble and assembled, adapted and optimized by us and others (Leenaars et al., 2011). The bottom was covered with sawdust from the home cages. Water and food were provided. (D) Example eyeblink traces of various representative mice per experimental group, showing raw eyeblink traces of (top) one mice per group without CRs during acquisition on day 1, (middle) one mouse per group with CRs during acquisition session 1 and (bottom) one mouse per group with CRs during acquisition session on day 5. The black trace is the average trace of all underlying traces. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(De Zeeuw & Ten Brinke, 2015; De Zeeuw & Yeo, 2005; Halverson, Khilkevich, & Mauk, 2015; Ohmae & Medina, 2015; ten Brinke et al., 2015; ten Brinke et al., 2017). Moreover, the advance of cell-specific genetics has allowed for functional dissection of the local network and forms of plasticity involved (Boele et al., 2018; Gao et al., 2012; ten Brinke et al., 2015). During EBC a neutral conditioned stimulus (CS), such as a LED-light, is presented at a fixed temporal interval co-terminating with an unconditioned stimulus (US) like an aversive air-puff to the eye (Fig. 1A). This US will automatically and consistently evoke an eyelid closure, also called unconditioned response (UR), right from the start. With every paired trial during the process of conditioning the subjects will learn to better associate the two stimuli (CS and US) and eventually generate a well-timed conditioned eyelid response (CR) such that the eyelid is optimally closed around the moment when the US was about to be presented (Heiney, Wohl, Chettih, Ruffolo, & Medina, 2014; Ivarsson & Svensson, 2000). Ultimately, providing the CS alone will be sufficient to evoke a CR.

After acquisition the memory needs to be stored. For decades, the location for memory storage of the conditioned eyelid response has been debated, with the cerebellar cortex and nuclei both being the major players (Cooke, Attwell, & Yeo, 2004; Krakauer & Shadmehr, 2006). Mounting evidence supports a critical time-window for the storage and consolidation of the CR. Temporary inhibition of lobule HVI following EBC narrowed the relevant time window for storage in the cerebellar cortex down to the first few hours after acquisition (Cooke et al., 2004). Presumably, during these first hours of acquisition, conditioning is facilitated by active inhibition of Purkinje cell activity through the molecular layer interneurons (ten Brinke et al., 2015) as well as by long-term depression of the parallel fiber to Purkinje cell synapse (Aiba et al., 1994; Ichise et al., 2000; Kishimoto et al., 2002; Koekkoek et al., 2003; Shibuki et al., 1996; Boele et al., 2018). Manipulation of cerebellar cortical processing after this critical period does not inhibit consolidation, suggesting that the site of storage is at least partially transferred to regions downstream (Clark, Zhang, & Lavond, 1992; Cooke et al., 2004; Freeman, Halverson, & Poremba, 2005; Krupa, Thompson, & Thompson, 1993; Krupa & Thompson, 1997; Medina, Garcia, & Mauk, 2001; Napier, Macrae, & Kehoe, 1992). Indeed, as learning progresses, changes in neuronal activity in the cerebellar nuclei emerge and these changes can be correlated to the level of expression of the memory (ten Brinke et al., 2017). Accordingly, we focused our research question here on whether sleep-deprivation during a critical period of up to 4 h after acquisition can affect cerebellum-dependent consolidation and/or retention of CRs.

EBC was performed on 3 groups of mice, including a control group with sleep *ad libitum* (Group 1), a group with sleep deprivation for four hours immediately after learning (Group 2), and a group with sleep deprivation for four hours starting 4 h after learning (Group 3; Fig. 1A–C). After acquisition, mice were retested for two weeks to evaluate consolidation and retention (Fig. 1A). After memory was extinguished, mice were presented with a re-acquisition protocol to test for the rate of re-learning compared to the rate of initial learning, highlighting the amount of savings (Medina et al., 2001). We found that consolidation of sleep-deprived mice of group 2 was affected in that both the percentage and amplitude of their CRs were smaller than those of controls. In addition, the degree of retention as well as of savings of especially the percentage of CRs was stronger in mice with sleep *ad libitum* compared to sleep-deprived mice. When we tested a subpopulation of mice that received the same treatment as group 3 mice, during a different day-night cycle we could not observe any deficits in consolidation. Moreover, to further investigate to what extent the depth and duration of sleep might affect the level of learning, we quantified sleep using electrocorticography (ECoG) in a subpopulation of group 1 mice after EBC. The more mice slept during the first four hours following EBC, the more conditioned responses they showed and the bigger the fraction of the corresponding eyelid closures. Together, our data suggests that sleep after EBC affects the occurrence of subsequent conditioned responses, indicating that sleep may have a modulatory role in facilitating consolidation and retrieval of associated memories.

2. Results

To establish the effects of sleep deprivation on mice that have learned the EBC task we compared the memory consolidation of eyeblink responses in mice that were allowed to have sleep *ad libitum* (Group 1, blue colored, Fig. 1A–C) with that in mice that were sleep-deprived 0–4 h directly after training (Group 2, red colored) and with that in mice that were sleep-deprived for 4 h starting 4 h after the training was finished (Group 3, orange colored). To make sure that potentially different outcomes of the two intervention groups (Groups 2 and 3) were not dominated by different distributions of sleep states at the moment when they were deprived of sleep, we first examined these distributions across the 12 h light phase in mice that had normal sleep *ad libitum*. As shown previously (Huber, Deboer, & Tobler, 2000; Genzel, Kroes, Dresler, & Battaglia, 2014), these mice did not show a significant difference in this respect; for example, the distribution of states 0–4 h after acquisition (10:30 AM-2:30 PM; NREM 46.90 ± 5.20%; REM 8.69 ± 2.27%; Wake 41.42 ± 4.85%) showed similar values as that 4–8 h after acquisition (2:30 PM-6:30 PM; NREM 46.74 ± 9.62%; REM 10.28 ± 2.35%; Wake 42.3 ± 10.95%). Likewise, as one cannot exclude that short localized sleep sometimes occurs

in a sleep deprivation box (Vyazovskiy & Harris, 2013), we investigated to what extent mice were sleeping during forced locomotion in our sleep deprivation boxes. As far as could be detected by recording ECoGs and EMGs, none of these additional control mice (N = 3) showed any clear sign of elongated sleep during the 4 h of sleep deprivation. These control data indicate that the baseline settings in terms of state of arousal before the interventions as well as during the interventions could be controlled in a consistent manner. To further warrant a stable starting point with similar levels of habituation, all mice were handled similarly before being subjected to the experimental procedures (Fig. 1A).

2.1. Effects of sleep deprivation immediately following eyeblink conditioning on CRs in the first acquisition week; the first signs of a critical period

To evaluate the effects of sleep deprivation on memory consolidation, we first trained mice for 5 consecutive days on the EBC setup (Fig. 1; acquisition week highlighted by green background) and tested their ability to learn the task. One training session consisted of 20 blocks of 1 US trial, 10 paired trials as well as 1 CS trial. Four hours after training all mice were retested (see 4.6 Training protocol) presenting 10 CS and 2 US trials to mice, so as to examine to what extent mice consolidated the learned behavior over 4 h. In this case, we only applied a limited amount of CS-only trials so as to not extinct the learned behavior (Medina, Nores, & Mauk, 2002; Siegel et al., 2015), and to keep the mice in the setup only for a limited amount of time. All mice included in the study were able to learn the task and to perform a conditioned eyeblink in response to the CS (Fig. 1D, Supplement (Suppl.) Figs. 1–4). Mice without conditioned eyeblinks (% CR < 10%) during the five days of acquisition were excluded from further analyses (this concerned one Group 1 and one Group 2 mouse). All three groups showed a significant increase in CR percentage and fraction of eyelid closure (FEC; 0 and 1 represent fully closed closed and fully open eyelids, respectively) over the course of 5 acquisition sessions during both CS-only (Figs. 2B and 3B) and paired CS-US (Figs. 2C and 3C) trials (Tables 1–4 for p-values, Suppl. Figs. 1 and 2 for individual data). The changes in the onset timing (Fig. 4) and peak timing (Fig. 5) of the CRs showed both signs of learning-dependent timing, but in line with the limited number of CS-only trials the changes in the CS-only trials appeared more noisy than those in the paired trials (Tables 5 and 6, and Suppl. Figs. 3 and 4 for individual data).

Before we looked at the impact of sleep deprivation on consolidation, we first studied the sleeping pattern using 4-second-long ECoG/EEG/EMG recordings directly after acquisition in a subpopulation of mice that had sleep *ad libitum* (Group 1 mice) and mice that were deprived of sleep (Group 2 mice) for four hours (Fig. 6). The first signs of short sleep occurred between 30 and 60 min after mice were placed back into their homecage. After 60 min mice showed frequently alternating wake and sleep stages including clear non-rapid eye movement (NREM) sleep (Group 1: Acquisition session (S)1, 51.08 ± 3.08%; S2, 47.20 ± 7.95%; S3, 51.24 ± 2.28%; S4, 52.21 ± 5.80%, and S5, 52.64 ± 6.15%; Group 2: S1, 44.44 ± 5.25%; S2, 50.31 ± 3.26%; S3, 41.06 ± 7.58%; S4, 56.76 ± 3.50%, and S5, 49.89 ± 6.68%) as well as rapid eye movement (REM) sleep (Group 1: S1, 9.81 ± 1.43%; S2, 13.74 ± 3.40%; S3, 10.06 ± 2.69%; S4, 9.61 ± 3.47%; and S5, 10.10 ± 2.08%; Group 2: S1, 8.77 ± 2.67%; S2, 9.64 ± 2.44%; S3, 10.52 ± 1.90%; S4, 9.88 ± 1.38%; and S5, 10.25 ± 1.80%).

Comparing mice that were sleep-deprived immediately following acquisition (Group 2, red) to mice that had sleep *ad libitum* (Group 1, blue) or that suffered from sleep deprivation later on (Group 3, orange), the quality and quantity of the CRs of Group 2 mice improved less. Sleep-deprived mice of Group 2 learned the task significantly less well in terms of percentage of CRs and FEC over the course of the 5 acquisition days (Tables 1–4; Figs. 2B-C and 3B–C). The changes in timing of CR onset and peak amplitude were less consistent and only showed some trends (Figs. 4 and 5). For example, Group 2 mice only did significantly worse than Group 1 and Group 3 mice in shortening the interval between CS and CR onsets on days 3 and 5 of the CS-only trials (Fig. 4B; Tables 5 and 6), and Group 2 mice only did significantly worse than Group 1 mice in increasing the CR peak time on days 3 and 4 for the paired trials (Fig. 5C; Tables 5 and 6).

Analyzing the short retest sessions following acquisition revealed that the performances of Groups 1 and 3 improved or remained relatively constant compared to those of the prior acquisition sessions, whereas the performance of Group 2 often decreased dramatically between the acquisition and subsequent retest session, in extreme cases from 80 to 0 and from 0.8 to 0.2 for percentage of CRs and FEC, respectively (even though the performance could return to relatively normal levels during the subsequent acquisition). Presumably due to the limited amount of 10 CS-only trials, the data were relatively noisy in all groups (Suppl. Figs. 1 and 2). Even so, Groups 1 and 3 mice improved their memory over 4 h of rest/sleep, whereas the retests of Group 2 mice revealed variable results (Suppl. Figs. 1 and 2). Behavioral observations of Group 2 mice support the possibility that the variability in the outcomes resulted from stress-related squeezing of the eye and reduced motivation to run after sleep deprivation via forced locomotion (see also Albergaria, Silva, Pritchett, & Carey, 2018).

To summarize, in all groups a small subpopulation of mice was able to acquire the basics of the task within one acquisition session, indicating that sleep is not obligatory for the acquisition of the occurrence of a conditioned eyeblink itself. Furthermore, Group 2 mice were able to learn the task albeit at a reduced rate; thus sleep directly following acquisition might fulfill a modulatory, yet not an obligatory, role in consolidating EBC.

2.2. Sleep-deprivation directly following EBC impairs memory consolidation and retention for weeks

To further establish the effects of sleep deprivation on long-term memory retention of the learned behavior, mice were subsequently exposed 6 days over 2 weeks to retests of 1 US and 10 CS-only trials, followed by 1 final US-only trial (Figs. 1–5). As mentioned above, also here the low amount of CS- and US-only trials were chosen to minimize the chances for extinction (Medina et al., 2002; Siegel et al., 2015), while at the same time having a sufficient number of trials to analyze the behavior and not retraining the animals with paired trials, which would make it difficult to draw conclusions. During these two retention weeks all groups of mice had a similar treatment with sleep *ad libitum*. Group 2 mice (red) performed significantly worse in terms of percentage of CRs (Fig. 2B) and FECs (Fig. 3B) during the 1st to 3rd retest sessions (D8, D10, D12); in particular the changes from the last acquisition day (D5) to the first retention day (D8) showed prominent differences with controls (Figs. 2B and 3B). These data support the idea that sleep deprivation immediately following acquisition affects consolidation of long-term memories (Tables 1–4). Group 3 mice retained the memory at a similar level as Group 1 mice. Only during retest 3 on day 12, Group 3 mice showed a significantly higher percentage of CRs compared to Group 1 mice, suggesting that on that particular single day these mice saved their memories even better than mice with sleep *ad libitum*. The FEC of Group 1 and 3 mice was similar throughout retest sessions and differed from that of Group 2 mice (Fig. 3B; Tables 3 and 4). Also, the timing of CR onset, but not that of the peak amplitude, of the CS-only trials of Group 2 mice was significantly worse compared to that of Group 3 mice during the first retest session (Figs. 4B and 5B, Tables 5 and 6). Afterwards the measurements of timing became even less reliable, because of the low percentage of CRs. Given that sleep-deprived mice of Group 2, but not Group 3, showed a higher rate of extinction of CRs over time (Fig. 2), the question remains to what extent these animals can or cannot re-learn their naturally extinguished conditioned eyeblink responses.

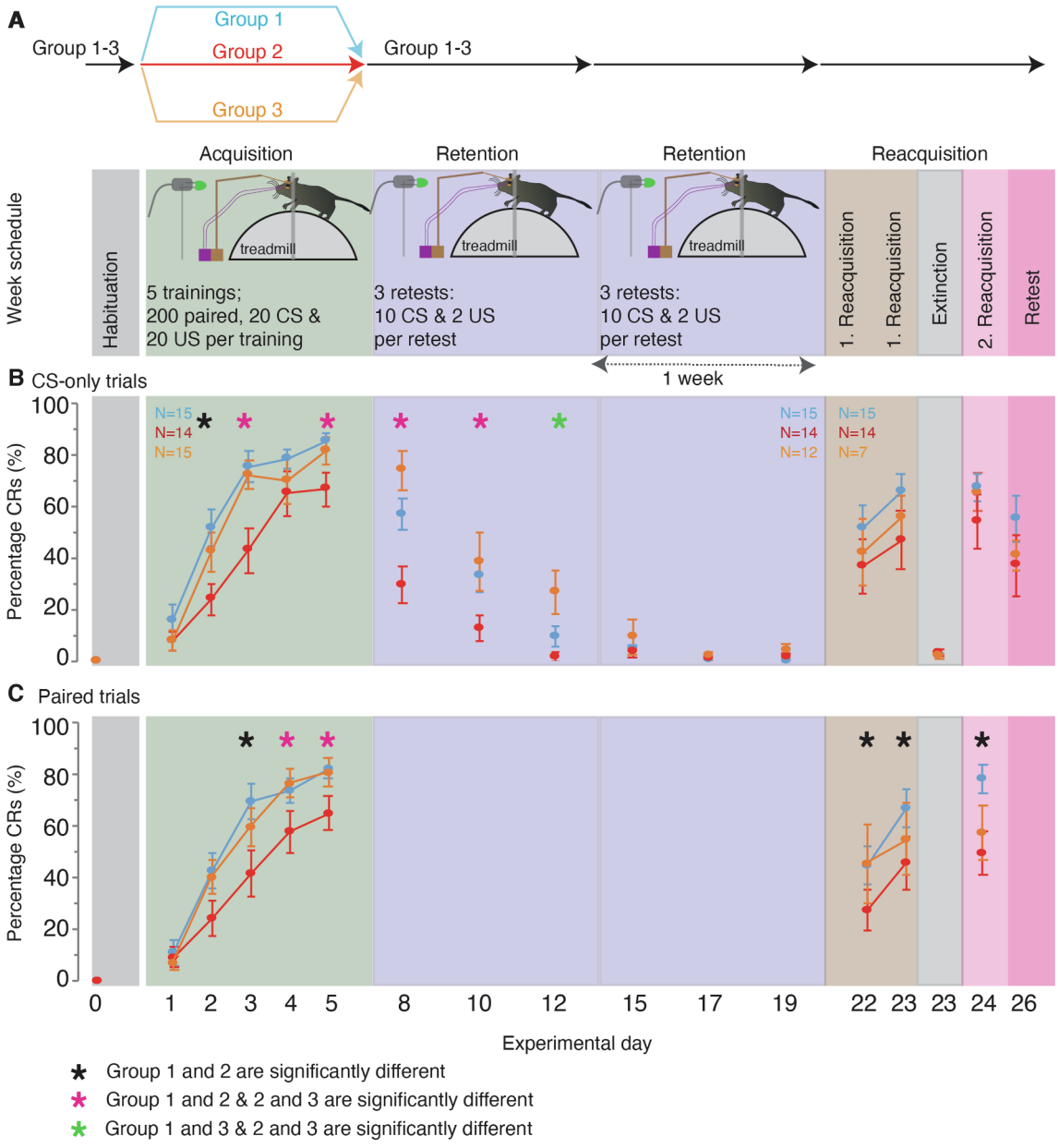

Fig. 2. Percentage of CRs (%) per session. (A) The experimental outline. (B and C) The percentage CRs per training day and session for (B) CS-only trials and (C) paired trials. The color coding of the asterisks is indicated at the bottom. Color coding of groups: Blue = Group 1, red = Group 2, orange = Group 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.3. Sleep deprivation immediately following learning affects the degree of savings even after prolonged extinction

In a next step we first investigated whether the three groups of mice showed similar re-learning after short, yet complete, extinction' of the memory that occurred after the retesting (Figs. 1–5). Upon this re-acquisition, Group 1 and Group 3 mice showed savings during CS-only and paired trials in that they jumped to a significantly higher level of CRs and FECs on the first day of reacquisition compared to the initial acquisition day, whereas group 2 mice did not jump to a significantly higher level of CRs during paired trials and also not to a significantly higher level of FEC during CS-only trials (CR percentage: Group 1 CS-only (CSo) CR % (1, 9) = 23.17, $p < 0.01$, paired (1,10) = 50.59, $p < 0.01$; Group 2 CSo CR % (1, 12) = 6.166, $p = 0.03$, paired (1,13) = 3.429, $p = 0.087$; Group 3 CSo (1,6) = 9.445, $p = 0.02$, paired (1,6) = 11.30, $p = 0.02$; FEC: Group 1 CSo (1,9) = 18.46, $p < 0.01$, paired (1,10) = 26.13, $p < 0.01$; Group 2 CSo (1, 12) = 3.29, $p = 0.10$, paired (1,13) = 5.67, $p = 0.03$; Group 3 CSo (1,6) = 12.60, $p = 0.01$ paired (1,6) = 6.91, $p = 0.04$) (compare day 22 with day 1 in Figs. 3 and 4). Group 2 mice also showed a significantly lower percentage of CRs compared to Group 1 and 3 mice in their paired trials during both the first and second session of re-acquisition (Table 2; Fig. 2). Thus, Group 2 mice showed less savings compared to Group 1 mice with continuous sleep *ad libitum*, even though these Group 2 mice were allowed to have sleep *ad libitum* after both of the reacquisition sessions and they started at the same null level of CRs at the beginning of the first reacquisition as the control mice. Regarding FEC, that of Group 2 mice was only significantly smaller than that of Group 3 mice (Tables 3 and 4; Fig. 3). The timing of almost all reacquired CRs did not change significantly compared to the initially acquired CRs on the first day = (CR onset time: Group 1 CSo (1, 4) = 3.19, $p = 0.15$, paired (1,5) = 1.28, $p = 0.31$; Group 2 CSo (1,3) = 6.32, $p = 0.09$, paired (1,7) = 1.97, $p = 0.20$; Group 3 CSo (1,3) = 63.72, $p < 0.01$, paired (1,4) = 289, $p = 0.16$; CR peak time:

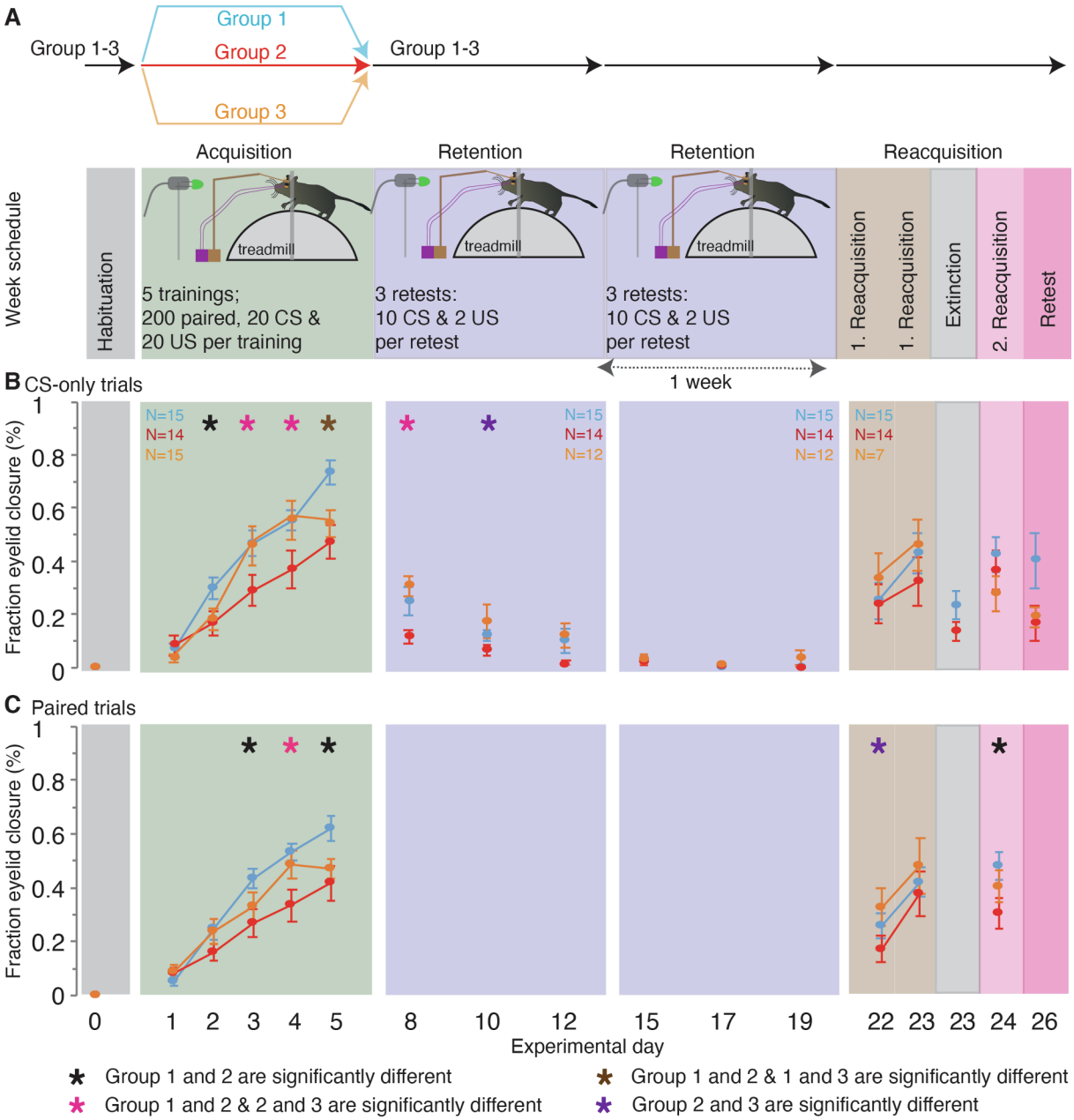

Fig. 3. Fraction eyelid closure per session. (A) The experimental outline. (B and C) The fraction of eyelid closure (FEC) per training day and session for (B) CS-only trials and (C) paired trials. The color coding of the asterisks is indicated at the bottom. Color coding of groups: Blue = Group 1, red = Group 2, orange = Group 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Group 1 CSo (1, 4) = 5.31 p = 0.08, paired (1,5) = 3.68, p = 0.11; Group 2 CSo (1,3) = 0.22, paired (1,6) = 5.42, p = 0.06; Group 3 CSo (1,3) = 0.01, p = 0.99, paired (1,4) = 2.05, p = 0.23).

Subsequently, we started to investigate to what extent reacquisition following prolonged induced extinction also showed differences among the 3 groups (Figs. 2–5). This prolonged extinction of the memory was induced by initially presenting 20 US-only and 220 CS-only trials (see Suppl. Figs. 1–4 for individual indici extinction data points). If CRs were still occasionally visible towards the end of the extinction protocol, another 5 US-only and 55 CS-only trials were presented until no CR was visible anymore. Here too, we observed that during the subsequent reacquisition on day 24 Group 2 mice showed significantly less savings during the paired trials, both in terms of percentage of CRs and eyelid closures, but not in terms of timing of these responses (Tables 1–6; Figs. 2–5). Thus, both after short and prolonged extinction, the savings of especially the CR percentages and FECs of the mice that were sleep-deprived directly following EBC during the initial acquisition week were worse than those of the control groups. Finally, to find out whether the consolidation impairments of Group 2 did not merely result from a shift in circadian rhythm, which might have been induced by the four hours of sleep deprivation at the beginning of the light cycle, we examined an additional control group (Suppl. Fig. 5). This control group followed the protocol of Group 3 except that the acquisition and resting period ended before light onset with the sleep deprivation period starting at the beginning of the light period. The data indicate that even though the performance was less during the acquisition period, the consolidation during the retention and reacquisition period was as high as that of Group 3 mice (Suppl. Fig. 5), suggesting that a slight shift in the circadian rhythm does not necessarily affect retention and reacquisition of procedural learning.

Table 1
Pavlovian eyeblink conditioning. The percentage of conditioned responses (CRs) per session and experimental group for CS-only trials.

CR percentage CS-only trials	Group 1 (*ad libitum* sleep)	Group 2 (SD 0–4 hrs after acquisition)	Group 3 (SD 4–8 hrs after acquisition)	Repeated measures (acquisition sessions)	ANOVA on LME model on all sessions	Group 1 vs. Group 2	Group 1 vs. Group 3	Group 2 vs. Group 3
Acquisition D1	15.81	7.79	8.13	Group 1	Condition * Session	p = 0.213	p = 0.263	p = 0.899
Acquisition D2	51.8	24	42.56	(4, 14.09) = 51.36	(23, 132.19) = 8.128	p = 0.003	p = 0.203	p = 0.060
Acquisition D3	75.6	43.07	72.42	p < 0.001	p < 0.001	p = 0.002	p = 0.640	p = 0.014
Acquisition D4	78.5	65.07	70	Group 2	Condition	p = 0.159	p = 0.367	p = 0.650
Acquisition D5	85.5	66.79	81.67	(4, 13) = 26.23	(2, 1554) = 7.189	p = 0.017	p = 0.575	p = 0.045
Retest 1 D8	57.13	29.71	74.17	p < 0.001	p < 0.01	p = 0.006	p = 0.156	p < 0.001
Retest 2 D10	33.32	12.85	38.70	Group 3	Session	p = 0.017	p = 0.495	p = 0.005
Retest 3 D12	9.5	1.77	26.92	(4, 12.68) = 35.14	(12, 165.89) = 86.11	p = 0.257	p = 0.030	p = 0.002
Retest 4 D15	4.56	3.54	9.38	p < 0.001	p < 0.001	p = 0.285	p = 0.268	p = 0.992
Retest 5 D17	0.38	0.85	2.08			N.A.	N.A.	N.A.
Retest 6 D19	0	0	4			N.A.	N.A.	N.A.
1.Reacquisition D22	51.44	36.71	46.5			p = 0.140	p = 0.484	p = 0.556
1.Reacquisition D23	65.69	47.11	58.36			p = 0.225	p = 0.800	p = 0.400
Extinction D23	2.70	2.91	3.07			N.A.	N.A.	N.A.
2.Reacquisition D24	67.29	54.41	65.25			p = 0.079	p = 0.575	p = 0.329
Retest D27	55.25	37.18	38.18			p = 0.225	p = 0.880	p = 0.400

Values represent mean.
Percentage CRs during CS-only trials, divided into sessions and experimental groups. (2nd–4th column) Mean percentage CRs per experimental mouse group and session. Statistics testing for the (5th column) changes in the percentages of CRs within one experimental group over the 5 acquisition days, and (6th–9th column) the differences between all experimental sessions and experimental groups.

2.4. Duration and depth of sleep between 1 and 4 h after acquisition can be correlated to level of EBC

To further assess the effects of sleep on learning, we correlated the levels of sleep and eyeblink conditioning parameters in a subpopulation of mice that had sleep *ad libitum* (Group 1 mice). These mice received various implants for EEG/ECoG/EMG (Fig. 6), similar to what all other mice received (N = 7). As determined following analyses of 4 s epochs of ECoG/EEG/EMG recordings, the first signs of short sleep occurred between 30 and 60 min after mice were placed back into their home-cage (for definitions of sleep stages, see 4.114.), while longer periods of non-rapid eye movement (NREM) sleep as well as rapid eye movement (REM) sleep emerged after 60 min. Because mice hardly slept within the first hour after acquisition, we focused on the level of sleep from the 2nd until the 4th hour post-acquisition. Pearson's analyses revealed slight trends in that the longer the mice slept, the more CRs and higher FECs they generally showed during the training session on subsequent days (for relations with different sleep stages including NREM, see Suppl. Tables 1–4 and Suppl. Fig. 6). Indeed, even though not all correlations were positive, the majority (52 out of 66; i.e., 79%) was, which is significant (p = 0.001, Fisher's Exact Test). Moreover, whereas none of the negative correlations were significant (not even without corrections for multiple comparisons), 10 out of the 52 positive correlations were. The probability that 10 out of all the 10 significant correlations were positive by chance equals 0.5^{10}, which is 0.00098. Finally, the average of all the positive correlations (r = 0.41) trended to be higher (p = 0.06; T-Test) with respect to that of all the negative correlations (r = -0.3) (in the latter comparison we turned all negative values positive, so as to merely compare the strength of the correlations). We conclude that sleep after learning may facilitate consolidation of procedural learning.

3. Discussion

To our knowledge this is the first study testing the effects of sleep deprivation directly after the formation of procedural memories on the consolidation and retention thereof in mice. Mice that were sleep-deprived in the first four hours directly after being subjected to an eyeblink conditioning (EBC) task were slower in consolidating their conditioned responses (CRs) than mice that had sleep *ad libitum*. These sleep-deprived mice still showed a reduced percentage of CRs and eyelid closures during retention, despite the fact that they had sleep *ad libitum* during this period of retention. Even when these animals re-acquired the task after prolonged extinction, the percentages of their CRs and FECs were significantly less than those of mice who had sleep *ad libitum* during their initial acquisition. In contrast, mice that were

Table 2
Pavlovian eyeblink conditioning. The percentage of conditioned responses (CRs) per session and experimental group for paired trials.

CR percentage paired trials	Group 1 (*ad libitum* sleep)	Group 2 (SD 0–4 hrs after acquisition)	Group 3 (SD 4–8 hrs after acquisition)	Repeated measures (acquisition sessions)	ANOVA on LME model on all sessions	Group 1 vs. Group 2	Group 1 vs. Group 3	Group 2 vs. Group 3
Acquisition D1	10.81	9.21	6.8	Group 1	Condition * Session	p = 0.694	p = 0.475	p = 0.749
Acquisition D2	42.56	24.28	40.2	(4, 14) = 48.25	(14, 34.02) = 1,72	p = 0.062	p = 0.782	p = 0.114
Acquisition D3	69.5	41.43	59.64	p < 0.001	p = 0.099	p = 0.004	p = 0.389	p = 0.052
Acquisition D4	73.69	57.86	76.58	Group 2	Condition	p = 0.012	p = 0.844	p = 0.011
Acquisition D5	82.38	64.92	80.77	(4, 13.01) = 29.42	(2, 41,25) = 3.78	p = 0.010	p = 0.722	p = 0.033
1.Reacquisition D22	44.77	27.48	45.24	p < 0.01	p < 0.05	p = 0.044	p = 0.753	p = 0.145
1.Reacquisition D23	66.79	45.69	48.04	Group 3	Session	p = 0.038	p = 0.385	p = 0.330
2.Reacquisition D24	78.26	49.48	57.21	(4, 24.91) = 56.96	(7, 34.42) = 70.16	p = 0.003	p = 0.102	p = 0.812
				p < 0.001	p < 0.001			

Values represent mean.
Percentage CRs during paired trials, divided into sessions and experimental groups. (2nd–4th column) Mean percentage CRs per experimental mouse group and session. Statistics testing for the (5th column) changes in the percentages of CRs within one experimental group over the 5 acquisition days, and (6th–9th column) the differences between all experimental sessions and experimental groups.

Table 3
Pavlovian eyeblink conditioning. The fraction eyelid closure (FEC) per session and experimental group for CS-only trials.

Fraction eyelid closure CS-only trials	Group 1 (*ad libitum* sleep)	Group 2 (SD 0–4 hrs after acquisition)	Group 3 (SD 4–8 hrs after acquisition)	Repeated measures (acquisition sessions)	ANOVA on LME model on all sessions	Group 1 vs. Group 2	Group 1 vs. Group 3	Group 2 vs. Group 3
Acquisition D1	0.07	0.08	0.03	Group 1	Condition * Session	p = 0.854	p = 0.788	p = 0.933
Acquisition D2	0.30	0.16	0.18	(4, 12.25) = 66.60	(23, 39.24) = 5.48	p = 0.045	p = 0.254	p = 0.347
Acquisition D3	0.47	0.29	0.46	p < 0.001	p < 0.001	p = 0.039	p = 0.933	p = 0.048
Acquisition D4	0.56	0.37	0.56	Group 2	Condition	p = 0.040	p = 0.853	p = 0.040
Acquisition D5	0.74	0.47	0.54	(4, 13.0) = 13.94	(2, 140.12) = 3.96	p = 0.002	p = 0.021	p = 0.380
Retest 1 D8	0.25	0.12	0.31	p < 0.001	p < 0.05	p = 0.030	p = 0.374	p = 0.005
Retest 2 D10	0.12	0.06	0.17	Group 3		p = 0.053	p = 0.099	p = 0.09
Retest 3 D12	0.10	0.01	0.12	(4, 13.24) = 28.01		p = 0.274	P = 0.294	p = 0.984
Retest 4 D15	0.03	0.02	0.03	p < 0.001		p = 0.262	p = 0.282	p = 0.996
Retest 5 D17	0	0.01	0.01			N.A.	N.A.	N.A.
Retest 6 D19	0	0.01	0.04			N.A.	N.A.	N.A.
1.Reacquisition D22	0.25	0.24	0.36			p = 0.623	p = 0.570	p = 0.313
1.Reacquisition D23	0.43	0.32	0.48			p = 0.231	p = 0.802	p = 0.203
Extinction D23	0.23	0.13	0.30			N.A.	N.A.	N.A.
2.Reacquisition D24	0.42	0.37	0.46			p = 0.737	p = 0.529	p = 0.365
Retest D27	0.40	0.17	0.41			N.A.	N.A.	N.A.

Values represent mean.
Fraction eyelid closure (FEC) CRs during CS-only trials, divided into sessions and experimental groups. (2nd–4th column) Mean FEC CRs per experimental mouse group and session. Statistics testing for the (5th column) changes in the FEC of CRs within one experimental group over the 5 acquisition days, and (6th–9th column) the differences between all experimental sessions and experimental groups.

deprived of sleep in a window later than four hours after acquisition, showed a similar level of consolidation and retention as mice with sleep *ad libitum*; this level of consolidation was even similar when we tested mice during a different circadian rhythm as an additional control. Moreover, mice with *sleep ad libitum* that slept longer directly following EBC showed a tendency to show more CRs on the subsequent days. Together, these data highlight that consolidation, retention and savings of cerebellar motor memories are supported by sleep, being most effective in the first four hours following learning.

3.1. Sleep deprivation affects memory consolidation, retention and savings

Mice that were allowed to have sleep *ad libitum* between daily training sessions over 5 consecutive days showed a better consolidated delayed EBC task compared to mice that were sleep-deprived directly after acquisition. Moreover, sleep-deprived mice also showed deficits in retention of the memory and savings after extinction and subsequent reacquisition. Our data are in line with the observation that activity in the cerebellar cortex can be correlated with the level of consolidation of procedural motor memories (Lewis, Couch, & Walker, 2011), while decreases in cerebellar gray-matter (Fogel et al., 2017) or deficits in cerebellar cortical processing (Galliano et al., 2013; Wulff et al., 2009) can be correlated with deficits thereof. It should be noted though that some mice were able to learn the EBC task within one training session before sleep deprivation and that all mice sleep-deprived directly after acquisition were able to learn the task sufficiently at the end of the five days of training. Together, these findings suggest that sleep during the first 4 h after acquisition is not obligatory for consolidation of EBC, but that instead it has a faciliatory role in this process.

Why does sleep loss after memory acquisition affect consolidation, retention and savings of cerebellum-dependent EBC? Sleep deprivation may impair synaptic plasticity (Cirelli, 2013; McDermott et al., 2003; Ravassard et al., 2009; Tartar et al., 2006), but the link between these two processes remains to be further elucidated at both the molecular and structural level (Longordo, Kopp, & Lüthi, 2009). With regard to the impact of sleep on consolidation of procedural memories, the cerebellar nuclei may be particularly relevant (Attwell, Cooke, & Yeo, 2002; Krupa & Thompson, 1997). Whereas acquiring eyeblink responses may largely depend on synaptic plasticity at the inputs to Purkinje cells and molecular layer interneurons (Boele et al., 2018; ten Brinke et al., 2015), consolidation may depend more on cell physiological and structural changes of synapses in the nuclei downstream (Canto, Broersen, & De Zeeuw, 2018; ten Brinke et al., 2017). Thus, it will be interesting to find out to what extent sleep and/or deprivation thereof can affect consolidation of EBC through synaptic effects in the cerebellar nuclei.

Table 4
Pavlovian eyeblink conditioning. The fraction eyelid closure (FEC) per session and experimental group for paired trials.

Fraction eyelid closure paired trials	Group 1 (*ad libitum* sleep)	Group 2 (SD 0–4 hrs after acquisition)	Group 3 (SD 4–8 hrs after acquisition)	Repeated measures (acquisition sessions)	ANOVA on LME model on all sessions	Group 1 vs. Group 2	Group 1 vs. Group 3	Group 2 vs. Group 3
Acquisition D1	0.05	0.08	0.09	Group 1	Condition * Session	p = 0.416	p = 0.245	p = 0.749
Acquisition D2	0.25	0.16	0.24	(4, 13.14) = 75.73	(14, 31.92) = 2.63	p = 0.087	p = 0.914	p = 0.108
Acquisition D3	0.43	0.27	0.33	p < 0.001	p < 0.05	p = 0.013	p = 0.151	p = 0.281
Acquisition D4	0.54	0.34	0.49	Group 2	Condition	p = 0.003	p = 0.448	p = 0.027
Acquisition D5	0.62	0.42	0.47	(4, 13.07) = 8.44	(2, 30.29) = 2.23	p = 0.007	p = 0.072	p = 0.354
1.Reacquisition D22	0.26	0.17	0.29	p < 0.01	p = 0.125	p = 0.994	p = 0.096	p = 0.049
1.Reacquisition D23	0.42	0.38	0.43	Group 3	Session	p = 0.066	p = 0.841	p = 0.154
2.Reacquisition D24	0.48	0.31	0.41	(4, 26.04) = 23.31	(7, 32.45) = 52.11	p = 0.023	p = 0.534	p = 0.162
				p < 0.001	p < 0.05			

Values represent mean.
Fraction eyelid closure (FEC) CRs during paired trials, divided into sessions and experimental groups. (2nd–4th column) Mean FEC CRs per experimental mouse group and session. Statistics testing for the (5th column) changes in the FEC of CRs within one experimental group over the 5 acquisition days, and (6th–9th column) the differences between all experimental sessions and experimental groups.

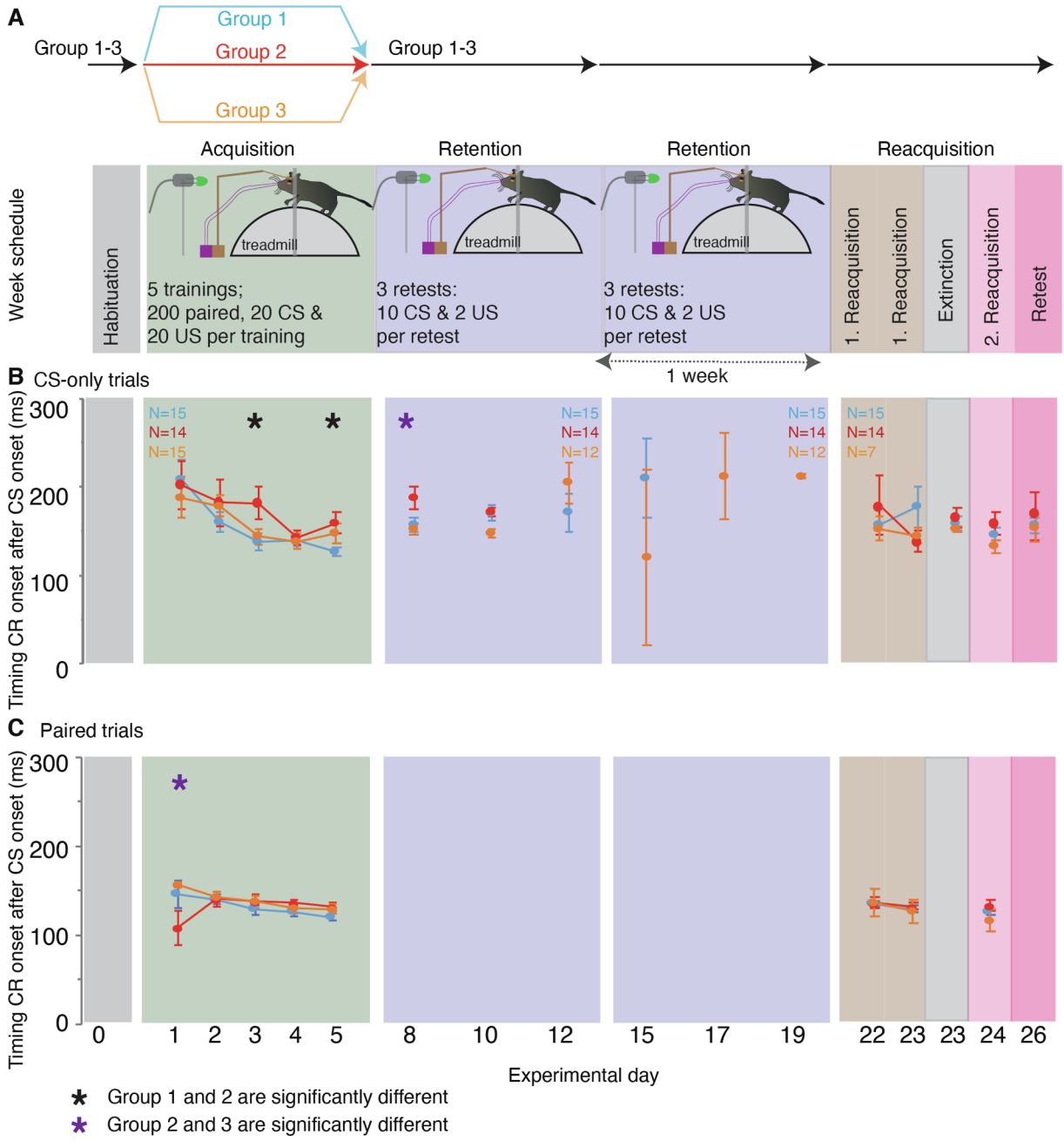

Fig. 4. CR onset times per session. (A) The experimental outline. (B and C) The timing of CRs onset times per training day and session for (B) CS-only trials and (C) paired trials. The color coding of the asterisks is indicated at the bottom. Color coding of groups: Blue = Group 1, red = Group 2, orange = Group 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Time-window dependent effect of sleep deprivation

Mice that were sleep-deprived between 4 and 8 h after training showed consolidation curves that were similar to those of mice with sleep *ad libitum*, independent from the shift in the circadian rhythm that presumably took place due to the sleep deprivation. These data indicate that the period of 0–4 h after acquisition may form a critical window for long-term consolidation and retention. Thus, similar to the effects of sleep on declarative memory (Graves, Heller, Pack, & Abel, 2003; Hagewoud et al., 2010; Havekes et al., 2014 and 2016; Prince et al., 2014), sleep after EBC is most effective during the first 4 h after training. This time-window dependent impact of sleep on EBC is supported by a similar positive impact of concurrent exercise such as locomotion activity (Albergaria, Silva, Pritchett, & Carey, 2018; Thomas et al., 2016) . Possibly, such exercise not only raises synergistic actions in cerebellar processing, but also improves the overall level of attention, which may facilitate learning in general.

3.3. Potential contribution of circadian rhythm on delayed eyeblink conditioning

We cannot exclude that the circadian rhythm disturbances due to sleep deprivation during different day-night cycles also contributed to our results. Even though the long-term consolidation and memory

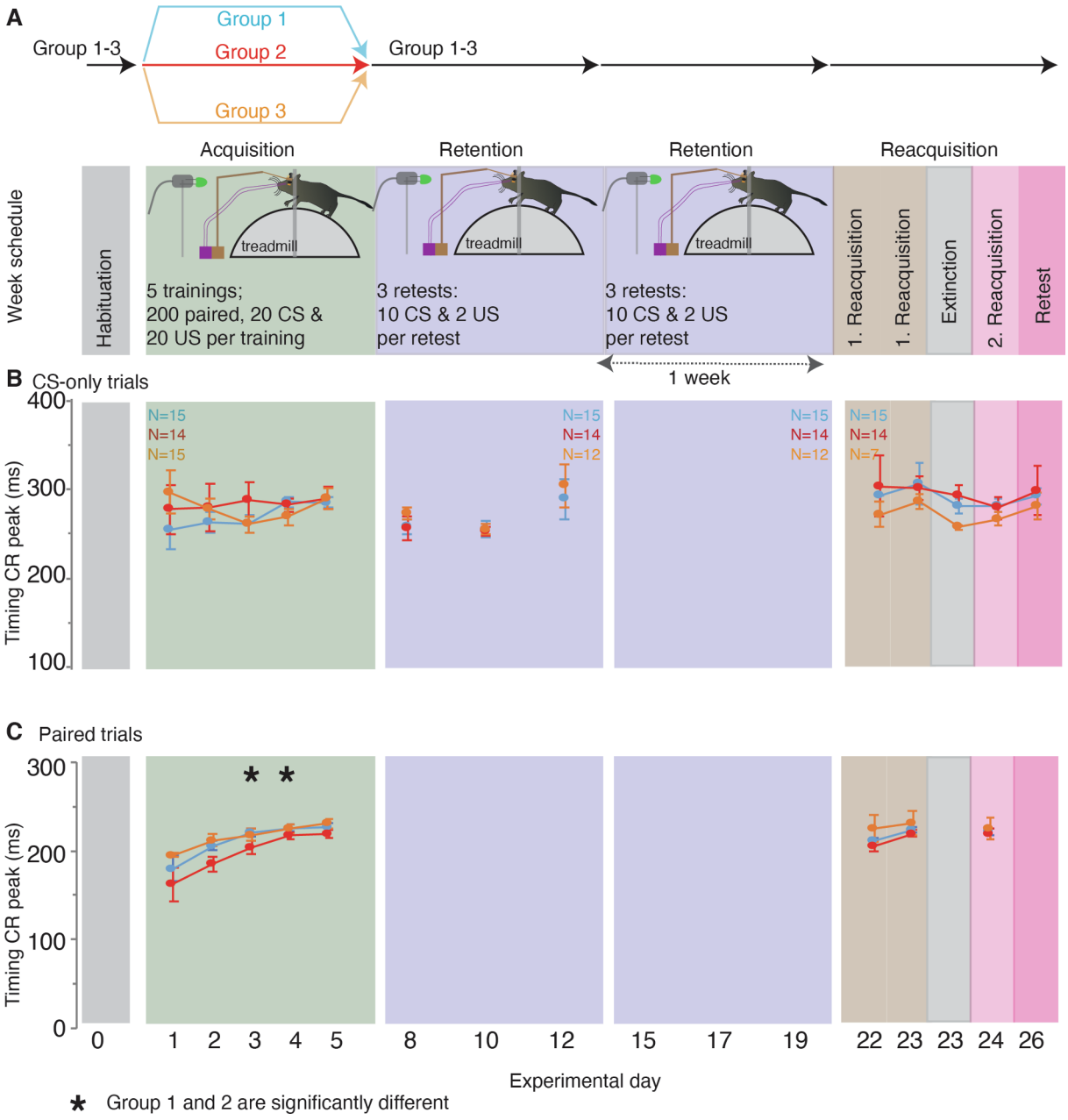

Fig. 5. CR peak time per session. (A) The experimental outline. (B and C) CR peak time per training day and session for (B) CS-only trials and (C) paired trials. The color coding of the asterisks is indicated at the bottom. Color coding of groups: Blue = Group 1, red = Group 2, orange = Group 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

retention were not significantly affected by general circadian rhythm problems in Group 3 mice, the initial acquisition appeared to be affected by them (Suppl. Fig. 5). Specialized studies are needed to further crystalize how eyeblink acquisition and consolidation differs between wake/sleep cycles and how important the duration and quality of sleep is for the acquisition and consolidation of memory.

3.4. Sleep-stage and site dependent consolidation

Specific sleep stages have been shown to affect motor sequence learning and procedural memory formation in human subjects (Barakat et al., 2013; Cousins et al., 2016). Accordingly, sleep stages can be correlated with the level of conditioned eyeblink responses in newborn infants and during quiet sleep infants are more likely to show conditioned eyeblink responses (Fifer et al., 2010; Tarullo et al., 2016), raising the possibility that NREM and REM sleep might play distinct roles during the acquisition, retention and savings of conditioned eyeblink responses. In our study in mice, we have been able to detect subtle correlations between sleep 1–3 h after acquisition and EBC performance on subsequent days. Our data suggest that it is especially the NREM period of sleep that may positively modulate EBC performance (see Suppl. Tables 3 and 4). When analyzing the impact of different sleep stages on the level of memory consolidation, one may also have to take the brain location into account. Indeed, as sleep deprivation can lead over time to differential effects on different parts of the brain, including differences among the left and right side (Achermann, Finelli, & Borbély, 2001), it may be interesting to analyze not only the stage, but also the precise location of the area with the electrophysiological sleep

Table 5
Pavlovian eyeblink conditioning. The timing and peak time of CRs per session and experimental group for CS-only trials.

CR Timing CS-only trials	Group 1 (*ad libitum* sleep)	Group 2 (SD 0–4 hrs after acquisition)	Group 3 (SD 4–8 hrs after acquisition)	Repeated measures (acquisition sessions)	ANOVA on LME model on all sessions	Group 1 vs. Group 2	Group 1 vs. Group 3	Group 2 vs. Group 3
Acquisition D1	209	202	187	Group 1	Condition	N.A	N.A.	N.A.
Acquisition D2	160	182	178	(4, 6.51) = 6.51	(2, 2.01) = 12.79	p = 0.391	p = 0.364	p = 0.945
Acquisition D3	138	181	143	p < 0.05	p = 0.0725	p = 0.024	p = 0.766	p = 0.062
Acquisition D4	139	143	138	Group 2	Session	p = 0.691	p = 0.986	p = 0.694
Acquisition D5	127	159	147	(4, 6.16) = 1157.10	(12, 9.63) = 17.28	p = 0.027	p = 0.143	p = 0.053
Retest 1 D8	157	187	151	p < 0.001	p < 0.001	p = 0.053	p = 0.676	p = 0.031
Retest 2 D10	170	171	147	Group 3		p = 0.542	p = 0.340	p = 0.273
Retest 3 D12	171	N.A.	205	(4, 11,09) = 0.06		N.A.	N.A	N.A
Retest 4 D15	N.A.	N.A.	N.A.	p < 0.001		N.A.	N.A.	N.A.
Retest 5 D17	N.A.	N.A.	N.A.			N.A.	N.A.	N.A.
Retest 6 D19	N.A.	N.A.	N.A.			N.A.	N.A.	N.A.
1.Reacquisition D22	157	179	152			p = 0.569	p = 0.873	p = 0.514
1.Reacquisition D23	177	138	145			p = 0.193	p = 0.296	p = 0.898
Extinction D23	158	165	152			N.A.	N.A.	N.A.
2.Reacquisition D24	146	159	132			p = 0.340	p = 0.332	p = 0.081
Retest D27	157	166	154			N.A.	N.A.	N.A.
CR peak time CS-only trials								
Acquisition D1	255	277	297	Group 1	Condition * Session,			
Acquisition D2	263	280	279	(4, 3) = 1.396	Condition,			
Acquisition D3	262	288	261	p = 0.41	Session			
Acquisition D4	286	283	269	Group 2	All not significant			
Acquisition D5	258	290	290	(4, 3) = 0.082				
Retest 1 D8	258	256	273	p = 0.966				
Retest 2 D10	255	253	256	Group 3				
Retest 3 D12	289	N.A.	303	(4, 11,09) = 3.422				
Retest 4 D15	N.A.	N.A.	N.A.	p = 0.093				
Retest 5 D17	N.A.	N.A.	N.A.					
Retest 6 D19	N.A.	N.A.	N.A.					
1.Reacquisition D22	292	303	272					
1.Reacquisition D23	305	301	286					
Extinction D23	281	293	258					
2.Reacquisition D24	281	279	267					
Retest D27	292	298	281					

Values represent mean.
Timing CRs and peak time of CRs during CS-only trials, divided into sessions and experimental groups. (2nd–4th column) Mean timing of CRs per experimental mouse group and session. Statistics testing for the (5th column) changes in the timing of CRs within one experimental group over the 5 acquisition days, and (6th–9th column) the differences between all experimental sessions and experimental groups.

characteristics involved. Thus, sleep appears to modulate eyeblink learning positively in general, but specific stages and sites might be more relevant than others.

3.5. Relation sleep and declarative memory formation

Whereas procedural memory formation depends mainly on the cerebellum, declarative memory formation depends to a large extent on areas like the hippocampus (Scoville & Milner, 1957). In line with our current findings, sleep also has a positive impact on memory consolidation of declarative memories (Cox et al., 2012; Diekelmann, 2014; Durmer & Dinges, 2005; Graves et al., 2003; Hagewoud et al., 2010; Havekes et al., 2016; Prince & Abel, 2013; Stern, 1971; Takashima et al., 2009; Talamini, Nieuwenhuis, Takashima, & Jensen, 2008; Van Der Werf, Van Der Helm, Schoonheim, Ridderikhoff, & Van Someren, 2009; Verweij et al., 2016). The effects of sleep deprivation on hippocampus-dependent declarative memory consolidation are also time-window dependent with sleep deprivation for up to 4 h after acquisition also being most effective (Graves et al., 2003; Hagewoud et al., 2010; Havekes et al., 2014 and 2016; Prince et al., 2014). Moreover, the related memory traces have been suggested to be transferred from the hippocampus to targets downstream in cerebral cortex during processes of consolidation (Kitamura et al., 2017). These data raise the possibility that some of the molecular and physiological mechanisms that underlie consolidation of procedural memory formation (Boele, Koekkoek, De Zeeuw, & Ruigrok, 2013; Okamoto, Endo, Shirao, & Nagao, 2011), may be analogous to those that are instrumental in consolidating declarative memories.

3.6. Clinical relevance

Understanding the effects of sleep and sleep deprivation on EBC will eventually allow us to evaluate whether EBC can also be used in the clinic to test for sleep-problem associated, cerebellum-dependent, memory deficits. Likewise, our data will allow us to extrapolate which time-windows might be of importance for cerebellum-dependent memory consolidation to occur and thus which are the time-intervals where sleeping conditions can interfere with cerebellum-dependent memory performance in our daily life. Indeed, sleep problems can lead to deficits in cerebellar learning (Fogel et al., 2017), whereas sleeping after motor learning or sports exercise may help to perform better (Dal Maso, Desormeau, Boudrias, & Roig, 2018). Interestingly, vice versa, cerebellar disorders can also contribute to sleep problems. For example, cerebellar lesions can lead to excessive daytime somnolence, REM sleep behavior disorders, and restless leg syndrome (Dang & Cunnington, 2010; Howell, Mahowald, & Gomez, 2006; Pedroso et al., 2011; Pedroso et al., 2011; Reimold et al., 2006). Accordingly, approximately 40–80 percent of autistic children, most of whom suffer from maldevelopment of the cerebellum (Stanfield et al., 2008; Stoodley, 2014; Wegiel et al., 2014), have sleeping problems (Canto, Onuki, Bruinsma, van der Werf, & De Zeeuw, 2017; Souders et al., 2017). Because of the mutual effects between sleep deficits and cerebellar deficits, it is not always clear to what extent the specific deficits involved are the cause and/or the consequence of the symptoms under investigation. The current study sheds some light on this question in that it revealed that sleeping directly after acquisition of a motor skill can positively affect the process of consolidation in both the short-term and long-term.

Table 6
Pavlovian eyeblink conditioning. The timing of CRs per session and experimental group for paired trials.

CR Timing paired trials	Group 1 (*ad libitum* sleep)	Group 2 (SD 0–4 hrs after acquisition)	Group 3 (SD 4–8 hrs after acquisition)	Repeated measures (acquisition sessions)	ANOVA on LME model on all sessions	Group 1 vs. Group 2	Group 1 vs. Group 3	Group 2 vs. Group 3
Acquisition D1	145	108	156	Group 1 (4, 8.43) = 7.65 p < 0.05 Group 2 (4, 4.16) = 3.61 p = 0.16 Group 3 (4, 21.07) = 2.568 p = 0.07	Condition * Session (14, 19.92) = 1.33 p = 0.276 Condition (2, 36.66) = 2.78 p = 0.075 Session (7, 20.03) = 10.880 p < 0.001	p = 0.160	p = 0.653	p = 0.045
Acquisition D2	139	141	142			p = 0.736	p = 0.630	p = 0.894
Acquisition D3	128	138	137			p = 0.202	p = 0.282	p = 0.839
Acquisition D4	125.	136	130			p = 0.200	p = 0.329	p = 0.181
Acquisition D5	120	132	129			p = 0.130	p = 0.094	p = 0.419
1.Reacquisition D22	134	136	136			p = 0.195	p = 0.584	p = 0.107
1.Reacquisition D23	129	132	127			p = 0.237	p = 0.809	p = 0.447
2.Reacquisition D24	125	133	116			p = 0.130	p = 0.613	p = 0.170
CR peak time paired trials								
Acquisition D1	180	162	195	Group 1 (4, 1) = 13.616 p = 0.2 Group 2 (4, 4) = 2554 p = 0.193 Group 3 (4, 6) =22.12 p = 0.001	Condition * Session (14, 11.30) = 1.53 p = 0.238 Condition (2, 12.75) = 2.52 p = 0.119 Session (2, 11.52) = 10.22 p < 0.001	p = 0.625	p = 0.387	p = 0.152
Acquisition D2	205	185	212			p = 0.81	p = 0.854	p = 0.074
Acquisition D3	221	204	218			p = 0.005	p = 0.146	p = 0.146
Acquisition D4	225	217	225			p = 0.028	p = 0.612	p = 0.091
Acquisition D5	226	219	231			p = 0.122	p = 0.932	p = 0.115
1.Reacquisition D22	211	205	205			p = 0.801	p = 0.593	p = 0.436
1.Reacquisition D23	129	132	127			p = 0.539	p = 0.211	p = 0.090
2.Reacquisition D24	221	219	116			p = 0.833	p = 0.519	p = 0.418

Values represent mean
Timing CRs and peak time of CRs during during paired trials, divided into sessions and experimental groups. (2nd–4th column) Mean timing of CRs per experimental mouse group and session. Statistics testing for the (5th column) changes in the timing of CRs within one experimental group over the 5 acquisition days, and (6th–9th column) the differences between all experimental sessions and experimental groups.

4. Materials and methods

4.1. Animals and regulations

Male C57BL/6J (Janvier) mice (N = 5750) 3–4 weeks of age (when handling started) were used for the experiments. All mice were born at the Institute and we always performed experiments with 3 male siblings simultaneously to reduce variability between mice. Mice were housed in a standard 12-hour light/12-hour dark cycle with access to food and water *ad libitum*. Acquisition, retests and reacquisitions were performed during the light cycle. The experiments started 1–2 h after light cycle started. The cage of mice was enriched with one running-wheel and nesting material. All experiments were approved by the institutional animal care and use committee of the Royal Netherlands Academy of Arts and Sciences and complied with all relevant ethical regulations.

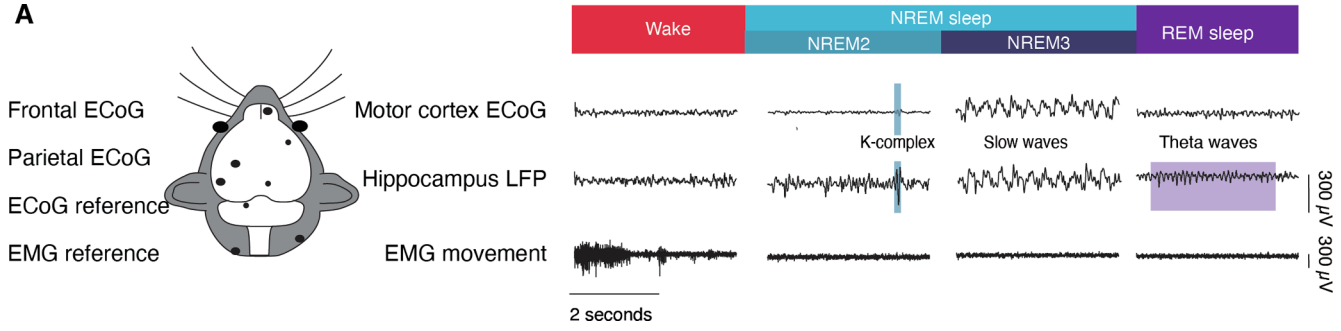

Fig. 6. Vigilance states of group 1 and 2 mice are similar after acquisition and sleep deprivation, respectively. (A) To the left approximate locations of ECoG, LFP and EMG recording electrodes with recordings of different arousal states to the right. We were able to discriminate between wake (red), non-rapid eye movement (NREM) sleep (blue), and REM sleep (purple) stages. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4.2. Handling

Prior to surgery mice were handled 6–10 times in 2 weeks to reduce stress. After surgery the animals were handled another 4–7 times to reduce stress prior to experiments.

4.3. Animal preparation

Mice were prepared for the experiments by placing 4 electrocorticographic (ECoG) electrodes, 1 local field potential (LFP) electrode, 2 electromyographic (EMG) electrodes and a pedestal under isolfuorane anesthesia (5% for induction, 1.5–2.3% in 0.5 L/min O_2 and 0.2 L/min air). The electrodes were placed on the motor cortex, frontal cortex, parietal cortex, hippocampus and cerebellum to study sleep, whereas the pedestal is needed to head-fix the mouse in the EBC setup. Mice were mounted into a stereotaxic head holder (David Kopf Instruments, USA) with a heating pad under the ventral surface of the mouse. Eyes were covered with Terra-cortril eye cream (Pfizer, USA) to prevent drying out. The skin on top of the head was shaved and cut sagittal to expose the bone, a scraper used to clear the periosteum. Three <2 mm diameter holes for the EEG and LFP electrodes, respectively, were drilled over the motor cortex (1.5 mm frontal and 2.0 mm lateral from bregma), one over the frontal and one over the parietal cortex, one over the hippocampus (2.0 mm caudal and 1.5 mm lateral from bregma) and one over the cerebellum. ECoG electrodes were made from silver wires that were bent at the end soldered to golden connectors (Multi-Contact Stäubli Group, Switzerland). LFP electrodes were polyamide coated stainless steel wires (0.05 mm diameter (PlasticOne, Germany)) with the ends being stripped of the coating to ensure electrical continuity. Each piece was soldered onto the legs of a golden IC connector (Multi-Contact; Stäubli Group, Switzerland). After ECoGs and LFPs were placed, primer and adhesive were applied according to manufacturer's specification (Kerr, Orange, USA). A pedestal was attached on top with dental acrylic (flowline or Tertric EvoFlow; Heraeus Kulzer, Germany). Animals received analgesia in the form of Metacam (AUV, 2 mg/kg) and were allowed to recover for at least 7 days.

4.4. EBC set up

Mice were conditioned in a sound proof light-isolated blink box (Blink 2.0 Neurasmus). They were head-fixed with a pedestal on the cylindrical treadmill. A green LED is used as the CS and stands 7 cm in front of the mouse. A plastic gauge needle tip (MPPI-3) positioned approximately 5 mm from the left eye delivers an air puff (US) at a pressure of 35–40 psi. The kinetic and frequency domain properties of conditioned and unconditioned eyelid responses was measured using a Magnetic Distance Measurement Technique (MDMT) (Boele et al., 2018; Koekkoek, Den Ouden, Perry, Highstein, & De Zeeuw, 2002). A small magnet (1.5 mm by 0.5 mm) was glued under the left eye. In the box a magneto-sensitive chip was placed above this magnet which measures the magnetic field.

4.5. Habituation protocol

Prior to training mice were habituated for 5 days in the blink box. The first day animals are placed in the setup for 10 min, the second day for 20 min and the third day for 30 min. The fourth and fifth day, animals receive 2 US-only and 20 CS-only trials and remain in the setup for 45 and 60 min, respectively.

4.6. Training protocol

Delay EBC was achieved by first shinning the LED (CS) for a duration of 260 ms, after an inter-stimulus interval (ISI) of 250 ms, an air puff (US) is delivered for 10 ms. Both the light and puff co-terminate after 260 ms. An inter-trial interval (ITI) of minimal 10 s is set between trials. All animals received one continuous training session per 24 h. The training was performed 1–2 h after the lights were turned on, which is the time point where the sleep pressure on mice is very high. Control group (C) (Suppl. Fig. 5) forms an exception, with the acquisition and the 4 hours sleep deprivation following acquisition ending before light onset. Every session consisted of 240 trials, of which 200 paired trials, with equally interspersed 20 CS-only and 20 US-only trials. Moreover, to quantitatively assess the impact of the training session involved as a whole, we inserted a quick retest of 10 CS-only and 2 US-only trials 4 h after each acquisition session. The acquisition / training was performed by experimenter 1. After the first training animals were randomly subdivided into the different experimental groups by experimenter 2. This was performed as a double-blind experiment.

4.7. Sleep deprivation device

A rotating drum (∅ 39 cm, height 37 cm), divided into 4 semicircular compartments by stationary central walls (Technicoplast, France) developed at the University of Grenoble and assembled, adapted and optimized at the Netherlands Institute for Neuroscience (NIN) (Leenaars et al., 2011) was used for sleep deprivation. We added circular openings in the walls and placed half-circular openings at the boundary between wall and platform to allow for social interaction between mice, each placed in one of the compartments of the device. Drum, wall and lids consist of Plexiglas. The bottom consists of an aluminum diamond plate to prevent mice from sliding. The bottom was covered with sawdust from the home cages. Water and food were provided. The devices are driven by a computer-controlled motor (MACDO-B1, JVL, Denmark), which runs bi-directionally. The motor is connected to the drum via a belt. During the sleep deprivation the device rotates 1 min clock-wise, 1 min counter-clockwise with a break of 10 s in between rotations at a speed of 2 rotations per minute.

4.8. Experimental groups

We subdivided mice into 4 experimental groups (Fig. 1). Group 1 was a control group of mice that were allowed to have *ad libitum* sleep in their home cage after acquisition. In a subpopulation of these mice we measured sleep. Mice were connected to the EEG recorder (adapted MEA60, Multichannel systems, Germany) via a counterbalanced swivel (Air Precision, France). All channels were sampled at 10,000 Hz. In some animals the wires broke during the recording or noise entered the recording system, which disabled us from analyzing those groups and those sleep sessions were excluded from the analysis. Group 2 was a group of mice that was sleep-deprived for 4 h after acquisition of the eyeblink task, whereas group 3 was sleep deprived 4 to 8 h after acquisition of the eyeblink task. The latter group was tested to study the effects of the sleep deprvation procedure during a different time interval on mice. Control group (C) followed the protocol of Group 3 except that the acquisition and resting period ended before light onset with the sleep deprivation period starting at the beginning of the light period.

4.9. Retention/retest protocol

After acquisition (D1-5) animals were retested 3 (D8), 5 (D10), 7 (D12), 10 (D15), 12 (D17), and 14 (D19) days after learning to test long-term memory consolidation. A retest/retention consisted of 1 × US trial, 10 × CS-only trials, and subsequently a 1 × US-only trial (this retest protocol can also be considered as a short extinction protocol). After another short extinction on D22, we tested the ability of the mice to reacquire the task (retention) with another (re)acquisition session on D23. Between those reacquisition sessions, mice were allowed to have sleep *ad libitum*. In the afternoon (PM) of D23 a prolonged extinction protocol (at least 20 blocks of 1 US-only trial and 11

CS-only trials, totaling 20 US-only and 220 CS-only trials) was provided to the mice until no trace of memory was visible anymore. In the morning of D24 (AM) mice were allowed to reacquire the task again (Reacquisition 2; test savings after prolonged extinction).

4.10. Analysis and Statistics

Eyelid traces were analyzed with custom LabView (National Instrument, USA) or Matlab (Mathworks, USA) scripts and as published before (Boele et al., 2018). Eyelid traces were excluded, based on several criteria. If the UR amplitude changed significantly from the beginning until the end of the session ($>5 \times$ standard deviation), leading to a large coefficient of variation, the whole session was excluded. Or if significant squinting of the eye or baseline eyelid activity occurred up to 500 ms prior to CS stimulation, trials were excluded. Sessions for which >75% of trials were excluded, were not considered. Finally, sessions with large variation in UR were excluded. CRs were detected as eyelid closures during the last 200 ms of the CS-US interval that exceeded 10% of average UR amplitude. Furthermore, we had to exclude data of some mice due to additional reasons. Group 1; Data points of mouse #2773 and #2774 were excluded after the second retention week, because the pedestal and electrodes detached (the primer can lose its strength after being open for too many weeks). Group 2; Mouse #2751 was excluded after the first retest, because the pedestal broke off. Group 3; we excluded mouse #2776, #2790 and #2793, because the electrodes broke off after the last training session. Mouse #2777, #2778, #2779, #2782, # 2783 were not trained longer than the retention period, because there was a problem with the power-supply to the eyeblink boxes during the reacquisition weeks."

After exclusion criteria were met, included trials were aligned and normalized to the average of the baseline period. For all valid trials we determined the maximum fraction eyelid closure (=FEC), the latency to CR onset, and the latency to CR peak. The average amplitude of all unconditioned blink responses was used to denote 100% eyelid closure. The eyelid closure was then calculated as a percentage of movement from baseline to 100% eyelid closure amplitude and eventually averaged for all trials (Boele et al., 2018). To calculate the CR onset and the CR peak time we only used the trials in which a CR was present during the CS-US interval. CR onset was determined as the first time point of a continuous positive eyelid velocity leading to up to the fifth percentile of the amplitude from baseline to CR peak. Eyelid movements larger than 0.1 and with a latency to CR onset between 50 and 250 ms and a latency to CR peak of 100 to 250 ms (both relative to CS onset) were considered as CRs. For CS-only trials, we used the exact same criteria except that the latency to CR peak time was set at 100 to 500 ms after CS onset (Boele et al., 2018). In the text and figure mean ± SEM are presented with p-values indicated accordingly. Statistical effects were calculated in SPSS 25 using a linear mixed models analysis with an unstructured repeated covariance type and maximum likelihood method and LSD post-hoc testing to determine the effect of group and session on parameters and an repeated measures analysis. To assess the relationship between sleep and behavior, Pearson's correlations were performed. Data were considered significant if $p < 0.05$. For bootstrapping and calculation of distribution of the correlation values we used Fisher's Exact Test.

4.11. Sleep analysis

The EEGs and EMGs were continuously recorded without filtering. To analyze and score sleep the open access program https://wonambi-python.github.io/introduction.html was used. Offline, EEG was band-pass filtered (0.5–30 Hz) and power density spectra were calculated with a Fast Fourier Transform within a frequency range of 0.25–30 Hz. EEG and EMG signals were integrated. Three vigilance states were determined; waking, NREM sleep and REM sleep based on EEG and EMG criteria for mice. The level of sleep was determined by analyzing 4-second-long ECoG/EEG/EMG recordings. Active and quiet waking were defined by a desynchronized low amplitude ECoG/EEG. Theta activity in the parietal lobe and hippocampus was visible during active walking. NREM sleep was defined by low EMG, synchronized and high-amplitude ECoGs/EEGs with defined K-complexes or slow waves in the hippocampus and cortex. REM sleep can be defined by low amplitude ECoG/EEG and EMG signals (Seibt et al., 2017). Epochs with long lasting artifacts were excluded from the analysis.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nlm.2020.107165.

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