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RESEARCH ARTICLE

Sleep and conditioning of the siphon withdrawal reflex in *Aplysia*

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ABSTRACT

Sleep is essential for memory consolidation after learning as shown in mammals and invertebrates such as bees and flies. *Aplysia californica* displays sleep, and sleep in this mollusk was also found to support memory for an operant conditioning task. Here, we investigated whether sleep in *Aplysia* is also required for memory consolidation in a simpler type of learning, i.e. the conditioning of the siphon withdrawal reflex. Two groups of animals (Wake, Sleep, each n=11) were conditioned on the siphon withdrawal reflex, with the training following a classical conditioning procedure where an electrical tail shock served as the unconditioned stimulus (US) and a tactile stimulus to the siphon as the conditioned stimulus (CS). Responses to the CS were tested before (pre-test), and 24 and 48 h after training. While Wake animals remained awake for 6 h after training, Sleep animals had undisturbed sleep. The 24 h test in both groups was combined with extinction training, i.e. the extended presentation of the CS alone over two blocks. At the 24 h test, siphon withdrawal duration in response to the CS was distinctly enhanced in both Sleep and Wake groups with no significant difference between groups, consistent with the view that consolidation of a simple conditioned reflex response does not require post-training sleep. Surprisingly, extinction training did not reverse the enhancement of responses to the CS. On the contrary, at the 48 h test, withdrawal duration in response to the CS was even further enhanced across both groups. This suggests that processes of sensitization, an even simpler non-associative type of learning, contributed to the withdrawal responses. Our study provides evidence for the hypothesis that sleep preferentially benefits consolidation of more complex learning paradigms than conditioning of simple reflexes.

KEY WORDS: Long-term memory, Classical conditioning, Sensitization, Sleep deprivation, Memory consolidation, Invertebrate, Mollusk

INTRODUCTION

Without doubt, sleep plays an important role in memory consolidation (Rasch and Born, 2013; Vorster and Born, 2015; Donlea, 2019). The formation of adaptive long-term memory appears to be one of the major functions of sleep (Klinzing et al., 2019). Sleep seems to support memory formation through an active systems consolidation process where the repeated neuronal reactivation of newly encoded memories transforms these memory representations such that they become more abstract and schema-like, and more easily retrievable in different conditions (Dudai et al., 2015; Klinzing et al., 2019). However, it is debatable whether the memory-enhancing effect of sleep applies to all kinds of memory. Whereas sleep seems to be fundamentally necessary for higher forms of learning including episodic and procedural memories as well as operant conditioning, it has been hypothesized that simpler types of associative and non-associative learning such as classical conditioning, sensitization and habituation might not benefit from sleep as they are primarily mediated through synaptic consolidation processes, and do not implicate any systems consolidation and trace transformation (Dudai, 2012; Dudai et al., 2015).

In rodents, consistent evidence has indeed accumulated that sleep supports complex types of learning (e.g. Sawangjit et al., 2020, 2018; Hunter, 2015; Melo and Ehrlich, 2016; Pace-Schott et al., 2009, 2012; Silvestri, 2005), whereas findings are inconclusive regarding the role of sleep in more simple forms of learning such as cued fear conditioning (Cai et al., 2009; Graves et al., 2003; Kumar and Jha, 2012). A similar picture is found in invertebrates. In *Drosophila*, operant courtship conditioning is enhanced by sleep (Ganguly-Fitzgerald et al., 2006; Donlea et al., 2011; Dissel et al., 2015; Dag et al., 2019), whereas effects of sleep are less consistent with simpler types of learning, such as classical conditioning (Le Glou et al., 2012). In honeybees, Hussaini et al. (2009) did not find a sleep dependency of the classically conditioned proboscis extension response. However, its extinction, a more complex form of memory, benefits from sleep. Sleep dependency was also found in a complex pathfinding task for bees (Beyaert et al., 2012), as well as in an odor-associated contextual memory task (Zwaka et al., 2015). The latter study closely paralleled human studies that used contextual odor cues during slow wave sleep to reactivate hippocampal circuits, thereby increasing episodic memory retention (Rasch et al., 2007). This suggests that similar patterns of recurrent activation during sleep might support an active systems consolidation process for complex memories in insects and vertebrates (Vorster and Born, 2018).

The mollusk *Aplysia californica* has been enormously helpful unraveling the synaptic and circuit mechanisms underlying the fundamental forms of learning and memory related to habituation, sensitization, classical conditioning and operant conditioning (Hawkins, 2019; Hawkins and Byrne, 2015; Levitan et al., 2012). The sea slug possesses a relatively simple neuronal network, composed of only about 20,000 neurons that are organized in 5 paired ganglia, which makes it an ideal model organism for studies of the cellular and circuit mechanisms underlying learning and memory (Akhmedov et al., 2014). Notably, sleep was found to be essential for memory consolidation of inhibitory operant conditioning in *Aplysia* (Vorster and Born, 2017; Krishnan et al., 2016a,b). However, simpler forms of learning such as classical conditioning have not been evaluated for their dependency on sleep.

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Classical conditioning in *Aplysia* was first described more than 40 years ago using the siphon withdrawal reflex (Carew et al., 1981a,b, 1983; Hawkins et al., 1986, 1989, 1998). We here asked whether the persistence of this classically conditioned reflex in *Aplysia* depends on sleep.

MATERIALS AND METHODS

Subjects, design and general procedure

Subjects were 30 *Aplysia californica* (James Graham Cooper 1863) (80–125 g; South Coast Bio-Marine, San Pedro, CA, USA), entrained to a 12 h:12 h light:dark cycle (lights on at 07:00 h). The *Aplysia* were assigned to a Sleep group (final n=11) or a Wake group (n=11). All were tested on three occasions: before (pre-test), and 24 and 48 h after training (24 h test and 48 h test). Each of the tests consisted of 7 presentations of the conditioned stimulus (CS, stimulation of the siphon with a chopstick, inter-trial interval 5 min; see below) and aimed to assess conditioning memory (see below). The pre-test took place at 14:45 h, and thus 75 min before the conditioning training, which started at 16:00 h (Fig. 1A). Conditioning in the Wake group was followed by sleep deprivation for 6 h. The 24 h test taking place at 16:00 h on the following day was extended into an extinction training. Both conditioning and extinction training were thereby timed such that they ended shortly before the dark phase. The 48 h test took place 48 h after conditioning and, thus, ∼24 h after extinction training. In order to explore the longevity of the induced memory, in a subset of Wake group animals an additional test was performed 72 h after conditioning (72 h test). Conditioning training was performed (by K.I.T.) with the experimenter blinded regarding the animal's experimental condition (Sleep versus Wake). Sleep deprivation and test sessions were performed by K.I.T. or A.P.A.V., with no differences in results between the experimenters.

Sleep deprivation

Wake group animals were sleep deprived during the first 6 h of the dark phase following conditioning training. A duration of 6 h of sleep deprivation was chosen as a compromise because, on the one hand, the period should be sufficiently long to effectively suppress consolidation but, on the other hand, it should stress the animal as little as possible. The animals were motivated to stay active during this period by presenting the odor of food and gentle handling. If animals did not show any motion for more than 1 min, they were gently displaced by means of a flexible plastic ruler (Vorster and Born, 2017, 2018), without touching the siphon. A maximum of 150 min of sleep during this initial 6 h period for Wake group animals and a minimum of 250 min of sleep for Sleep group animals

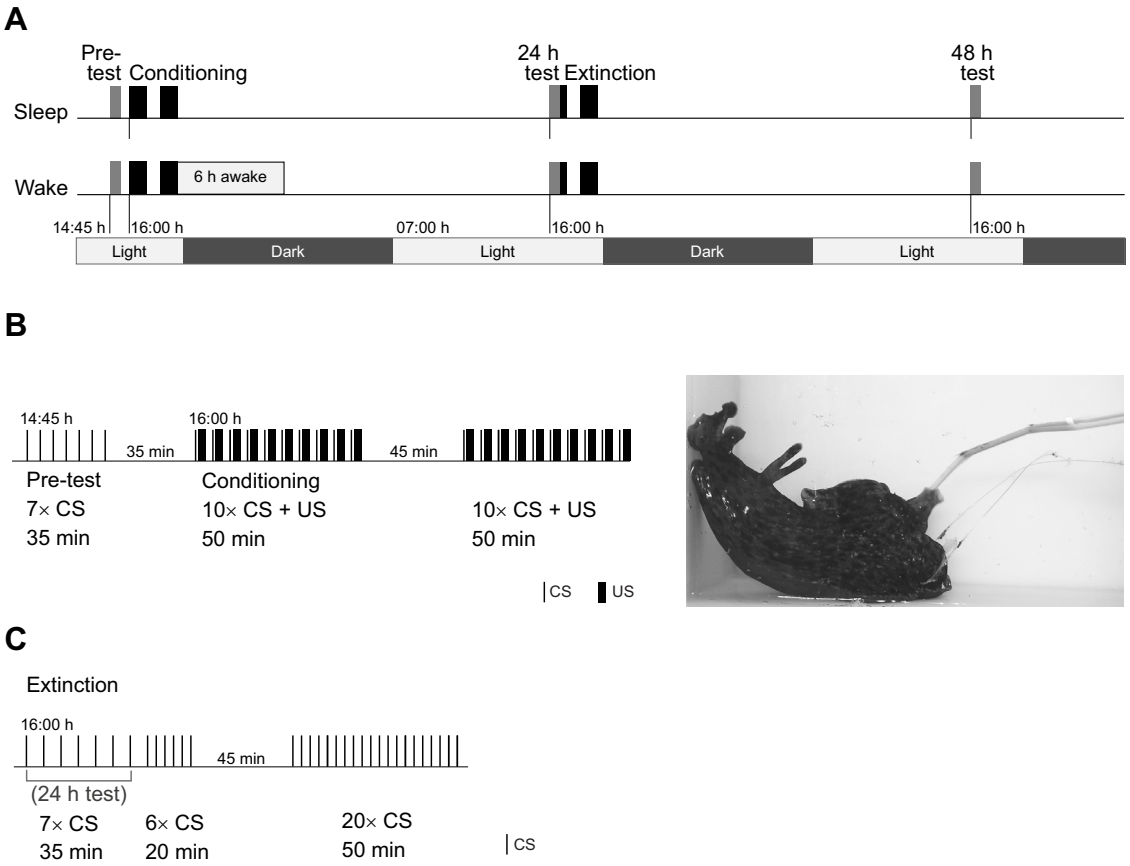

Fig. 1. Experimental design and procedures. (A) *Aplysia* were allocated to either the Sleep or Wake group (n=11 for each group) and tested on three occasions: before (pre-test), and 24 and 48 h after training (24 h and 48 h test). Each of the tests consisted of 7 presentations of the conditioned stimulus (CS, stimulation of the siphon with a chopstick; see photo) with an inter-trial interval of 5 min. The pre-test took place at 14:45 h, 75 min before conditioning training starting at 16:00 h. Conditioning in the Wake group was followed by a 6 h period of wakefulness induced through gentle handling. The 24 h test was extended into an extinction training. Both conditioning and extinction training were timed such that they ended shortly before the dark phase (19:00 h). (B) Conditioning started at 16:00 h (∼35 min after the last pre-test trial) and comprised 2 blocks of 10 trials (paired stimulations), separated by 45 min. The inter-trial interval was 5 min; the inter-stimulus interval between the CS and unconditioned stimulus (US, electric shock) presentation was 0.5 s. (C) Extinction training started at 16:00 h and integrated the 7 trials of the 24 h test (inter-trial interval 5 min) followed by 6 CS presentations with an inter-trial interval of 2.5 min and – after a 45 min break – a second block of 20 CS presentations (inter-trial interval of 2.5 min).

was chosen as *a priori* sleep criteria. In the Wake group, all subjects met the criterion; in the Sleep group, one animal was excluded from analyses, as it did not meet the criterion.

Surgery for siphon withdrawal reflex conditioning

As in the original experiment by Carew et al. (1981b), parapodia were clipped to attain visibility of the siphon in any relaxed or withdrawn state (parapodectomy). For this, the animals first underwent cold anesthesia, i.e. *Aplysia* were immersed for 7 min in iced artificial sea water at −2.5°C until the siphon withdrawal reflex could not be elicited. Cold-anesthetized animals were then laid on one side and the upper parapodial skin was lifted with forceps. A hemostat compressed the skin below the cutting edge, while a stripe of parapodial tissue was cut off, starting from the junction of the parapodia and tail (pseudosiphon). This junction is easily mistaken for the siphon, as both feature bright markings. Cutting as low as possible is thus important to avoid confusion (Fischer et al., 2000). As a guideline for cutting, the white, shiny marking at the upper part of the parapodia was used (Fig. S1C). We took care not to cut much below this area, as within the fleshier part of the parapodia a substantial amount of hemolymph might drain from a resulting skin defect. The procedure was repeated for the other parapodia. Subsequently, *Aplysia* were returned to the home tank, regaining normal body temperature. After the cold anesthesia, animals showed no inking or other aversive reactions.

To deliver shocks at equal current in all animals, the snails were implanted with stainless steel wires (15 cm, SS-5T/A, SS-8T/A, SS-10T/HH, Science Products GmbH) serving as electrodes (thickness: 125, 200 or 150 µm, 'flexible' or 'half hard'). Because of delivery retardation, it was not possible to use the identical wire in all animals. However, it was ensured that the resulting current flow did not vary between animals. All wires were coated with Teflon; this provided insulation from sea water. Fig. S1D depicts details of the implantation process. A 5 mm section of the insulation was stripped, 2 cm from one end of each wire, to create a contact zone. The wire was implanted to a depth of 4 cm into the left tail region of the anesthetized, relaxed animal. When animals were back in the home tank and at normal body temperature, they contracted. Thereby, the distance of the electrodes was reduced to approximately 1.5 cm. A change in distance, depending on the muscle tonus of the *Aplysia*, was also reported by Carew et al. (1981b). The wire was inserted into the skin of the slug's tail by means of hypodermic (hollow) needles. To secure the wire, it was bent in the contact area and both ends stuck out of the animal's skin. In this way, there was no contact of the bare wire with the sea water and current could only flow between the two wires inside the tissue. Hot glue was put on either end to prevent the electrode from being pulled out accidentally (Fig. S1D).

Conditioning

A classical conditioning paradigm was applied as described by Carew et al. (1981b) that aimed at enhancing the pre-existing siphon withdrawal reflex (Fig. 1B). An electric shock (2.8 mA, 1 s, 7 V AC; power supply: EA-3051B, EA Elektro-Automatik) delivered to the animal's tail via implanted stainless steel electrodes (SS-5T/A, Science Products GmbH) served as an unconditioned stimulus (US). The unconditioned response (UR) to the shock is a prolonged siphon withdrawal. A constant current level for each animal was ensured by continuously monitoring the current via an amperemeter (MS8229, MASTECH) during US delivery (Fig. S1B). To stabilize the flow of current, a 1 kΩ series resistor was included in the circuit. This intensity was determined in preliminary experiments as the minimal strength that still resulted in reliable conditioning. Current flow was activated by manually holding a pressure switch that was guided by an audio signal. As the CS, a tactile stimulation of the siphon with a wooden chopstick (Fackelmann) was used, which itself provokes only a very brief siphon withdrawal. The chopsticks were bent near the top to form hooks of different sizes and angles in order to best reach the siphon in whatever position the slug was at the time. In detail, the chopstick was inserted into the siphon, touching the inner, upper part and the stick was pulled upwards. Thereby, the inner part of the siphon skin was touched the whole way (roughly 1.5 cm) to the top. The whole procedure lasted about 0.5 s and was performed similarly to Carew et al. (1981b). The change in behavior after successful conditioning is indicated by a distinctly prolonged duration of the reflexive withdrawal response to the CS (Movie 1). For conditioning (starting 3 h before the dark period), we followed the scheme of Hawkins et al. (1989). Long-term sensitization of the siphon withdrawal is known to be greater when animals are trained and tested during the day (Fernandez et al., 2003), whereas learning is impaired during the night (Levy et al., 2016). Therefore, we trained and tested our animals prior to the onset of the dark period at 19:00 h (corresponding to Zeitgeber time 9), a time that is marked by increased locomotor activity and arousal (Vorster et al., 2014). We administered two training blocks of 10 CS–US pairings, with an interval of 45 min between the blocks. Each tactile CS was followed after 0.5 s by the electric shock (US). The inter-trial interval was 5 min. Siphon withdrawal was observed for a maximum period of 180 s. To achieve comparability of the two experimental groups with regard to training performance, animals that exhibited 5 or more trials of ≥180 s siphon withdrawal duration during conditioning training were excluded from analysis (n=7).

Extinction training

All *Aplysia* underwent extinction training 24 h after conditioning. Extinction training started at 16:00 h and overall consisted of 33 presentations of the CS. The first 7 of these 33 trials were the CS presentations used for the 24 h test (inter-trial interval 5 min). These were followed by 6 CS presentations with an interval-trial interval of 2.5 min and – after a 45 min break – a second block of 20 CS presentations (inter-trial interval 2.5 min; Fig. 1C).

Behavioral analysis

All test and training sessions were video-recorded and analyzed off-line. The start of the withdrawal duration was defined as the moment of deepest retraction, and its end when the siphon had reappeared by 80%. All videos were visually scored by the experimenter (K.I.T.), and the results of the pre-test, and 24 h and 48 h tests were verified by a second scorer who was blinded as to the experimental conditions (Verena Koppe). Activity was continuously monitored by an infrared camera accompanied by infrared light invisible to the animals (850 nm, SAL 35, B&S Technology) and analyzed semi-automatically (EthoVision XT 13 Noldus Information Technology, Wageningen, The Netherlands). The sleep-like state was scored whenever the animal exhibited no movements for at least 2 min, with the exception of slight rhinophore movements or respiratory pumping and siphon movement. Animals were scored as active when body movements were present, especially in the head and neck region. Total light intensity was <5 lx during the dark period and >100 lx during the light period.

Statistical analysis

For the test occasions, the median withdrawal time for an individual animal was used for analysis. Absolute values as well as difference

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values (the individual pre-test value subtracted from the 24 h or 48 h test value) were compared between the Sleep and Wake groups, using unpaired *t*-tests (with Welch's correction for unequal variances) for comparisons between groups, and paired *t*-tests for additional comparisons within groups. For differences in variance between groups, an *F*-test was performed. Additional ANOVA with Šidák's multiple comparisons were run to assess temporal dynamics of conditioning memory in the two groups. Basically, ANOVA included a group factor (Sleep/Wake) and repeated measures factors for the time points of testing (pre-test, 24 h test, 48 h test). Correlations were calculated using the Pearson's product moment correlation coefficient. A P-value <0.05 was considered significant.

RESULTS

We asked whether sleep in *Aplysia* supports consolidation of a classical conditioning memory. Both the Sleep and Wake group showed a prolonged siphon withdrawal at the 24 h test in comparison with the pre-test level. The mean (±s.e.m.) increase in withdrawal time (with reference to pre-test levels) for the Sleep

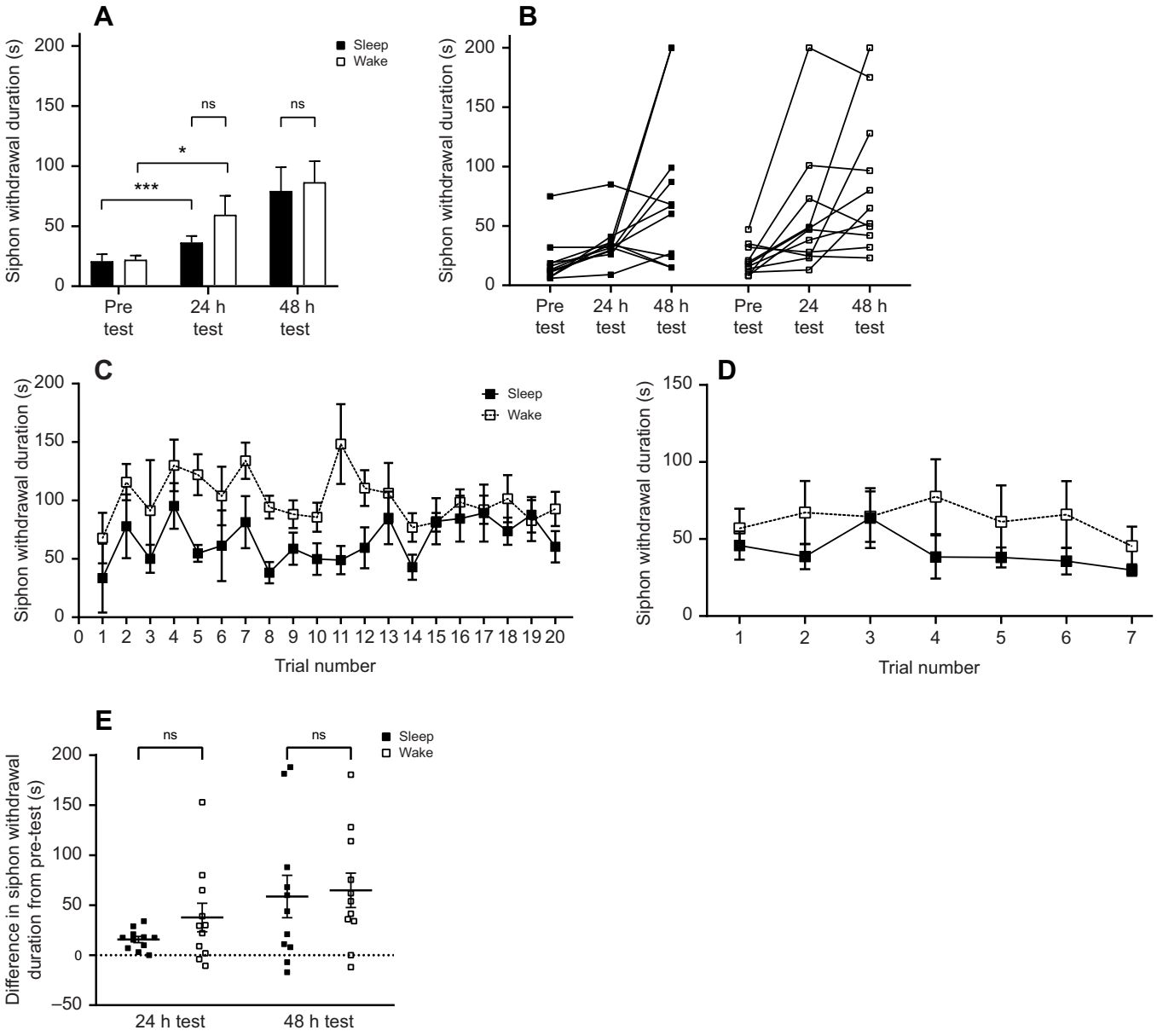

Fig. 2. Siphon withdrawal conditioning induces long-term memory at the 24 h test which survives extinction and is still present at the 48 h test. (A) Both Sleep and Wake group animals show prolonged siphon withdrawal duration after conditioning training (means±s.e.m. for pre-test, and 24 h and 48 h test). (B) Individual animal's withdrawal durations at the pre-test, and 24 h and 48 h test. Each data point represents the median of 7 withdrawal durations measured at each test. (C,D) Mean±s.e.m. siphon withdrawal duration in the Sleep and Wake groups for each trial of the conditioning training (C) and of the extinction training at the 24 h test (D). There were no significant differences between groups or significant changes over trials during conditioning or extinction (P>0.05; see Results). (E) Sleep and Wake groups show similar increases in withdrawal duration at the 24 h and 48 h tests compared with pre-test values. Data points show the difference in median withdrawal duration, with pre-test values subtracted from median durations at the 24 h and 48 h test for each animal. Group means (horizontal lines) ±s.e.m. (whiskers) are indicated. Withdrawal durations were further prolonged at the 48 h test compared with the 24 h test (P=0.016, see Results). Differences between groups were not significant (ns).

group was 15.7±3.1 s, and for the Wake group was 37.7±14.3 s, indicating that a persistent memory of the conditioned response was present in both groups (Fig. 2A,B). Although the Wake group, on average, displayed a greater increase in siphon withdrawal duration, neither this increase (P=0.16) nor absolute withdrawal duration (P=0.19) differed between groups. This appeared to be partly due to the Wake group showing a remarkable increase in variability in withdrawal duration among the animals at the 24 h test (F=20.95, P<0.0001, for the difference in variances between groups), while no such difference in variance was found in the pre-test of the siphon withdrawal between the Sleep and Wake group (F=1.213, P=0.7660). During conditioning, the Wake group showed a slightly longer withdrawal response across trials than the Sleep group (mean±s.e.m. absolute time, Sleep 65.73±4.20 s versus Wake 101.2±4.60 s; mean±s.e.m. difference of conditioning minus pre-test, Wake 76.6±8.4 s versus Sleep 50.4±11.9 s). However, this difference failed to reach significance (P=0.0902; Fig. 2C).

Whereas the amount of sleep in Wake group animals was greatly reduced to an average of 80 min during the 6 h interval after conditioning (range: 0–147 min), Sleep group animals achieved 317 min of sleep (range: 260–360 min; Fig. 3A). Still, Wake group animals did not catch up on sleep loss in the remaining time until the 24 h test. In fact, the difference in the amount of sleep persisted up to the 24 h test (mean±s.e.m. sleep time, Sleep 829±31 min, Wake 514 ±62 min, P=0.0004). We also did not find any significant correlation between the amount of sleep (6 h after training) and memory outcome measures at 24 h. These correlations were for the increase in siphon withdrawal duration (pre-test to 24 h test) across both groups (r=−0.3069, P=0.1647), and separately for the Sleep and Wake groups (r=−0.2704, P=0.4212 and r=0.0001, P=0.9997) (Fig. 3B).

Starting with the 7 test trials of the 24 h test, extinction training took place comprising two blocks of 13 and 20 trials, respectively. Responses during extinction training did not show systematic changes in the course of the training (linear regression across trials: Sleep r^2=0.0263, P=0.1733; Wake r^2=0.0025, P=0.6932) and also did not differ between groups (P=0.4020, 0.1680 and 0.6451, for time and group main effects and time×group interaction, respectively, in an ANOVA on the extinction trials; Fig. 2D). Responses at the 48 h test indicated that conditioning memory survived extinction training. At the 48 h test, both the Sleep and Wake group still showed a prolonged siphon withdrawal in comparison with pre-test levels (mean±s.e.m., Sleep 58.7±21.2 s, Wake 64.9±17.2 s; Fig. 2A,B), with no differences between groups (P=0.8229 and 0.7862, for the increase from pre-test levels and for absolute durations, respectively). Contrary to our expectation, siphon withdrawal duration did not decrease, but had further increased 24 h after the extinction training, i.e. at the 48 h test in comparison with the 24 h test ($F_{1,20}$=6.931, P=0.016, for effect of time in a 24 h test/48 h test×Sleep/Wake ANOVA, P=0.4252 and 0.5582, for main effect of Sleep/Wake and Sleep/Wake×24 h test/48 test interaction, respectively). The absolute increase in withdrawal duration from the 24 h test to the 48 h test was comparable in the two groups (mean±s.e.m. difference from 24 h to 48 h test, Sleep 43.0 ±20.7 s, Wake 27.1±16.8 s; P=0.5585, Welch's t-test). Given the unexpected persistence of the conditioning memory, we tested 9 Wake group animals a third time, 72 h after conditioning (Fig. 3C). Still, memory persisted in the form of increased siphon withdrawal duration, in comparison with pre-test levels (mean±s.e.m. increase from pre-test level, 66.2±11.0 s, P=0.0054, for difference between pre-test and 72 h test). Sleep during the 24 h following extinction training was comparable between the Sleep group (797 min, range: 540–961 min) and the Wake group (826 min, range: 401–1190 min; P=0.7434; Fig. 3A) which implies that the Wake group also did not recover lost sleep during this period. Accordingly, cumulative sleep duration over the total 48 h was still different between groups (mean ±s.e.m.: Sleep 1626±67 min, Wake 1339±96 min; P=0.0247).

DISCUSSION

Here, for studying the effects of sleep on memory consolidation, we adopted a landmark paradigm of classical conditioning of the siphon withdrawal reflex in *Aplysia californica*, which was developed in the 1980s. We found signs of an enhanced memory for the conditioned siphon withdrawal (i.e. prolonged withdrawal time) at a test 24 h after conditioning training, which did not significantly differ between the animals that slept or were kept awake during the 6 h after conditioning training. Contrary to our expectations, an extinction training introduced 24 h after conditioning did not diminish the duration of withdrawal responses to the CS. This failure of extinction was observed in both groups, i.e. independently of whether the original conditioning training was followed by sleep or wakefulness. Instead, in both groups at a test 48 h after conditioning and 24 h after extinction, withdrawal responses to the CS were even further enhanced. Another unexpected finding was that the *Aplysia* of the Wake group did not recover sleep (lost during a 6 h post-conditioning period of sleep deprivation) during the following 42 h.

Our findings confirm previous evidence that conditioning of the siphon withdrawal reflex by pairing a tactile stimulus to the siphon (CS) with an electric shock (US) leads to a prolonged siphon withdrawal duration in response to the CS, with this conditioning memory persisting up to 72 h (Carew et al., 1981b). Notably, in the present study, we used a distinctly lower intensity of the electric shock, which was less than a 10th of that used by Carew et al. (1981b) (3 mA, 1 s in our study versus 50 mA, 1.5 s in the previous study). In pilot studies, we found that shocks with current strengths as used in these early experiments harmed the animals and persistently elicited aversive inking. Such an aversive response typically occurring to life threatening stimuli is to be avoided as it can strongly enhance sensitization over classical conditioning. Sensitization refers to an increase in response amplitude occurring solely due to the repetitive presentation of the stimulus. As described by Carew et al. (1981a, 1983), classical conditioning of the siphon withdrawal reflex always holds such a non-associative sensitization component, which can mask the conditioning effect. Sensitization effects have to be considered particularly against the backdrop that, unlike in other classical conditioning paradigms (used in other species), siphon withdrawal conditioning uses a CS that itself invokes an, although only brief, (unconditioned) siphon withdrawal response. In a differential conditioning paradigm, Hawkins et al. (1989) showed that in animals trained at higher shock intensity, the sensitization component clearly exceeded the conditioning effect. Against this background, a confounding effect of sensitization processes cannot be excluded for the present findings. Clearly dissociating sensitization from conditioning effects would have required an additional control condition comprising the repetitive presentation of only the shock or, alternatively, a differential conditioning procedure including a CS^- applied to a different part of the animal, which we did not perform here. Accordingly, it might have been processes of sensitization, rather than classical conditioning, that were enhanced after the conditioning training in our animals, independently of sleep or wakefulness. Considering evidence that sensitization is enhanced in conditions of generally increased brain activity and excitability (Barbas et al., 2003; Sutton and Opp, 2014),

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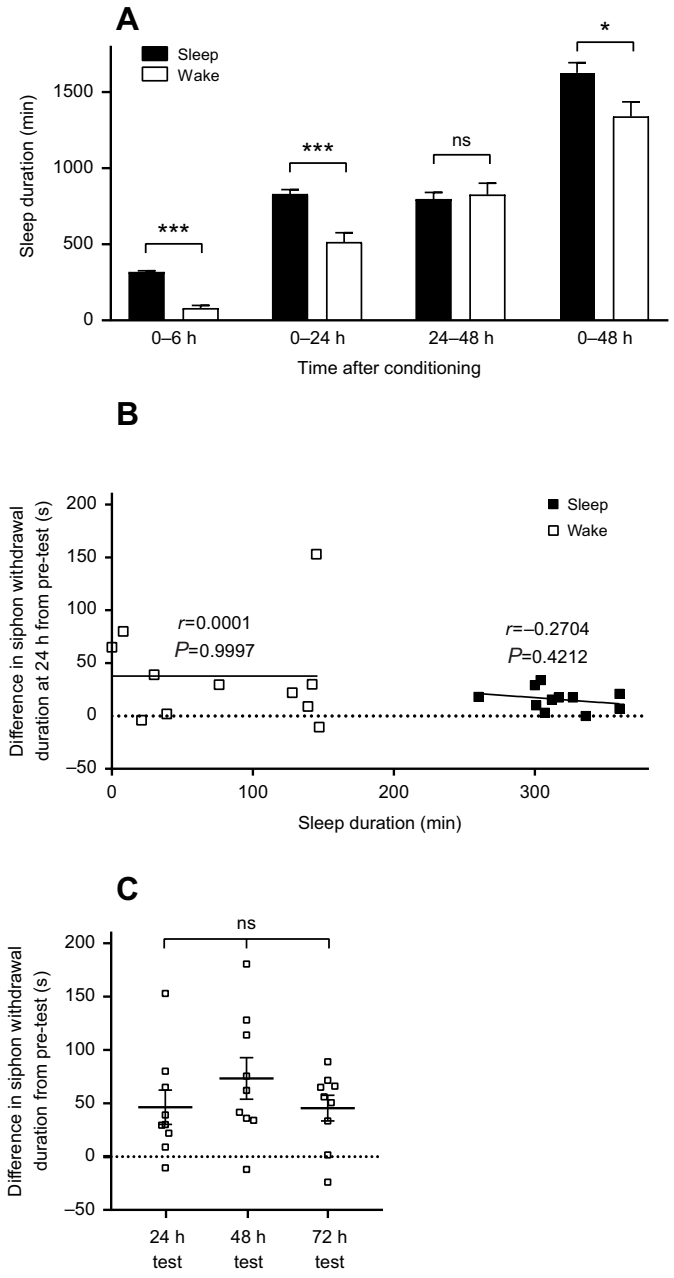

Fig. 3. Sleep after conditioning and extinction training. (A) Mean±s.e.m. sleep duration in the Sleep and Wake group animals. Wake animals were effectively kept awake during the 6 h interval after conditioning (0–6 h). The difference in sleep duration between Sleep and Wake groups persisted for the 24 h interval following conditioning (0–24 h) as well as for the 48 h interval following conditioning (0–48 h), with the latter including the 24 h interval following extinction, where sleep duration was comparable between groups. ***P<0.001, *P<0.05. (B) Correlation between sleep duration and siphon withdrawal duration (24 h test minus pre-test level) separately for the Sleep and Wake group. (C) Exploratory follow-up 72 h test of conditioning memory in a Wake sub-group of n=9 animals. Withdrawal duration (measured as the difference from pre-test levels) remained enhanced in this group at all tests (24 h, 48 h and 72 h). Means (horizontal lines) ±s.e.m. (whiskers) are indicated. Response durations differ significantly from pre-test levels at all three tests, but do not differ between each other (P>0.0675, see Results).

it might be further argued that sleep deprivation served as an additional sensitizing stressor, strengthening the withdrawal reflex memory in our animals. However, although here we did not examine effects of sleep deprivation itself on the unconditioned withdrawal reflex, previous studies employing even longer deprivation intervals (17 h) did not provide hints (such as changes in feeding behavior) that the animals were particularly stressed by the sleep deprivation, in particular when testing took place after a period of recovery sleep (Vorster and Born, 2017).

In an attempt to diminish confounding sensitization processes, we used distinctly lower shock intensities in comparison with those used by Carew et al. (1981b). Indeed, in pilot studies we did not find robust conditioning if we further reduced shock intensity. Nevertheless, this does not exclude the presence of sensitization, which, moreover, could have been selectively enhanced by post-condition wakefulness whereas sleep might have selectively profited from classical conditioning. The use of distinctly lower shock intensities might be related to another divergence: unlike Carew et al. (1981b), we did not observe a systematic increase in the duration of the siphon withdrawal response over the course of the conditioning training (Fig. 2C). A related factor that might be of relevance in this context is the pre-test phase, which comprised 7 presentations of the CS and was introduced to follow the original protocol by Carew et al. (1981b). It could be argued that this pre-test induced habituation, thereby altering subsequent conditioning/sensitization and respective consolidation processes. However, exploratory analyses of siphon withdrawal times during the pre-test did not provide any evidence for a systematic decrease in response during this phase (Fig. S2), making it unlikely that habituation substantially added to or interfered with performance during conditioning training.

Our finding that the siphon withdrawal duration at the 24 h test did not differ between the Sleep and Wake groups is consistent with our hypothesis that simple forms of memory do not need sleep to be consolidated (Vorster and Born, 2015). On average, increases in the duration of the conditioned withdrawal response observed at the 24 h test were even slightly higher in the Wake than Sleep group animals, making a statistical type I error, i.e. the false rejection of the null hypothesis based on a too small sample size, highly unlikely. In favor of our conclusion, we also did not reveal a positive association between sleep duration 6 h after training and siphon withdrawal performance at the 24 h test (Fig. 3B). Data on the effect of sleep on classical conditioning memory in invertebrates is scarce, yet our results are in line with findings in honeybees where classical conditioning of the proboscis extension response was consolidated independently of sleep following conditioning, whereas extinction of the response was enhanced by subsequent sleep (Hussaini et al., 2009).

A sleep dependency of classically conditioned responses was found in *Drosophila* (Le Glou et al., 2012), although, in that study, testing additionally required memory transfer of the conditioning context. Thus, those findings appear to be in line with the view that sleep comes into play when more complex representations including contextual aspects are to be formed. Indeed, a sleep dependency of memory consolidation has also been demonstrated in *Aplysia* in learning paradigms, such as inhibitory operant conditioning of food intake, that are distinctly more complex than the simple classical conditioning of the siphon withdrawal reflex (Vorster and Born, 2018, 2017). This is not to say that classical conditioning is generally less complex than operant conditioning. In fact, with respect to its constituting sub-processes (self- and world-learning) there are operant conditioning paradigms that are probably simpler than the typical classical conditioning task (Brembs et al., 2002; Brembs, 2008; Colomb and Brembs, 2010). Regarding conditioning paradigms, complexity may pertain to differences in the types of behaviors and contexts examined, rather than to the

different type of training (operant versus classical conditioning) used. Thus, feeding behavior is inherently a more complex behavior than a withdrawal reflex, and for this reason one might expect that, unlike the withdrawal reflex, classical conditioning of feeding is a memory that does profit from sleep in *Aplysia* (Lechner et al., 2000). Similarly, classical conditioning to a discrete and simple cue may be considered less complex than context conditioning where the same behavior becomes associated with a contextual configuration of multiple stimulus features. In fact, there is evidence from studies in mammals that sleep preferentially supports the consolidation of contextually integrated memories whereas memories for simple cue–response associations only indirectly profit, if at all, from sleep through their being embedded in a certain context (Sawangjit et al., 2018; Latchoumane et al., 2017). The preferential strengthening of contextually integrated memory is thought to be achieved during sleep by a systems consolidation process that involves a reorganization and displacement of the engram, and that probably can also occur in snails such as *Aplysia* (Levitan et al., 2008; Hatakeyama et al., 2006; Braun and Lukowiak, 2011). Assuming that sleep enhances memory through such active systems consolidation (Klinzing et al., 2019; Diekelmann and Born, 2010), no sleep effect would be expected in simple classical conditioning or sensitization of the siphon withdrawal reflex that occurs unconnected to any further contextual stimuli, as it is represented by a few rather local synaptic enhancements without engram reorganization or displacement (Glanzman, 2013).

A straightforward interpretation of our findings, in terms of a sleep independency of the consolidation of classical conditioning memory, is hampered by two unexpected findings. First, the Wake animals did not show a rebound of sleep after the 6 h period of sleep deprivation. Consequently, at the 24 h, test they might have been less well rested, leading to respective changes in conditioning behavior. For example, the distinctly increased variance in withdrawal responses in the animals of the Wake group could reflect such ongoing effects of insufficient sleep. In fact, the missing rebound sleep after sleep deprivation, diverging from previous findings (Vorster et al., 2014), is difficult to explain. As total sleep time remained reduced in the Wake animals in comparison with the Sleep animals, also across the whole 48 h post-conditioning period, it might point to an overestimation of the actual presence of sleep in our assessment of sleep (by scoring resting behavior). In this case, the putative rebound sleep in the Wake animals could have occurred during times when the Sleep animals just rested but physiologically were not asleep. To confirm this explanation, physiological recordings complementing behavioral recordings are needed. However, if true, insufficient sleep at the 24 h test could be excluded as a factor confounding performance at this test. More generally, it could also be questioned in this context that preventing sleep for 6 h was enough to prevent or disturb the consolidation process. The Wake animals slept on average 80 min during this deprivation period. However, although sleep cannot be completely prevented through sleep deprivation, as even continuous handling will allow for brief periods of local sleep (Vyazovskiy et al., 2011), strong reductions in sleep time comparable with those in the present experiments have been repeatedly shown to effectively suppress memory consolidation (e.g. Vorster and Born, 2017; Djonlagic et al., 2012).

The second and more important finding questioning our conclusion that consolidation of the classically conditioned siphon withdrawal reflex is independent of sleep, is the unexpected failure of the extinction training. This finding contrasts with those of Carew et al. (1981b) who report a successful extinction of the classical conditioned siphon withdrawal duration. However, unlike in the present experiments, extinction training by Carew et al. (1981b) occurred directly after conditioning. Specifically, they reported an immediate decrease of the siphon withdrawal duration within 10 extinction trials, which was also not observed here in either the Sleep or Wake group (Fig. 2D). Possibly, consolidation during the 24 h interval before the extinction training made the conditioning memory more resistant to extinction. However, extinction training 24 h after conditioning was found to be successful in other classical conditioning paradigms in *Aplysia* (Colwill et al., 1988, 1997) as well as in an operant conditioning paradigm (Vorster and Born, 2018). Notably, our extinction training not only failed to induce a decrease in the duration of conditioned withdrawal responses but also further enhanced withdrawal duration. The extinction failure in combination with the increase in withdrawal response with repetitive CS presentations could be taken to argue that siphon withdrawal performance at the 24 h test as well as at the 48 h test was primarily determined by a sensitization process, rather than reflecting an associative conditioning process. It is important to note here, however, that in comparison with associative classical conditioning, sensitization is an even simpler form of non-associative learning which is assumed to be exclusively mediated by synaptic consolidation processes, rather than involving any systems consolidation processes. Hence, regardless of whether the enhanced withdrawal duration at the 24 h test in *Aplysia* is a consequence of sensitization or associative conditioning processes, the finding can be taken as evidence in support of our basic hypothesis (Vorster and Born, 2015) that sleep is not necessary for the consolidation of memories that originate from simple types of learning and merely involve synaptic consolidation mechanisms.

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Competing interests
The authors declare no competing or financial interests.

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Conceptualization: K.I.T., J.B., A.P.V.; Methodology: K.I.T., J.B., A.P.V.; Software: A.P.V.; Validation: K.I.T.; Formal analysis: K.I.T.; Investigation: K.I.T., A.P.V.; Resources: J.B.; Data curation: K.I.T.; Writing - original draft: K.I.T., A.P.V.; Writing - review & editing: K.I.T., J.B., A.P.V.; Visualization: K.I.T., A.P.V.; Supervision: J.B., A.P.V.; Project administration: J.B.; Funding acquisition: J.B.

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