Soc. Horticult. Sci. Proc. **35,** 83–85 (1938).
9. Hofmeyr, J. D. J. Some genetic and breeding aspects of *Carica papaya*. *Agronomia Tropical* **17,** 345–351 (1967).
10. Storey, W. B. in *The Evolution of Crop Plants* (ed. Simmonds, N. W.) 21–24 (Longman, London, 1976).
11. Horovitz, S. & Jiminez, H. Cruzamientos interespecificos e intergenericos en Caricaceaes y sus implicationes fitotecnias. *Agronomia Tropical* **17,** 353–359 (1967).
12. Sondur, S. N., Manshardt, R. M. & Stiles, J. I. A genetic linkage map of papaya based on randomly amplified polymorphic DNA markers. *Theor. Appl. Genet.* **93,** 547–553 (1996).
13. Ma, H. *et al.* High-density linkage mapping revealed suppression of recombination at the sex determination locus in papaya. *Genetics* (in the press).
14. Deputy, J. C. *et al.* Molecular marker for sex determination in papaya (*Carica papaya* L.). *Theor. Appl. Genet.* **106,** 107–111 (2002).
15. Ming, R. *et al.* Construction and characterization of a papaya BAC library as a foundation for molecular dissection of a tree-fruit genome. *Theor. Appl. Genet.* **102,** 892–899 (2001).
16. Storey, W. B. The botany and sex relations of the papaya. *Hawaii Agricult. Exp. Station Bull* **87,** 5–22 (1941).
17. Tanksley, S. D., Miller, J. C., Paterson, A. H. & Bernatzky, R. *Proc. 18th Stadler Genet. Symp.* 157–173 (Plenum, New York, 1988).
18. Charlesworth, B. The evolution of sex chromosomes. *Science* **251,** 1030–1033 (1991).
19. Lahn, B. T. & Page, D. C. Four evolutionary strata on the human X chromosome. *Science* **286,** 964–967 (1999).
20. Iwase, M. *et al.* The *amelogenin* loci span an ancient pseudoautosomal boundary in diverse mammalian species. *Proc. Natl Acad. Sci. USA* **100,** 5258–5263 (2003).
21. Charlesworth, D. Plant sex determination and sex chromosomes. *Heredity* **88,** 94–101 (2002).
22. Filatov, D. A., Moneger, F., Negrutiu, I. & Charlesworth, D. Low variability in a Y-linked plant gene and its implications for Y-chromosome evolution. *Nature* **404,** 388–390 (2000).
23. Chiu, C. T. *Study on Sex Inheritance and Horticultural Characteristics of Hermaphrodite Papaya* Thesis, Nat. Pingtung Univ. Sci. Technol., Taiwan (2000).
24. Storey, W. B. Genetics of the papaya. *J. Hered.* **44,** 70–78 (1953).
25. Okada, S. *et al.* The Y chromosome in the liverwort *Marchantia polymorpha* has accumulated unique repeat sequences harboring a male-specific gene. *Proc. Natl Acad. Sci. USA* **98,** 9454–9459 (2001).
26. Bachtrog, D. Adaptation shapes patterns of genome evolution on sexual and asexual chromosomes in *Drosophila*. *Nature Genet.* **34,** 215–219 (2003).
27. Charlesworth, B. & Charlesworth, D. A model for the evolution of dioecy and gynodioecy. *Am. Nat.* **112,** 975–997 (1978).
28. Yang, Z. N. & Mirkov, T. E. Isolation of large terminal sequences of BAC inserts based on double restriction enzyme digestion followed by anchored PCR. *Genome* **43,** 412–415 (2000).
29. Siroky, J., Lysak, M. A., Dolezel, J., Kejnovsky, E. & Vyskot, B. Heterogeneity of rDNA distribution and genome size in *Silene* spp. *Chromosome Res.* **9,** 387–393 (2001).
30. Lengerova, M., Moore, R. C., Grant, S. R. & Vyskot, B. The sex chromosomes of *Silene latifolia* revisited and revised. *Genetics* **165,** 935–938 (2003).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank D. Charlesworth for comments on the manuscript; R. Perl-Treves for discussions; S. Ancheta, G. Asmus and L. Poland for technical assistance; R. Manshardt for providing an F_2 population for fine-mapping; and H. Albert, M. Moore, R. Osgood, B. Vyskot and S. Whalen for reviewing the manuscript. This work was supported by a United States Department of Agriculture Agricultural Research Service (USDA-ARS) Cooperative Agreement with the Hawaii Agriculture Research Center, and a subaward to R. M. and A.H.P. to produce and to characterize the BAC library.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to R.M. (rming@harc-hspa.com).
The sequences are deposited in GenBank under accession codes CG680451–CG681045 and AY428881–AY428946.

Sleep inspires insight

Ullrich Wagner[1], Steffen Gais[1], Hilde Haider[2], Rolf Verleger[3] & Jan Born[1]

[1]*Department of Neuroendocrinology, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany*
[2]*Institute of Psychology, University of Cologne, Gronewaldstrasse 2, 50931 Cologne, Germany*
[3]*Department of Neurology, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany*

Insight denotes a mental restructuring that leads to a sudden gain of explicit knowledge allowing qualitatively changed behaviour[1,2]. Anecdotal reports on scientific discovery suggest that pivotal insights can be gained through sleep[3]. Sleep consolidates recent memories[4–6] and, concomitantly, could allow insight by changing their representational structure. Here we show a facilitating role of sleep in a process of insight. Subjects performed a cognitive task requiring the learning of stimulus–response sequences, in which they improved gradually by increasing response speed across task blocks. However, they could also improve abruptly after gaining insight into a hidden abstract rule underlying all sequences. Initial training establishing a task representation was followed by 8 h of nocturnal sleep, nocturnal wakefulness, or daytime wakefulness. At subsequent retesting, more than twice as many subjects gained insight into the hidden rule after sleep as after wakefulness, regardless of time of day. Sleep did not enhance insight in the absence of initial training. A characteristic antecedent of sleep-related insight was revealed in a slowing of reaction times across sleep. We conclude that sleep, by restructuring new memory representations, facilitates extraction of explicit knowledge and insightful behaviour.

The idea that sleep can trigger the gain of insight is associated with the names of famous scientific discoverers[3]. For example, Nobel prize winner Loewi reported that he woke up with the essential idea for an experimental confirmation of his theory of chemical neurotransmission. Mendeleyev, who laid out the periodic table of chemical elements, reported that his understanding of the critical rule underlying it emerged out of a dream following unsuccessful puzzling with the symbols of the elements. Recent studies in animals and humans provided evidence for the concept that neuronal representations of task stimuli and responses acquired during wakefulness become reactivated during subsequent sleep[7–10]. This reprocessing of representations is considered to underlie the consolidating effect of sleep on memory[4,11–15], but could also be accompanied by restructuring these representations in memory to enable insight. Here, we tested whether sleep influences task representations in memory such that the gain of insight is facilitated. This was expressed in the extraction of explicit knowledge of a hidden abstract rule in stimulus–response sequences learned previously under implicit conditions.

To grasp the inherently unpredictable phenomenon of insight experimentally, we used a modified version of the Number Reduction Task (NRT; Fig. 1a) originally developed by Thurstone and Thurstone[16–18]. Based on continuous monitoring of subjects' behavioural responses, the task allows the exact determination of the time point when insight occurs, that is, when explicit knowledge of a hidden abstract rule is gained, leading to an abrupt, qualitative shift in responding. On each trial of the task, subjects were asked to transform a given string of eight digits into a new string through a stepwise digit-by-digit application of two simple rules to reach a specific digit indicating the final solution to this string. With increasing practice, the subject's responses become gradually faster on this task. Most important, however, a hidden rule was implemented in the digit strings, which was not mentioned to the subjects and was therefore initially processed at an implicit level without awareness. The time point when a subject gained insight into this rule could be determined precisely because at this time he/she would begin to cut short sequential responding to confirm the final solution in advance. All subjects were first trained on three task blocks to induce mental representations of the task that still remained implicit with regard to the hidden rule during this period. The training period was then followed by an 8-h interval of (1) nocturnal sleep, (2) nocturnal wakefulness, or (3) daytime wakefulness (Fig. 1b). Subsequently, subjects were retested on ten blocks.

Sleep more than doubled the probability of gaining insight into the hidden rule compared to wakefulness. In the sleep group, thirteen out of 22 subjects (59.1%) gained insight at retesting, compared to five subjects (22.7%) in either wake group ($\chi^2 = 8.54$, degrees of freedom (d.f.) $= 2$, $P = 0.014$; Fig. 2). For subjects gaining insight, the time point of its occurrence (number of blocks after beginning of retesting) did not differ significantly between groups (sleep, 4.5 ± 0.8 (mean $\pm$ s.e.m.); wake-night, 6.8 ± 1.5;

©2004 Nature Publishing Group

wake-day, 6.0 ± 1.0; $P > 0.28$). The two nocturnal and daytime wake conditions excluded the possibility that inferior performance after wake intervals resulted from sleep deprivation or variations in circadian rhythm. Subjective ratings obtained at the beginning of retesting revealed that, as expected, subjects in the sleep condition were less tired compared to subjects tested following a wakeful night (five-point scale: 2.95 ± 0.22 versus 3.64 ± 0.25, $P < 0.05$), but not compared to those tested after a period of daytime wakefulness (2.41 ± 0.22, $P > 0.11$). This pattern excludes that the inferior performance in the wake conditions resulted from unspecific effects of tiredness.

To ensure that the influence of sleep was on memory representations established during training before sleep rather than a proactive influence on performance at retesting, in supplementary experiments subjects were tested on 13 continuous blocks either in the morning after sleep (7:00 h) or in the evening after daytime wakefulness (19:00 h), with only ten blocks included in the analysis for the direct comparison with the retest situation of the main experiment. Since, unlike in the main experiment, subjects had no training before these test periods, off-line restructuring of initially acquired task representations could not occur during the periods of sleep or wakefulness, respectively, preceding task performance. Five out of 20 subjects (25.0%) gained insight in each of these two conditions, a level nearly identical to that of the wake conditions of the main experiment and substantially lower than in the sleep condition of the main experiment ($\chi^2 = 7.07$, d.f. = 2, $P = 0.029$; Fig. 2; the same result, $P < 0.01$, was obtained in this comparison when, as a control for overall task practice, the last rather than the first 10 of the 13 blocks were analysed). Thus, compared to subjects lacking initial training, subjects of the main experiment benefited from the 8-h post-training interval only if it was spent asleep. Critically, the sleep group of the main experiment also performed substantially better than subjects tested in the early morning after sleep without having established a task representation before sleep, ruling out an unspecific proactive influence of sleep on subsequent task performance.

Insightful behaviour can announce itself by characteristic antecedents[19,20]. Our task allowed us to identify sleep-related behavioural antecedents of insight in characteristic changes of reaction time patterns across the sleep and wake intervals in the main experiment. Following previous work with the NRT[18], we analysed the subjects' seven reaction times of each response string separately for the initial digit (response 1), the following three digits undetermined by the task structure (responses 2–4), and the last three digits (responses 5–7), which were fully determined because they were always mirroring responses 2–4 (Fig. 1a). We identified changes in the three response types from the last block of initial training to the first block of retesting separately for 'solvers' (subjects who later gained insight into the hidden rule) and 'nonsolvers' (subjects not gaining insight). Overall, reaction times decreased across both sleep and wakefulness. However, this decrease differed saliently between solvers and nonsolvers in the sleep and wake conditions (the data of the two wake conditions did not differ and were combined in this analysis). Notably, in the sleep condition the solvers showed only marginal speeding of reaction times across sleep as compared to the profound decrease in the nonsolvers' reaction times (overall speeding 29.4 ± 27.2 ms in solvers versus 206.4 ± 35.1 ms in nonsolvers; F-test variance ratio ($F_{1,20}$) = 16.35, $P < 0.001$, for solver/nonsolver main effect; Fig. 3a). This differential influence of sleep was most obvious for the first response, which in solvers even slowed down across sleep

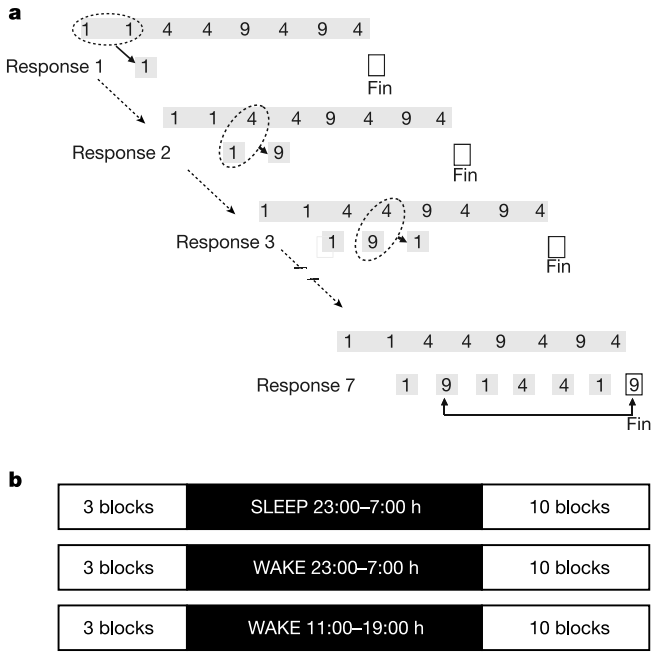

Figure 1 Task and experimental design. **a**, Number Reduction Task (NRT), illustrated by an example trial. On each trial, a different string of eight digits was presented. Each string was composed of the digits '1', '4', and '9'. For each string, subjects had to determine a digit defined as the 'final solution' of the task trial (Fin). This could be achieved by sequentially processing the digits pairwise from left to right according to two simple rules. One, the 'same rule', states that the result of two identical digits is just this digit (for example, '1' and '1' results in '1', as in response 1 here). The other rule, the 'different rule', states that the result of two non-identical digits is the remaining third digit of this three-digit system (for example, '1' and '4' results in '9' as in response 2 here). After the first response, comparisons are made between the preceding result and the next digit. The seventh response indicates the final solution, to be confirmed by pressing a separate key. Instructions stated that only this final solution was to be determined and this could be done at any time. Not mentioned to the subjects, the strings were generated in such a way that the last three responses always mirrored the previous three responses. This implies that in each trial the second response coincided with the final solution (arrow). Subjects who gain insight into this hidden rule abruptly cut short sequential responding by pressing the solution key immediately after the second response. **b**, Experimental design (main experiment). An 8-h period of nocturnal sleep, nocturnal wakefulness, or daytime wakefulness separated an initial training phase (three blocks) from later retesting (ten blocks).

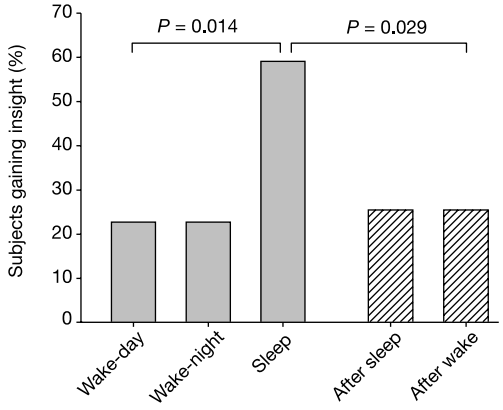

Figure 2 Effects of sleep and wakefulness on the occurrence of insight. Columns indicate percentage of subjects gaining insight into the hidden rule in the three experimental conditions of the main experiment (grey), in which subjects either slept (at night) or remained awake (at night or during daytime) between initial training and retesting, and in two supplementary conditions (hatched), where subjects were tested after nocturnal sleep or daytime wakefulness in the absence of initial training before these periods.

©2004 Nature Publishing Group

($F_{2,40} = 5.25$, $P < 0.01$, for solver/nonsolver × response type interaction). There were no similar differences between solvers and nonsolvers across the wake intervals, resulting generally in intermediate gains in reaction times ($P > 0.44$ and 0.14, for respective main effect and interaction; Fig. 3b). Independent of the sleep/wake conditions, the first response in later solvers as the only response was delayed compared to nonsolvers already in the last block of the initial training ($F_{2,124} = 6.91$, $P < 0.005$, for response type × solver/nonsolver interaction). The slowing of the initial response might be related to processes of search and task analysis[21], originating from an incipient representation of the hidden rule. Becoming particularly pronounced at retesting after sleep, this slowing could thus mean that sleep amplifies previously germinated antecedents of insight.

In conclusion, our results show that sleep acts on newly acquired mental representations of a task such that insight into hidden task structures is facilitated. Control conditions excluded the idea that this effect was due to non-specific effects of sleep deprivation, circadian rhythm, or proactive influences of sleep on subsequent capabilities of problem solving or divergent thinking[22–24]. Our reaction time data argue against the view that the qualitative change evolves from a sleep-dependent strengthening of procedural (implicit) memories, which in sequential motor tasks expresses itself in distinctly accelerated reaction times[25]. Here, sleep profoundly accelerated reaction times only in nonsolvers, but not in solvers, indicating that restructuring originates from an effect of sleep on memory representations different from those underlying procedural motor learning. Specifically, the slowing of reaction times in solvers appears to reflect the presence of an incipient representation of the rule overlapping with that required for implicit task performance. By amplification during sleep, this novel representation could eventually start to dominate the implicit memory representation, a process expressing itself in insightful behaviour as a consequence of an overall restructured representation.

The task representations associated with sleep-dependent gain of insight may be restructured by activity of the hippocampus and related medial temporal lobe structures, which, in connection with prefrontal cortical areas, are considered to play an essential role for generating awareness in memory[26–28]. Reactivation of hippocampal cell assemblies during sleep[6–8] is regarded as a mechanism by which recently encoded materials stored temporarily in autoassociative hippocampal networks are played back to the neocortex where they are gradually incorporated into preexisting knowledge representations[15,29]. This process of incorporation underlying long-term storage of previously acquired memories then forms the basis for a remodelling and qualitative restructuring of memory representations. Thus, our data support the concept that sleep, by hippocampal-neocortical replay, not only strengthens memory traces quantitatively, but can also 'catalyse' mental restructuring, thereby setting the stage for the emergence of insight. □

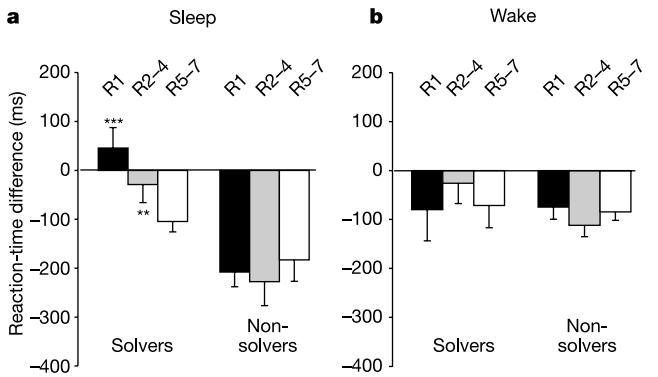

Figure 3 Reaction time analysis. **a**, **b**, Columns indicate changes in reaction times from the last block of initial training to the first block of retesting after eight hours of sleep (**a**) and wakefulness (**b**) for the initial response (R1, black), subsequent responses 2–4 undetermined by the task structure (R2–4, grey), and responses 5–7, which were determined by the task structure (R5–7, white), separately for solvers (who later gained insight) and nonsolvers (not gaining insight). ***$P < 0.001$, **$P < 0.01$, for pairwise contrasts between solvers and nonsolvers.

Methods

Sixty-six healthy subjects (age 18–31 yr) recruited at the University of Lübeck participated in the main experiment (twenty-two in each condition) and forty (age 20–32 yr) in the supplementary experiment (twenty in each condition). There were equal numbers of women and men in all five groups. The Number Reduction Task (explained in Fig. 1a) was adopted from ref. 18. Before the experiment proper, subjects had to perform without mistakes on ten practice strings to assure correct understanding of the 'same' and 'different' rule. Each task block consisted of 30 trials.

The hidden rule was abstract, that is, dependent on relational patterns rather than on fixed stimulus–stimulus or stimulus–response repetitions as in classical conditioning or in typical serial reaction-time tasks. In principle, insight into the hidden rule could be gained in different ways, which, however, were behaviourally equivalent and not treated separately here. The gain of insight reduced the total time to reach the final solution for a string abruptly from 8.73 ± 0.59 s to 2.39 ± 0.17 s. Post-experimental questionnaires confirmed the gain of explicit knowledge of the hidden rule in all subjects identified as solvers on the basis of their behavioural change.

In the design of the main experiment (Fig. 1b), three blocks were chosen as an initial training period based on previous work[17,18] and on pilot experiments indicating that at this level of task difficulty only a few subjects were able to recognize the hidden rule within this early phase. Here, five subjects gained insight into the hidden rule already during initial training and, thus had to be replaced by additional subjects. To exclude that insight into the hidden rule had taken place spontaneously between initial training and retesting, subjects were asked in a control questionnaire before retesting whether any thoughts or mentations regarding the task had occurred after initial training. No subject reported any relevant thoughts or dreams pertinent to the task.

Subjects in the sleep condition of the main experiment spent a habituation night in the sleep laboratory before participation. Sleep was monitored by standard polysomnography including electroencephalogram (from left and right central electrodes), vertical and horizontal electrooculogram, and electromyogram (from chin electrodes). Recordings were scored off-line according to standard criteria[30], revealing normal sleep architecture for the experimental nights (total sleep time, 480.5 ± 3.9 min; sleep onset, 12.9 ± 1.5 min; wake, 0.2 ± 0.1%; sleep stage 1, 4.5 ± 0.7%; sleep stage 2, 58.6 ± 1.8%; slow-wave sleep, 17.2 ± 1.5%; rapid-eye-movement sleep, 17.7 ± 1.2%).

Reaction time analyses across periods of sleep versus wakefulness (Fig. 3) were performed for correct response strings by a global 2 (sleep/wake) × 2 (solver/nonsolver) × 3 (response type) analysis of variance (ANOVA) and subsequent separate 2 (solver/nonsolver) × 3 (response type) ANOVA for sleep and wake subjects. Pairwise contrasts were specified by *t*-tests for statistically significant main effects and interactions. Degrees of freedom were adjusted using the Greenhouse–Geisser correction.

Received 21 August; accepted 17 November 2003; doi:10.1038/nature02223.

1. Köhler, W. *Gestalt Psychology: An Introduction to New Concepts in Modern Psychology* (Liveright, New York, 1947).
2. Sternberg, R. J. & Davidson, J. E. *The Nature of Insight* (Bradford Books/MIT Press, Cambridge, MA, 1995).
3. Mazzarello, P. What dreams may come? *Nature* **408,** 523 (2000).
4. Maquet, P. The role of sleep in learning and memory. *Science* **294,** 1048–1052 (2001).
5. Smith, C. Sleep states and memory processes. *Behav. Brain Res.* **69,** 137–145 (1995).
6. Buzsáki, G. Memory consolidation during sleep: a neurophysiological perspective. *J. Sleep Res.* **7** (Suppl. 1), 17–23 (1998).
7. Pavlides, C. & Winson, J. Influences of hippocampal place cell firing in the awake state on the activity of these cells during subsequent sleep episodes. *J. Neurosci.* **9,** 2907–2918 (1989).
8. Wilson, M. A. & McNaughton, B. L. Reactivation of hippocampal ensemble memories during sleep. *Science* **265,** 676–679 (1994).
9. Maquet, P. *et al.* Experience-dependent changes in cerebral activation during human REM sleep. *Nature Neurosci.* **3,** 831–836 (2000).
10. Stickgold, R., Hobson, J. A., Fosse, R. & Fosse, M. Sleep, learning, and dreams: off-line memory reprocessing. *Science* **294,** 1052–1057 (2001).
11. Fishbein, W. Disruptive effects of rapid eye movement sleep deprivation on long-term memory. *Physiol. Behav.* **6,** 279–282 (1971).
12. Gais, S., Plihal, W., Wagner, U. & Born, J. Early sleep triggers memory for early visual discrimination skills. *Nature Neurosci.* **3,** 1335–1339 (2000).
13. Plihal, W. & Born, J. Effects of early and late nocturnal sleep on declarative and procedural memory. *J. Cogn. Neurosci.* **9,** 534–547 (1997).
14. Wagner, U., Gais, S. & Born, J. Emotional memory formation is enhanced across sleep intervals with high amounts of rapid eye movement sleep. *Learn. Mem.* **8,** 112–119 (2001).
15. McNaughton, B. L., *et al.* in *Sleep and Brain Plasticity* (eds Maquet, P., Stickgold, R. & Smith, C.) 225–246 (Oxford Press, Oxford, UK, 2003).
16. Thurstone, L. L. & Thurstone, T. G. Factorial studies of intelligence. *Psychometr. Monogr.* **2,** 94 (1941).
17. Frensch, P., *et al.* in *Attention and Implicit Learning* (ed. Jiménez, L.) 335–366 (John Benjamins, Amsterdam/Philadelphia, 2002).

©2004 Nature Publishing Group

18. Rose, M., Haider, H., Weiller, C. & Büchel, C. The role of medial temporal lobe structures in implicit learning: an event-related FMRI study. *Neuron* **36,** 1221–1231 (2002).
19. Epstein, R., Kirshnit, C. E., Lanza, R. P. & Rubin, L. C. 'Insight' in the pigeon: antecedents and determinants of an intelligent performance. *Nature* **308,** 61–62 (1984).
20. Durso, F. T., Rea, C. B. & Dayton, T. Graph-theoretic confirmation of restructuring during insight. *Psychol. Sci.* **5,** 94–98 (1994).
21. Jiménez, L., Méndez, C. & Cleeremans, A. Comparing direct and indirect measures of sequence learning. *J. Exp. Psychol. Learn. Mem. Cogn.* **22,** 948–969 (1996).
22. Cartwright, R. D. Problem solving: waking and dreaming. *J. Abnorm. Psychol.* **83,** 451–455 (1974).
23. Horne, J. A. Sleep loss and "divergent" thinking ability. *Sleep* **11,** 528–536 (1988).
24. Walker, M. P., Liston, C., Hobson, J. A. & Stickgold, R. Cognitive flexibility across the sleep-wake cycle: REM-sleep enhancement of anagram problem solving. *Brain Res. Cogn. Brain Res.* **14,** 317–324 (2002).
25. Fischer, S., Hallschmid, M., Elsner, A. L. & Born, J. Sleep forms memory for finger skills. *Proc. Natl Acad. Sci. USA* **99,** 11987–11991 (2002).
26. Clark, R. E. & Squire, L. R. Classical conditioning and brain systems: the role of awareness. *Science* **280,** 77–81 (1998).
27. Luo, J. & Niki, K. Function of hippocampus in "insight" of problem solving. *Hippocampus* **13,** 316–323 (2003).
28. McIntosh, A. R., Rajah, M. N. & Lobaugh, N. J. Functional connectivity of the medial temporal lobe relates to learning and awareness. *J. Neurosci.* **23,** 6520–6528 (2003).
29. McClelland, J. L., McNaughton, B. L. & O'Reilly, R. C. Why there are complementary learning systems in the hippocampus and neocortex: insights from the successes and failures of connectionist models of learning and memory. *Psychol. Rev.* **102,** 419–457 (1995).
30. Rechtschaffen, A. & Kales, A. *A Manual of Standardized Terminology, Techniques and Scoring System for Sleep Stages of Human Subjects* (NIH Publ. No. 204, Maryland, 1968).

Acknowledgements We thank S. Sabban, C. Benedict, M. Degirmenci, L. Hecking, A. Otterbein, and M. Rose for their assistance. This work was supported by grants from the Deutsche Forschungsgemeinschaft to J.B. and R.V.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to U.W. (wagner@kfg.uni-luebeck.de) or J.B. (born@kfg.uni-luebeck.de).

Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1

Mehrdad Matloubian[1,2], Charles G. Lo[1], Guy Cinamon[1], Matthew J. Lesneski[1], Ying Xu[1], Volker Brinkmann[3], Maria L. Allende[4], Richard L. Proia[4] & Jason G. Cyster[1]

[1]*Howard Hughes Medical Institute and Departments of Microbiology, Immunology and* [2]*Medicine, University of California San Francisco, 513 Parnassus Avenue, San Francisco, California 94143-0414, USA*
[3]*Transplantation & Immunology, Novartis Institutes for BioMedical Research, WSJ-386.101, CH-4002 Basel, Switzerland*
[4]*Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, Maryland 20892-1821, USA*

Adaptive immunity depends on T-cell exit from the thymus and T and B cells travelling between secondary lymphoid organs to survey for antigens. After activation in lymphoid organs, T cells must again return to circulation to reach sites of infection; however, the mechanisms regulating lymphoid organ exit are unknown. An immunosuppressant drug, FTY720, inhibits lymphocyte emigration from lymphoid organs, and phosphorylated FTY720 binds and activates four of the five known sphingosine-1-phosphate (S1P) receptors[1–4]. However, the role of S1P receptors in normal immune cell trafficking is unclear. Here we show that in mice whose haematopoietic cells lack a single S1P receptor ($S1P_1$; also known as Edg1) there are no T cells in the periphery because mature T cells are unable to exit the thymus. Although B cells are present in peripheral lymphoid organs, they are severely deficient in blood and lymph. Adoptive cell transfer experiments establish an intrinsic requirement for $S1P_1$ in T and B cells for lymphoid organ egress. Furthermore, $S1P_1$-dependent chemotactic responsiveness is strongly upregulated in T-cell development before exit from the thymus, whereas $S1P_1$ is downregulated during peripheral lymphocyte activation, and this is associated with retention in lymphoid organs. We find that FTY720 treatment downregulates $S1P_1$, creating a temporary pharmacological $S1P_1$-null state in lymphocytes, providing an explanation for the mechanism of FTY720-induced lymphocyte sequestration. These findings establish that $S1P_1$ is essential for lymphocyte recirculation and that it regulates egress from both thymus and peripheral lymphoid organs.

The G-protein-coupled receptors that are engaged by the lysophospholipid S1P are characterized most prominently for their functions in endothelial cells and for their roles in heart and vascular development[5]. However, $S1P_1$ and $S1P_4$ are also highly expressed in T and B lymphocytes, and sphingosine kinase is present in lymphoid organs[6,7]. To test whether $S1P_1$ has an intrinsic role within lymphocytes, we generated fetal liver chimaeras, transplanting lethally irradiated wild-type mice with day 12.5 fetal liver cells from $S1P_1$ knockout donors[8]. Analysis of peripheral blood from reconstituted animals revealed an almost complete absence of peripheral T cells as well as reduced numbers of B cells in $S1P_1$-deficient ($S1P_1^{-/-}$) fetal liver chimaeras when compared with $S1P_1^{+/+}$ control chimaeras (Fig. 1a, b, g). CD4 and CD8 T cells were also absent from spleen, lymph nodes and Peyer's patches (Fig. 1c), and were not found in liver or lungs (data not shown). In contrast with this peripheral T-cell deficiency, the thymus contained normal numbers of immature CD4 and CD8 double-positive thymocytes (Fig. 1d, f) but had an increased proportion of CD4 and CD8 single-positive cells (Fig. 1d). Further analysis of the single-positive populations revealed a marked increase in the number of mature L-selectin^{hi} cells but unchanged numbers of the immature L-selectin^{lo} cells (Fig. 1e, f; see also Supplementary Fig. 1). This thymic accumulation of mature single-positive T cells is reminiscent of that seen in mice expressing the G_i-inhibiting subunit of pertussis toxin within thymocytes[9] and in animals treated with the immunosuppressive drug FTY720 (refs 2, 10).

In addition to high expression of L-selectin there were also greater numbers of cells expressing β7 integrin and Qa2 and expressing reduced levels of CD24 (Fig. 1e; see also Supplementary Fig. 1), additional phenotypic changes typical of mature single-positive thymocytes[11,12]. CD69 was expressed at intermediate levels on the $S1P_1^{-/-}$ single-positive thymocytes rather than being fully downregulated (Fig. 1e). In this regard it is notable that FTY720 treatment was recently found to downregulate CD69 on single-positive thymocytes[10] and $S1P_1$ was identified in a screen for negative regulators of CD69 induction in activated Jurkat T cells[13]. Therefore, $S1P_1$ signalling may normally promote downregulation of CD69 in maturing single-positive thymocytes. By immunohistochemical analysis, thymic architecture appeared grossly normal in $S1P_1^{-/-}$ fetal liver chimaeras (data not shown).

In contrast with the peripheral T-cell deficiency, $S1P_1^{-/-}$ B cells were found in spleen, lymph nodes and Peyer's patches, and although they were altered in their proportions in these organs compared with wild-type controls, the total peripheral B-cell numbers were similar (Fig. 1h). The phenotype of peripheral B cells was normal with the exception that CD69 expression was elevated (Supplementary Fig. 2). In the bone marrow, pro/pre- and immature B cells were present at their usual frequency, whereas mature B cells that recirculate from blood to bone marrow were reduced (Fig. 1i). Therefore, peripheral B cells appeared to have accumulated in secondary lymphoid organs but seemed to be recirculating poorly. This possibility was tested further by quantification of lymphocyte numbers in lymph fluid isolated from the cisterna chyli (Fig. 1g and Methods). B-cell numbers were significantly reduced in lymph from the $S1P_1^{-/-}$ fetal liver chimaeras

©2004 Nature Publishing Group