nature neuroscience

Humans can learn new information during sleep

Anat Arzi[1], Limor Shedlesky[1], Mor Ben-Shaul[1], Khitam Nasser[2], Arie Oksenberg[2], Ilana S Hairston[3] & Noam Sobel[1]

During sleep, humans can strengthen previously acquired memories, but whether they can acquire entirely new information remains unknown. The nonverbal nature of the olfactory sniff response, in which pleasant odors drive stronger sniffs and unpleasant odors drive weaker sniffs, allowed us to test learning in humans during sleep. Using partial-reinforcement trace conditioning, we paired pleasant and unpleasant odors with different tones during sleep and then measured the sniff response to tones alone during the same nights' sleep and during ensuing wake. We found that sleeping subjects learned novel associations between tones and odors such that they then sniffed in response to tones alone. Moreover, these newly learned tone-induced sniffs differed according to the odor pleasantness that was previously associated with the tone during sleep. This acquired behavior persisted throughout the night and into ensuing wake, without later awareness of the learning process. Thus, humans learned new information during sleep.

Sleep is a rapidly reversible state that is characterized by a loss of consciousness and reduced responsiveness to external stimuli. There is compelling evidence, however, that the sleeping brain is by no means incapable of processing sensory information[1–5]. For example, presentation of meaningful stimuli, such as one's own name, during sleep elicits different brain responses than presentation of meaningless names or tones[2]. Moreover, sensory information presented during sleep can strengthen previously acquired memories. For example, when an odor was presented during the acquisition of a memory while awake, later presentation of the same odor during sleep resulted in enhanced recall at ensuing wake[3]. In addition, delay conditioning, a form of hippocampal-independent learning that does not require awareness[6], has been observed in sleeping rats[7,8], infants[9], and during drug-induced[10] or slow-wave sleep (SWS)[11] in humans. In contrast, efforts to use typical hippocampal-dependent tasks (for example, word pairing) to teach humans new information during natural sleep have been largely unsuccessful[12–18].

The unique interaction between sleep and smell allowed us to revisit the question of learning during sleep by applying differential partial-reinforcement trace conditioning[19] between tones and odors during sleep. This protocol is particularly attractive for probing learning during sleep for several reasons. First, although non-trigeminal odors presented during sleep do not wake[20–23], they nevertheless modulate the sensory-motor component of olfaction, namely sniffing[20]. Second, the sniff response, an odorant-specific change in nasal airflow in which pleasant odors drive stronger sniffs and unpleasant odors drive weaker sniffs[24,25], provides a nonverbal implicit measure of processing. Third, during wake, the sniff response can be conditioned to a tone, such that different tones then drive different sniffs[26]. Finally, trace conditioning is considered to be a marker for hippocampal-dependent learning[6]. These conditions combine to provide an ideal setting for asking whether humans can learn new information during sleep. We paired different tones with pleasant and unpleasant odors during sleep (**Fig. 1**) and then tested whether these tones alone, without an ensuing odor, would induce stronger or weaker sniffs in accordance with the odor pleasantness with which they were previously associated. We tested for such learned tone-induced sniffs during the same night's sleep and in ensuing wake.

RESULTS

Odors do not wake

Several studies have indicated that non-trigeminal odorants presented during sleep do not wake[20–23]. To verify that our stimuli did not wake, an experienced sleep technician, blind to experimental aims and conditions, applied polysomnography standards for arousal and wake[27]. Of 1,256 reinforced trials in 28 subjects, 81 trials (6.4%) were followed by an observable arousal or wake within 30 s of tone onset. Six subjects had no arousals surrounding any reinforced trial, and the remaining subjects had between one and eight arousals (mean = 2.9 ± 2). All trials preceded or followed by an arousal or wake were omitted from ensuing analyses.

To further characterize the brain response to the stimuli, we analyzed the electroencephalogram (EEG) spectral properties (**Fig. 2**). Because the 1-s tone generated an inevitable evoked response (ERP; **Fig. 2a**), consistent with previous studies[3], we removed this 1-s period from initial analysis. An analysis of variance (ANOVA) applied to 29-s epochs before tone onset compared with 29-s epochs after tone offset in the 1,175 retained trials, with conditions of frequency band (delta, 0.5–4 Hz; theta, 4–8 Hz; alpha, 8–12 Hz; sigma, 11–15 Hz; beta, 12–24 Hz; gamma, 24–100 Hz), stimulus presentation (before tone versus after tone) and odor pleasantness (pleasant versus unpleasant), revealed a main effect of frequency band ($F_{4,135} = 52.9$, $P < 0.00001$), no main effect of stimulus presentation ($F_{1,27} = 3.17$, $P > 0.05$), no main effect of odor pleasantness ($F_{1,27} = 0.10$, $P > 0.74$),

[1]Department of Neurobiology, Weizmann Institute of Science, Rehovot, Israel. [2]Sleep Disorders Unit, Loewenstein Rehabilitation Hospital, Raanana, Israel. [3]School of Behavioral Sciences, Academic College of Tel Aviv - Jaffa, Jaffa, Israel. Correspondence should be addressed to A.A. (anat.arzi@gmail.com).

Received 16 April; accepted 27 July; published online 26 August 2012; doi:10.1038/nn.3193

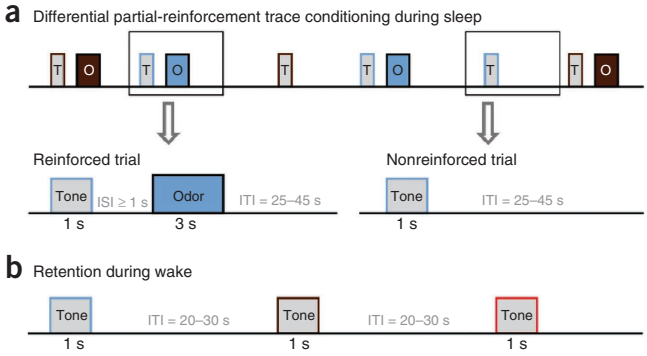

Figure 1 Partial-reinforcement trace conditioning between tones and odors during sleep. (**a**) Stimuli were generated in blocks of six trials: two reinforced trials with pleasant odor (blue), two reinforced trials with unpleasant odor (brown) and two nonreinforced trials (tone alone), one of each tone. On reinforced trials, each auditory stimulus (1,200 or 400 Hz) was paired with either a pleasant (deodorant or shampoo) or unpleasant (rotten fish or carrion) odor (left). On nonreinforced trials, a tone was generated without an odor (right). If sleep was not disturbed, five blocks were presented during NREM and five blocks during REM sleep (Online Methods). T, tone; O, odor; ISI, interstimulus interval; ITI, inter trial interval. (**b**) An awake retention procedure with three auditory stimuli (1,200 Hz, 400 Hz and a novel 800-Hz tone, eight repetitions each), but no odors presented.

and a significant interaction between frequency band and stimulus presentation ($F_{5,135} = 3.21$, $P < 0.01$). Follow-up comparisons (Bonferroni corrected, critical $t = 3.06$) revealed that this significant interaction reflected opposing trends, but we found no significant alteration in any frequency band (all $t_{27} < 2$, all $P >$ Bonferroni α; **Fig. 2c**,**d**).

We repeated this analysis three more times, using 30-s, 10-s and 5-s epochs, and included the 1-s tone ERP-containing data. Adding the ERP period resulted in main effects of frequency band (all $F_{5,135} >$ 56.8, all $P < 0.00001$), main effects of stimulus presentation (all $F_{1,27} >$ 5. 4, all $P < 0.05$), no main effects of odor pleasantness (all $F_{1,27} < 2.9$, all $P > 0.1$), and interactions between frequency band and stimulus presentation (all $F_{5,135} > 5.6$, all $P < 0.0005$). Follow-up comparisons revealed that this reflected an increase in delta power after stimulus onset (significant for 5 and 30 s, both $t_{27} > 3.4$, both $P < 0.005$, but not for 10 s, $t_{27} = 2.3$, $P >$ Bonferroni α), but we found no stimulus-related alteration in the other frequency bands (all $t_{27} <$ 2.2, all $P >$ Bonferroni α), including the arousal indicating alpha and theta[27]. In addition, there was a significant interaction between odor pleasantness and frequency band in the 5-s epochs ($F_{5,135} = 2.4$, all $P < 0.05$), but not in the longer epochs (both $F_{5,135} < 0.1$, both $P > 0.9$). The significant interaction in the 5-s epoch reflected opposing trends, but we found no significant alteration in any frequency band (all $t_{27} < 1.9$, all $P >$ Bonferroni α).

Although these analyses suggest that the stimuli did not wake, the ERP reflects a clear brain response to the stimuli (**Fig. 2a**). Moreover, the frequency power plot indicates a brief post–tone onset increase in delta power, followed by a peak in sigma (**Fig. 2b**). In that sigma has been related to both sleep spindles[28] and sleep-dependent memory consolidation[29], we again repeated the EEG spectral analysis for an epoch from seconds 2–5, which includes odor, but not tone. We found a main effect of frequency band ($F_{5,135} = 58.6$, $P < 0.00001$), no main effect of stimulus presentation ($F_{1,27} = 2.7$, $P > 0.1$), no main effect of odor pleasantness ($F_{1,27} = 1.2$, $P > 0.2$), and a significant interaction between frequency band and stimulus presentation ($F_{5,135} = 3.0$, $P < 0.05$). Follow-up comparisons revealed that this interaction reflected opposing trends, but we found no significant alteration in any frequency band (all $t_{27} < 1.9$, all $P >$ Bonferroni α).

Taken together, these analyses suggest that, insofar as stimuli influenced sleep architecture at all, they increased slow-wave activity, that is, increased sleep depth. This finding is consistent with previous findings implying odorant-induced improvements in sleep quality[30].

Odor pleasantness is processed during sleep

Our study relies on the untested premise that the brain processes pleasantness of olfactory stimuli presented during sleep. To verify that the selected odorants were perceived as intended during wake, we asked subjects to rank odorant pleasantness on a visual analog scale (VAS), both on the evening before and on the morning after sleep. Deodorant and shampoo were significantly more pleasant than rotten fish and carrion (average ranking (0–14 cm VAS) across evening and morning; pleasant odors, 11.1 ± 2.2 cm; unpleasant odors, 3.7 ± 2.3 cm; $t_{27} = 10.13$, $P < 0.00001$). In other words, we had achieved the intended psychophysical framework.

The nonverbal nature of the sniff response allowed us to test whether this perceptual framework persisted in sleep. To ensure our analysis was based on odor processing during sleep alone, we analyzed the 1,175 reinforced trials from 28 subjects that contained no arousals within 30 s of tone onset. Sniffs are nasal inhalations that subserve olfaction. In rodents, one can dissociate an exploratory sniff from a

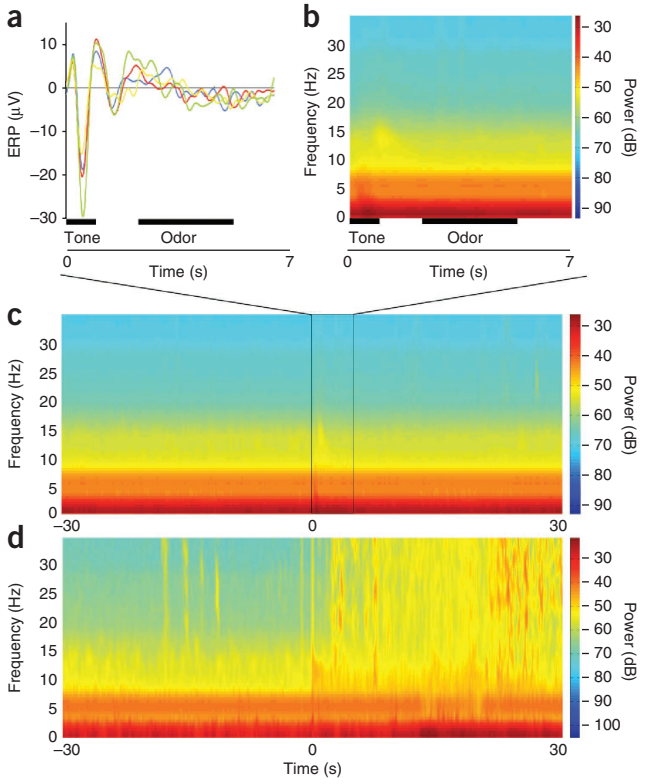

Figure 2 EEG spectral analysis verified sleep during learning. (**a**) Auditory ERPs to a tone followed by a pleasant odor (blue), a tone followed by an unpleasant odor (red), a tone (alone) previously paired with a pleasant odor (yellow) and a tone (alone) previously paired with an unpleasant odor (green). (**b**–**d**) EEG spectral analysis of a 7-s window from tone onset (**b**), and a 60-s window surrounding the tone in all retained trials ($n = 1,175$, **c**) and all excluded trials that contain wakes or arousals ($n = 81$, **d**). Subjects were not aroused or woken during learning.

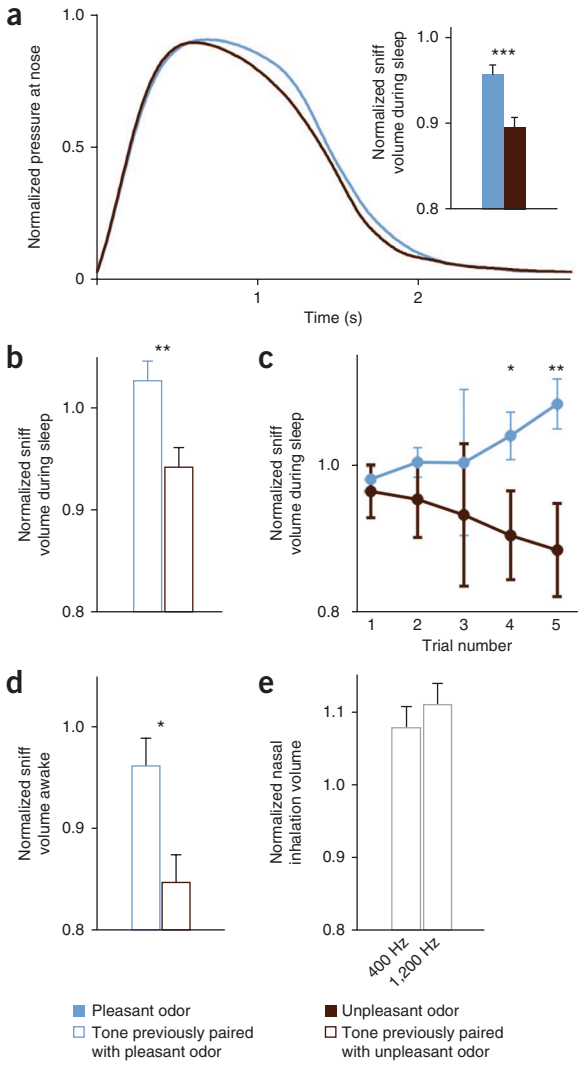

Figure 3 The sniff response revealed learning during sleep. (**a**) The averaged normalized sniff trace and (inset) sniff volume during sleep following a pleasant (blue) or unpleasant (brown) odor ($n = 28$). (**b**) The averaged normalized sniff volume during sleep following a tone (alone) previously paired during sleep with a pleasant odor (blue outline) and a tone (alone) previously paired during sleep with an unpleasant odor (brown outline) ($n = 20$). (**c**) The average learning curve across five continuous repetitions of tones (alone) previously paired with a pleasant odor (blue outline), or five continuous repetitions of tones (alone) previously paired with an unpleasant (brown outline) odor (total = 10 trials). (**d**) The averaged normalized sniff volume awake following a tone (alone) previously paired during sleep with a pleasant odor (blue outline) and a tone (alone) previously paired during sleep with an unpleasant odor (brown outline) ($n = 6$). (**e**) The averaged wake nasal inhalation volume following tones (400 and 12,00 Hz) in a control group that was not conditioned during sleep ($n = 10$). Statistical analysis was conducted using two-tailed t test (**a**,**b**,**d**,**e**) and one-tailed t test (**c**). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$. Y-axis units are normalized volume units (nvu). Error bars represent s.e.m. Subjects learned novel tone-odor association during sleep; this learning persisted in the same nights' sleep and during ensuing wake.

respiratory inhalation on the basis of frequency[31], but human sniffs are not similarly rhythmic[32], and there are no a priori behavioral measures that allow us to dissociate a respiratory nasal inhalation from an olfactory sniff. Thus, we entered into our analyses the single nasal inhalation that started after odorant onset (**Fig. 3**). We refer to this odor-ridden nasal inhalation as a sniff. We found that sniff volume during sleep was greater following pleasant odorants than unpleasant odorants (normalized volume: pleasant, 0.96 ± 0.09 normalized volume units (nvu); unpleasant, 0.90 ± 0.12 nvu; $t_{27} = 3.7$, $P < 0.001$; **Fig. 3a** and **Supplementary Fig. 1a**). In other words, the implicit nonverbal sniff response revealed that odor pleasantness is processed during sleep in a pattern resembling that during wake.

Novel tone-odor associations are learned during sleep

Pleasantness-specific sniff responses during sleep allowed us to ask whether subjects learned during sleep. After pairing tones with odors during sleep, we measured sniffs following tones alone on the same night. To ensure that the learning occurred during sleep only, we excluded subjects with a single wake or arousal within 30 s of tone onset in any of the first 18 reinforced trials. For subjects who had an arousal or wake in a reinforced trial beyond the 18th trial, we excluded data beyond that point. In other words, this analysis considered data that was obtained without a single instance of a reinforced trial in a state other than sleep. These strict criteria retained 20 subjects and 290 of 439 nonreinforced trials presented to these subjects.

We found that sniff volume during sleep was larger after a tone that was previously paired during sleep with a pleasant odor than after a tone that was previously paired during sleep with an unpleasant odor (normalized volume: tone paired with pleasant, 1.02 ± 0.11 nvu; tone paired with unpleasant, 0.94 ± 0.15 nvu; $t_{19} = 2.9$, $P < 0.01$; **Fig. 3b** and **Supplementary Fig. 1b**). We repeated this analysis including the previously excluded subjects and trials, and found the same effects ($n = 28$, $t_{27} = 2.6$, $P < 0.05$). In other words, participants learned a novel association and acted on this learning, all during sleep.

To observe how learning evolved over time, we plotted the learning curve up to the tenth nonreinforced trial or first arousal, whichever came first. Given typical sleep architecture, these trials were presented mainly during non–rapid eye movement (NREM) sleep. The slopes of these curves were significantly different by the fourth trial of each tone (one-tailed paired t test, $t_{15} = 1.8$, $P < 0.05$; **Fig. 3c**) and remained significantly different overall (slope, 0.02 ± 0.04 versus -0.04 ± 0.09, tone paired with pleasant odor and tone paired with unpleasant odor, respectively; one-tailed paired t test, $t_{15} = 2.8$, $P < 0.01$). To address the possibility that subjects with an arousal before the tenth nonreinforced trial skewed this result, we cropped the data at the first point that retained a sufficient number of subjects for analysis, yet contained no arousals. This occurred at the eighth nonreinforced trial, where 12 subjects with no arousals prior to this point retained the same effect (one-tailed paired t test, $t_{11} = 2.2$, $P < 0.05$). This indicates that successful trace conditioning between tones and odors occurred during sleep relatively quickly.

Learning persisted during NREM and REM sleep

Typically, the first half of a night is rich in SWS and the second half is rich in rapid eye movement (REM) sleep[27]. Thus, in the above experiment, REM conditioning trials always occurred after SWS or stage II conditioning trials, preventing conclusions regarding REM sleep alone (**Supplementary Tables 1** and **2**). To disentangle the relevant contribution of sleep states to learning, we studied an additional 27 subjects and began the experiment during either NREM or REM sleep (randomly assigned). This yielded 12 subjects who experienced the procedure in REM sleep and 15 subjects who experienced the procedure in NREM sleep. Moreover, the initial data can be cropped at first REM onset, combining to provide an NREM-only data set containing 43 subjects (28 from experiment 1 and 15 from experiment 2).

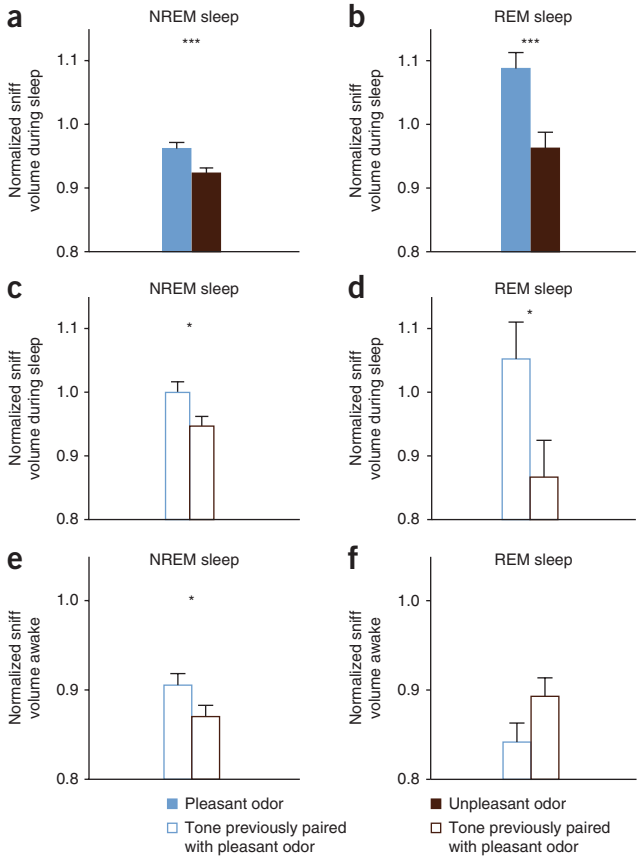

Figure 4 The sniff response revealed learning during NREM and REM sleep. (**a**) The averaged normalized sniff volume during NREM sleep following a pleasant odor (blue) and an unpleasant odor (brown) ($n = 43$). (**b**) The averaged normalized sniff volume during REM sleep following a pleasant odor (blue) and an unpleasant odor (brown) ($n = 12$). (**c**) The averaged normalized sniff volume during NREM sleep following a tone (alone) previously paired during NREM sleep only with a pleasant odor (blue outline) and a tone (alone) previously paired during NREM sleep only with an unpleasant odor (brown outline) ($n = 28$). (**d**) The averaged normalized sniff volume during sleep following a tone (alone) previously paired during REM sleep only with a pleasant odor (blue outline) and a tone (alone) previously paired during REM sleep only with an unpleasant odor (brown outline) ($n = 6$). (**e**) The averaged normalized sniff volume awake following a tone (alone) previously paired during NREM sleep only with a pleasant odor (blue outline) and a tone (alone) previously paired during NREM sleep only with an unpleasant odor (brown outline) ($n = 13$). (**f**) The averaged normalized sniff volume awake following a tone (alone) previously paired during REM sleep only with a pleasant odor (blue outline) and a tone (alone) previously paired during REM sleep only with an unpleasant odor (brown) ($n = 11$). Statistical analysis was conducted using one-tailed t test. $^{*}P < 0.05$, $^{***}P < 0.005$. Y-axis units are normalized volume units (nvu). Error bars represent s.e.m. Formation of tone-odor associations persisted in both NREM and REM sleep.

In the analysis of this second experiment, we had clear a priori hypotheses based on the results of the first experiment. This allowed us to use smaller samples and apply one-tailed tests. First, we asked whether odor pleasantness processing persisted across sleep stages. An ANOVA with conditions of odor pleasantness (pleasant versus unpleasant) and sleep stage (NREM versus REM) revealed a significant main effect of odor pleasantness ($F_{1,53} = 28.4$, $P < 0.00001$), reflecting larger normalized sniff volume following pleasant versus unpleasant odors across sleep stages (normalized volume: pleasant, 0.99 ± 0.10 nvu; unpleasant, 0.93 ± 0.11 nvu; **Fig. 4a**,**b**). Planned comparisons reveled the same effect in each sleep stage separately (one-tailed paired t tests, all $t > 3.4$, all $P < 0.005$; **Fig. 4a**,**b**). There was also a significant main effect of sleep stage ($F_{1,53} = 7.0$, $P < 0.01$), reflecting smaller normalized sniff volume in NREM sleep compared with REM sleep across odors (normalized volume: NREM, 0.94 ± 0.10 nvu; REM, 1.02 ± 0.15 nvu; $t_{53} = 2.6$, $P < 0.02$; **Fig. 4a**,**b**). This difference in normalized values reflected smaller variance in nasal inspiration during NREM versus REM sleep (s.d.: NREM, 5.4 ± 2.5 nvu; REM, 10.9 ± 4.6 nvu; $t_{25} = 3.9$, $P < 0.001$), and a trend toward larger inspiration volume in NREM sleep (inspiration volume: NREM, 237 ± 91 ml; REM, 178 ± 97 ml; $t_{25} = 1.8$, $P = 0.08$; note that this is nasal inspiration and not overall respiration). In addition, there was a significant interaction between odor pleasantness and sleep stage ($F_{1,53} = 7.2$, $P < 0.01$), reflecting a smaller difference in sniff volume between pleasant and unpleasant odors in NREM sleep compared with REM sleep (average difference: NREM, 0.04 ± 0.09 nvu; REM, 0.13 ± 0.13 nvu; $t_{53} = 2.7$, $P < 0.01$; **Fig. 4a**,**b**). In other words, pleasantness processing persisted in both sleep stages but its behavioral manifestation was more pronounced during REM sleep.

We then asked whether learning novel tone-odor associations differed across sleep stages. An ANOVA with conditions of conditioning (tone alone, previously paired with pleasant odor, versus tone alone, previously paired with unpleasant odor) and sleep stage (NREM versus REM) revealed a significant main effect of conditioning ($F_{1,32} = 14.0$, $P < 0.001$), reflecting larger sniffs for a tone (alone) previously paired with a pleasant odor compared with a tone (alone) previously paired with an unpleasant odor, across sleep stages (normalized volume: pleasant, 1.00 ± 0.10 nvu; unpleasant, 0.93 ± 0.16 nvu; **Fig. 4c**,**d**). Planned comparisons revealed the same effect in each sleep stage separately (one-tailed paired t test, all $t > 1.8$, all $P < 0.05$; **Fig. 4c**,**d**). There was no main effect of sleep stage ($F_{1,32} = 0.05$, $P > 0.82$), but there was a significant interaction between conditioning and sleep stage ($F_{1,32} = 4.2$, $P < 0.05$), reflecting a smaller difference in normalized sniff volume between tones (alone) paired with pleasant and unpleasant odors during NREM than during REM (average difference: NREM, 0.05 ± 0.13 nvu; REM, 0.18 ± 0.20 nvu; $t_{32} = 2.0$, $P < 0.05$; **Fig. 4c**,**d**). In other words, the second experiment also revealed that learning of new tone-odor associations occurred during sleep. Moreover, learning persisted in both sleep stages, yet the learned tone-specific sniff response was more pronounced during REM.

Learned associations persisted in ensuing wake

First, we asked whether novel information learned during sleep was retained in the ensuing wake. To test this, upon morning awakening, we measured the sniff response to tones alone. We also included a third, novel tone, presented awake only. We initially analyzed the subjects from the first experiment, who experienced the procedure in both NREM and REM. When we included only the six subjects who did not have a single arousal or wake within 30 s of tone onset during all reinforced trials in the analysis, a one-way ANOVA on tone (400, 800 and 1,200 Hz) did not reveal a difference across tones ($F_{2,5} = 2.72$, $P > 0.11$). In turn, planned comparisons revealed a significant effect ($t_5 = 2.9$, $P < 0.05$), reflecting larger morning sniffs for tones previously paired during sleep with pleasant odors than for tones previously paired during sleep with unpleasant odors (normalized volume: pleasant, 0.96 ± 0.21 nvu; unpleasant, 0.85 ± 0.16 nvu; **Fig. 3d** and **Supplementary Fig. 1c**). Repeating the analysis with all 28 subjects revealed the same effects (normalized volume: pleasant, 0.96 ± 0.23 nvu; unpleasant, 0.89 ± 0.19 nvu; $t_{27} = 2.2$, $P < 0.05$). Following systematic

morning debriefing, all subjects who learned during sleep and had zero wakes or arousals during all reinforced trials later professed no knowledge that sounds or odors were presented during sleep. In other words, subjects acted during wake on what they learned during sleep, despite reporting no awareness of the learning process.

Next, we set out to ask whether retained morning response depended on sleep stage during learning. The small number of subjects who had zero wakes or arousals throughout all of the reinforced trials (two in NREM only, four in REM only) prevented us from testing this with the strict exclusions applied so far. In turn, an ANOVA including all subjects, with conditions of conditioning (tone alone, previously paired with pleasant odor, versus tone alone, previously paired with unpleasant odor) and sleep stage (NREM versus REM) revealed no main effect of conditioning ($F_{1,22} = 0.25$, $P > 0.6$; **Fig. 4e**,**f**), no main effect of sleep stage ($F_{1,22} = 0.13$, $P > 0.7$) and a significant interaction ($F_{1,22} = 4.7$, $P < 0.05$), reflecting a significant difference following learning during NREM sleep (normalized sniff volume: tone (alone) previously paired with pleasant odor, 0.91 ± 0.18 nvu; tone (alone) previously paired with unpleasant odor, 0.87 ± 0.14 nvu; one-tailed paired t test: $t_{12} = 1.9$, $P < 0.05$; **Fig. 4e**), but no significant difference following learning during REM sleep (normalized sniff volume: tone (alone) previously paired with pleasant odor, 0.84 ± 0.11 nvu; tone (alone) previously paired with unpleasant odor, 0.89 ± 0.14 nvu; one-tailed paired t test, $t_{10} = 1.65$, $P > 0.11$; **Fig. 4f**). In other words, novel information learned during NREM alone was retained in ensuing wake, but information learned during REM alone was not. The power of this dissociation, however, remains limited by the inclusion of subjects who had instances of wake or arousal in this analysis.

Finally, to verify that previously unpaired tones alone do not elicit variable responses on the basis of tone frequency, we presented a control group with the retention protocol awake, without conditioning during sleep. We found no difference in nasal inhalation volume between 400- and 1,200-Hz tones (normalized volume: 400 Hz, 1.08 ± 0.17 nvu; 1,200 Hz, 1.11 ± 0.19 nvu; $t_9 = 0.72$, $P > 0.49$, $n = 10$; **Fig. 3e**).

DISCUSSION

We found that partial trace conditioning between tones and odors with varying pleasantness during sleep resulted in learning of a new behavior, namely pleasantness-dependent tone-induced sniffs, which persisted throughout the night and into ensuing wake. These effects materialized despite application of strict exclusion criteria for arousal, and again after omitting exclusions and including all subjects. Moreover, these effects were replicated across two experiments, which together contained 55 sleeping subjects.

Conditioning during either NREM or REM sleep alone implied that, although night-time effects were stronger during REM sleep, transfer to wake was absent following REM sleep–only conditioning. Although failed transfer from REM only contrasts with findings from rodents[8], the remaining results dovetail nicely with emerging views on processing across sleep stages[33]. Specifically, the greater REM over NREM sniff response during the night is consistent with stronger conditioning or potentiation of hippocampal responses during paradoxical sleep over SWS[7,8,34], and with the notion of primary olfactory (piriform) cortex going offline during SWS[35]. In addition, the stronger transfer from NREM learning to wake is consistent with the expanding literature regarding the role of SWS in memory consolidation of general[29,36,37] and olfactory-specific[38,39] information. The stronger transfer from NREM learning to wake may also be linked to the previously observed increased functional connectivity between olfactory and neocortical areas during slow-wave activity[40], and the currently observed stimulus-induced increase in EEG delta power, which in itself has been related to improved consolidation[41]. Finally, the flip-side of stronger transfer from NREM alone, namely absence of transfer from REM alone, may be viewed as consistent with the rapid forgetting of REM-related memories (dream amnesia)[42].

Learning occurred here during sleep following trace conditioning, a form of learning associated with a conscious declarative link between the conditioned and unconditioned stimulus[6]. However, demonstrations of trace conditioning in cases such as the vegetative state[43] and neonatal sleep[44], combined with our findings, suggest that trace conditioning can be acquired in states of altered consciousness as well. Although systematic debriefing revealed that subjects were unaware of the procedure in ensuing wake, one may argue that brief episodes of unremembered wake and awareness may have occurred during conditioning. Although we found no global EEG evidence for such wake or arousal, recent studies have highlighted local rather than global aspects of sleep architecture. For example, human electrophysiology uncovered local, rather than global, cortical sleep patterns[45], such that one may speculate that the phenomenon of local sleep[46] may be mirrored by a phenomenon of local wake, with no global change in EEG. Thus, although we can state that subjects were later unaware of the learning process, our statements on lack of awareness during learning are limited by the resolution of global EEG.

Several aspects of olfaction may have rendered it particularly effective for studying learning during sleep. First, non-trigeminal odorants do not wake[20–23]. In fact, the stimuli-induced increase in delta power that we observed, combined with observations of improvement in sleep quality following odorant presentation[30], suggest an odorant-induced sleep-protective response. Second, odorants are powerful reinforcers, and a few trials are therefore sufficient to establish new learning[47]. Third, the sniff response provides a nonverbal implicit index of learning, rendering it particularly attractive for sleep research. Finally, cortical processing of olfaction does not rely on a thalamic relay[48] (although the auditory stimulus clearly does[49]) and, although a thalamic-type gating function may be implemented in primary olfactory cortex itself[50], the thalamic circumvention may nevertheless provide special status for olfactory information obtained in sleep. It is likely the combination of these factors that optimized a setting for learning during sleep.

Our study has several limitations. One is the very limited scope of the wake retention procedure. Because testing retention in itself causes extinction (we presented tones without odors), the test was therefore limited to a single time point. Thus, our measure of retention likely constitutes an underestimation of the transfer to wake. A second limitation is the small number of subjects with zero wakes or arousals following the procedure in either NREM or REM sleep alone. Considering spontaneous wakes and arousals during a normal night, combined with presentation of a large number of trials, this is unsurprising, yet it dampens the power of our conclusions regarding the differences in morning retention following learning during NREM or REM sleep alone.

Despite these limitations, our results reveal learning of novel information during natural human sleep and implementation of this new learning in sleep and ensuing wake. Moreover, this learning occurred without later awareness of the learning process. This implies that, beyond the general health advantages associated with good sleep, humans may be able to utilize toward learning new information a state in which they spend about a third of their lives.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

We would like to thank R. Paz for advice. This work was supported by the James S. McDonnell Foundation.

AUTHOR CONTRIBUTIONS

A.A. conceived the idea. A.A. and N.S. designed experiments. A.A., L.S. and M.B.-S. carried out the experiments. A.A. analyzed the data. K.N., A.O. and A.A. carried out sleep scoring. A.A., I.S.H., A.O. and N.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nn.3193.
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ONLINE METHODS

All the raw data of this manuscript are available for download at: http://www.weizmann.ac.il/neurobiology/worg/materials.html.

Participants. We used 69 healthy participants with no use of medication (mean age = 25.2 ± 3.0 years, 24 females), screened for sleep disorders, abnormal sleep habits and history of nasal insults, and who gave informed consent to procedures approved by Helsinki committee. Subjects knew that they might or might not receive sounds or odors during the night, but they were unaware of specific experimental aims and conditions. An additional ten subjects participated in a wake retention procedure only, without participating in the conditioning procedure during sleep (mean age = 26.7 ± 2.9 years, 7 females). Overall, 14 subjects were excluded as a result of a priori–defined insufficient sleeping time ($n = 8$), technical problems with stimulus delivery ($n = 5$) or poor polysomnography ($n = 1$).

Stimuli. Tones (400, 800 and 1,200 Hz, duration = 1 s, at a non-arousing 40 dB; ref. 51) were presented by loudspeaker ~2 m from participants' heads. Odorants (pleasant, shampoo or deodorant; unpleasant, rotten fish or carrion; Sensale; all delivered at low, non-trigeminal concentrations) were presented in a nasal mask by computer-controlled air-dilution olfactometer from an adjacent room, with no visual, auditory, tactile, humidity or thermal cues as to the alteration between odor and clean air[52] (stimulus duration = 3 s, constant flow = 6 l per minute).

Polysomnography. Sleep was recorded by standard polysomnography[27]. EEG (obtained from C3 and C4, referenced to opposite mastoid), electro-oculogram (placed 1 cm above or below and laterally of each eye, referenced to opposite mastoid), electromyogram (located bilaterally adjacent to the submentalis muscles), respiration and oximetry were all recorded (Power-Lab 16SP and Octal Bio Amp ML138, ADInstruments) at 1 kHz[20]. Nasal respiration was measured using a spirometer (ML141, ADInstruments) and high-sensitivity pneumotachometer (#4719, Hans Rudolph) in line with the vent ports of the nasal mask[53].

EEG analysis. EEG absolute power spectral analysis in the delta (0.5–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), sigma (11–15 Hz), beta (12–24 Hz) and gamma (24–100 Hz) ranges of all reinforced trails that met study criteria was conducted using Matlab functions for Fast Fourier Transform of 29-s windows before tone onset and after tone offset. The first 1 s of every trial was spared in this analysis to exclude confounding influences from event-related EEG changes linked to processing of the tone. The same analysis was then repeated including the previously omitted 1-s tone period for 30-, 10-, 5- and 3-s epochs. Because there was no difference in effects between C3 and C4, data were collapsed across electrodes for final analysis and presentation.

Nasal airflow analysis. Nasal airflow is sensitive to sleep stage. To prevent sleep stage bias and to enable a comparison of the sniff response between sleep stages, we normalized the nasal inhalation volume. During sleep, for each block (six trials), we calculated the baseline sniff volume by averaging the volume of 15 nasal inhalations preceding block onset. We then divided the sniff response for each trial in the block by the block baseline. In the wake retention procedure, the sniff volume of each of the 24-tone presentations was divided by baseline nasal inhalation volume (averaged volume of 15 nasal inhalations preceding retention procedure onset). Trials differing by 4 s.d. or containing technical problems with stimuli delivery or respiration recording were excluded. The sniff response was typically evident in the inspiration following tone onset, yet was occasionally evident in the tail of the inspiration coinciding with tone onset (set as more than 20% change in the average sniff volume in response to odor stimulation).

Statistical analysis. EEG statistical analysis was conducted using an ANOVA with conditions of frequency band, stimulus presentation and odor pleasantness. Differences in sniff response between pleasant and unpleasant odors and tones in experiment 1 were estimated using two-tailed t tests, and differences in sniff response between pleasant and unpleasant odors and tones as a function of sleep stage in experiment 2 were estimated using an ANOVA with conditions of sleep stage and stimulus presentation, followed by one-tailed t tests.

Procedures. Subjects arrived at the olfactory sleep laboratory at a self-selected time, based on their usual sleep pattern, typically at 11:00 pm. The experimental room was coated in stainless steel to prevent ambient odor adhesion and was subserved by high-efficiency particulate air and carbon filtration to further assure an odor-free environment. After fitting of the polysomnography devices, subjects rated the intensity and pleasantness of the odorant using a VAS. Subjects were left alone in the darkened room to be observed from the neighboring control room via infrared video camera and one-way observation window. The experimenters observed the real-time polysomnography reading and, at least 20 min after they determined that the subject had entered stable sleep, they initiated the experimental protocol. In the first experiment, differential trace conditioning was initiated ~20 min after sleep onset in 34 subjects (28 (16 females) retained following exclusions, mean age = 24.8 ± 3.5 years). The conditioned and nonconditioned stimuli were partially reinforced at a ratio of 2:1 (average of 68 ± 19 trials; **Fig. 1**); on reinforced trials (two-thirds of trials), each 1-s auditory conditioned stimulus (either 1,200 Hz or 400 Hz) was triggered by inhalation and paired with a 3-s olfactory unconditioned stimulus (either pleasant or unpleasant). Trace duration (the time between tone offset and odor perception onset) was variable (2.7 ± 0.8 s) because of triggering off of inhalation, which is intrinsically variable across subjects (**Supplementary Fig. 2**). On nonreinforced trials (one-third of trials), a tone was generated without an odorant (tone alone). Stimuli were generated in blocks of six trials (two reinforced trials with pleasant odor, two with unpleasant odor and two nonreinforced trials, one of each tone, randomized between blocks), with an ITI of 34 ± 2 s and an interblock interval (IBI) of 14 ± 30 min, culminating in 23 ± 6 presentations per odorant and 11 ± 3 presentations per tone per night. Tone-odor contingencies were counter-balanced across subjects. The conditioned response was measured by the sniff-response magnitude to tones alone. In a night without arousals/wakes within a window of 30 s from tone onset, five blocks were presented in NREM sleep, then the procedure was halted up to stable REM sleep, at which point an additional five blocks were presented (total 60 trials; **Supplementary Tables 1** and **2**). If an arousal/wake was detected in the ongoing polysomnographic recording, the experiment was immediately stopped until stable sleep was resumed and then continued up to maximally 18 blocks. Because the experiment was halted following arousal or wake, different subjects had different numbers of trials with different inter-block-interval durations (non-normalized distributed with a median of 2.2 min). Notably, the experienced technician who halted and started the experiment online was not the same technician who later blindly scored sleep off-line. About half an hour after spontaneous morning wake, conditioned response was tested in a retention procedure: three auditory stimuli were presented, 1,200 Hz and 400 Hz, which were presented during the night, and a new 800-Hz tone (eight repetitions each, ISI = 21–30 s, duration = 1 s), which was presented while nasal respiration was recorded. Subjects again rated the intensity and pleasantness of the odorants, and were then debriefed and paid for participation.

To distinguish between the contributions of sleep states, we studied an additional 35 subjects (27 (8 females) retained following exclusions, mean age = 25.7 ± 2.6) in a second experiment with the same protocol as the first experiment, only now the procedure was triggered during either NREM sleep only (20 subjects, 15 after exclusions) or REM sleep only (15 subjects, 12 after exclusions). In NREM sleep, ITI was 34 ± 1 s, IBI was 16 ± 10 min, and reinforced trials accounted for 14 ± 4 presentations per odorant and 7 ± 2 presentations per tone per night. In REM sleep, ITI was 35 ± 1 s, IBI was 11 ± 7 min, and reinforced trials accounted for 14 ± 4 presentations per odorant and 7 ± 2 presentations per tone per night. The morning retention procedure was identical to the first experiment. Retention procedure data from three subjects ($n = 1$ in REM and $n = 2$ in NREM) was lost as a result of technical error.

Inclusion/exclusion criteria. An independent experienced sleep technician blind to experimental conditions and to stimulus onset/offset times scored the data off-line according to American Academy of Sleep Medicine criteria[27]. We then used these blindly obtained scorings to include subjects and/or trials as a function of the question at hand. This is of course critical, as if the participants were awake, then it would be unsurprising that they learned. Thus, we used strict inclusion criteria for each question.

First, to test whether odor pleasantness was processed during sleep, we included only trials without wake or arousal within 30 s of tone onset

doi:10.1038/nn.3193

(experiment 1, $n = 28$; experiment 2: NREM, $n = 43$ (28 from experiment 1 and 15 from experiment 2); REM, $n = 12$).

Second, to test whether the association between the tone and odor was learned and implemented during sleep, we included subjects with more than 18 reinforced trials before any instance of wake/arousal, and then only used trials up to the point of arousal/wake (experiment 1, $n = 20$; experiment 2: NREM, $n = 28$ (19 from experiment 1 and 9 from experiment 2); REM, $n = 6$).

Finally, to test whether information learned during sleep transferred to wake, we included subjects without a single instance of wake/arousal throughout the night within 30 s from tone onset in reinforced trials (experiment 1, $n = 6$). Notably, when abandoning exclusion criteria and including all 28 subjects and all trials, significant effects of learning during sleep persisted. Thus, the reported effects did not emerge as a reflection of the exclusion/inclusion criteria we applied alone. The small number of subjects who had zero wakes or arousals throughout all reinforced trials in experiment 2 (two in NREM only, four in REM only) prevented us from testing this with the strict exclusions applied in experiment 1.

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