The endogenous cannabinoid system controls extinction of aversive memories

Article *in* Nature · September 2002

DOI: 10.1038/nature00839 · Source: PubMed

12 authors, including:

Giovanni Marsicano
French Institute of Health and Medical Research
178 PUBLICATIONS **14,861** CITATIONS
SEE PROFILE

Carsten T. Wotjak
Max Planck Institute of Psychiatry
231 PUBLICATIONS **12,209** CITATIONS
SEE PROFILE

Tiziana Bisogno
Italian National Research Council
156 PUBLICATIONS **17,925** CITATIONS
SEE PROFILE

Gerhard Rammes
Technische Universität München
135 PUBLICATIONS **5,743** CITATIONS
SEE PROFILE

20. Ritchie, M. E. Scale-dependent foraging and patch choice in fractal environments. *Evol. Ecol.* **12,** 309–330 (1998).
21. Ritchie, M. E. & Olff, H. Spatial scaling laws yield a synthetic theory of biodiversity. *Nature* **400,** 557–560 (2000).
22. Lawler, G., Schramm, O. & Werner, W. The dimension of the planar brownian frontier is 4/3. *Math. Res. Lett.* **8,** 13–25 (2001).
23. Milne, B. T. Spatial aggregation and neutral models in fractal landscapes. *Am. Nat.* **139,** 32–57 (1992).
24. Kunin, W. E. Extrapolating species abundance across spatial scales. *Science* **281,** 1513–1515 (1998).
25. Mandelbrot, B. B. *The Fractal Geometry of Nature* (Freeman, New York, 1982).
26. Olff, H. & Ritchie, M. E. Fragmented nature: consequences for biodiversity. *Landscape Urban Planning* **858,** 1–10 (2001).
27. Kotler, N. B. & Wiens, J. A. Multiple scales of patchiness and patch structure: a hierarchical framework for the study of heterogeneity. *Oikos* **59,** 253–260 (1990).
28. Poole, A. & Gill, F. (eds) *The Birds of North America* (The Birds of North America, Philadelphia, 1996–2000).
29. Dunning, J. B. *CRC Handbook of Avian Body Masses* (CRC, Boca Raton, 1993).
30. Brown, J. H. in *Experimental Ecology* (eds Resetarits, W. J. Jr & Bernardo, J.) 71–95 (Oxford Univ. Press, New York, 1998).

Acknowledgements

We thank J. Brown, B. Enquist, J. Damuth and G. Belovsky. Work on this model began during the Fractals in Biology Meeting at the Santa Fe Institute, New Mexico. J.P.H. is supported by an NSF Graduate Research Fellowship.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to J.P.H. (e-mail: jhaskell@cc.usu.edu).

The endogenous cannabinoid system controls extinction of aversive memories

Giovanni Marsicano*☆, Carsten T. Wotjak†☆, Shahnaz C. Azad‡§, Tiziana Bisogno‖, Gerhard Rammes‡, Maria Grazia Cascio‖, Heike Hermann*, Jianrong Tang†, Clementine Hofmann¶#, Walter Zieglgänsberger‡, Vincenzo Di Marzo‖ & Beat Lutz*

** Molecular Genetics of Behaviour, † Neuronal Plasticity/Mouse Behaviour and ‡ Neuropharmacology Group, Max Planck Institute of Psychiatry, Kraepelinstrasse 2-10, 80804 Munich, Germany*
§ Clinic of Anaesthesiology, Klinikum Grosshadern, Ludwig-Maximilians-University, Marchioninistrasse 15, 81377 Munich, Germany
‖ Endocannabinoid Research Group, Institute of Biomolecular Chemistry, CNR, Via Campi Flegrei 34, 80078 Pozzuoli (Napoli), Italy
¶ Institute of Mammalian Genetics, GSF National Research Center for Environment and Health, Ingolstädter Landstrasse 1, 85764 Neuherberg-Munich, Germany
☆ These authors contributed equally to this work

Acquisition and storage of aversive memories is one of the basic principles of central nervous systems throughout the animal kingdom[1]. In the absence of reinforcement, the resulting behavioural response will gradually diminish to be finally extinct. Despite the importance of extinction[2], its cellular mechanisms are largely unknown. The cannabinoid receptor 1 (CB1)[3] and endocannabinoids[4] are present in memory-related brain areas[5,6] and modulate memory[7,8]. Here we show that the endogenous cannabinoid system has a central function in extinction of aversive memories. CB1-deficient mice showed strongly impaired short-term and long-term extinction in auditory fear-conditioning tests, with unaffected memory acquisition and consolidation. Treatment of wild-type mice with the CB1 antagonist SR141716A mimicked the phenotype of CB1-deficient mice, revealing that CB1 is required at the moment of memory extinction. Consistently, tone presentation during extinction trials resulted in elevated levels of endocannabinoids in the basolateral amygdala complex, a region known to control extinction of aversive memories[9]. In the basolateral amygdala, endocannabinoids and CB1 were crucially involved in long-term depression of GABA (γ-aminobutyric acid)-mediated inhibitory currents. We propose that endocannabinoids facilitate extinction of aversive memories through their selective inhibitory effects on local inhibitory networks in the amygdala.

To study the involvement of the endogenous cannabinoid system in memory processing, we generated CB1-deficient mice (*CB1* $^{-/-}$; see Supplementary Information). *CB1* $^{-/-}$ mice and *CB1* $^{+/+}$ littermates were tested in auditory fear conditioning, which is highly dependent on the amygdala[1] and enables the dissection of different phases of memory formation, including acquisition, consolidation and extinction. Mice were trained to associate a tone with a foot-shock (conditioning). After conditioning, animals froze when

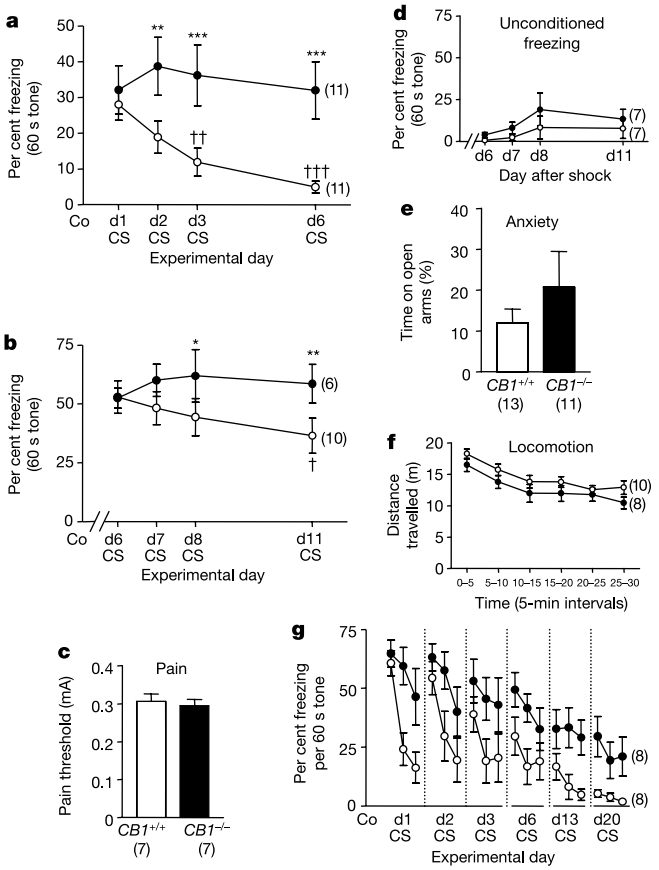


Figure 1 Impaired extinction of aversive memory in an auditory fear-conditioning task of *CB1* $^{-/-}$ mice (filled circles) as compared to their *CB1* $^{+/+}$ littermates (open circles). **a**, **b**, After conditioning (Co) animals were repeatedly exposed to 60 s tones (conditioned stimulus, CS) starting 24 h after conditioning (**a**) (d1) or after a 6-day consolidation period (**b**) (d6). **c**–**f**, *CB1* $^{-/-}$ and *CB1* $^{+/+}$ mice did not differ in their sensory-motor abilities, as assessed by sensitivity to rising electric foot-shock (**c**), unspecific freezing to a tone after shock application (**d**), anxiety-related behaviour on the elevated plus maze (**e**) and horizontal locomotion in an open field (**f**). **g**, *CB1* $^{-/-}$ mice showed memory extinction in response to a stronger extinction protocol (3 min tones until day 20; analysed in 60-s intervals), but still froze more than *CB1* $^{+/+}$ controls. Means ± s.e.m. are shown; number of animals are indicated in parentheses. Asterisk, $P < 0.05$; double asterisk, $P < 0.01$; triple asterisk, $P < 0.001$ (compared with *CB1* $^{+/+}$); dagger, $P < 0.05$; double dagger, $P < 0.01$; triple dagger, $P < 0.001$ (compared with day 1).

Present address: Molecular Neurogenetics Group, Max Planck Institute of Psychiatry, Kraepelinstrasse 2-10, 80804 Munich, Germany.

re-exposed to the tone. This response served as an indicator of aversive memory, and is gradually extinguished on repeated tone presentations. As the amygdala has a crucial role for extinction of aversive memories[9,10], we studied amygdala-dependent memory performance in the absence of possible confounding influences of the hippocampus by re-exposing the mice to the tone in an environment different from the conditioning context[1]. In this environment, neither $CB1^{-/-}$ nor $CB1^{+/+}$ mice showed freezing without tone presentation 24 h after conditioning (data not shown). During the subsequent tone presentation, however, animals of both groups showed the same amount of freezing (Fig. 1a; d1, $P > 0.05$), pointing to an equally successful tone–foot-shock association. On repeated exposure to the tone, however, $CB1^{+/+}$ and $CB1^{-/-}$ mice differed significantly in their freezing behaviour (genotype: $F_{1,20} = 5.81$, $P < 0.05$; genotype × day interaction: $F_{3,60} = 4.86$, $P < 0.005$; Fig. 1a). In fact, $CB1^{+/+}$ mice ($F_{3,10} = 9.70$, $P < 0.0005$), but not $CB1^{-/-}$ ($F_{3,10} = 0.94$, $P = 0.433$), showed extinction of freezing.

The identical behavioural performance of the two genotypes on day 1 indicates that acquisition and early consolidation processes do not involve CB1. However, it is possible that memory consolidation processes were not completed 24 h after conditioning, leaving open a potential involvement of CB1 in later phases of memory consolidation. To test this hypothesis, new groups of animals remained undisturbed after conditioning for 6 days, and mice from these groups were then exposed to the 60-s tones (Fig. 1b). Again, $CB1^{-/-}$ and $CB1^{+/+}$ mice did not differ in their initial freezing response, but behaved in a significantly different way in the course of repeated tone presentations (genotype × day interaction: $F_{3,42} = 3.03$, $P < 0.05$). Whereas $CB1^{+/+}$ mice showed a decrease in freezing behaviour until day 11 ($F_{3,27} = 3.73$, $P < 0.05$), $CB1^{-/-}$ mice failed to extinguish the freezing response ($F_{3,15} = 1.03$, $P = 0.404$). A more detailed analysis of the freezing response in 20-s intervals confirmed the difference in extinction (genotype × 20-s bin interaction: $F_{11,154} = 2.60$, $P < 0.005$; Supplementary Information). These differences were due to altered short-term and long-term extinction in $CB1^{-/-}$ mice but not to increased spontaneous recovery of the freezing response (genotype: $F_{1,14} = 0.18$, $P = 0.675$; genotype × day interaction: $F_{2,28} = 1.61$, $P = 0.217$; Supplementary Information).

We next analysed whether the differences in memory extinction between the two genotypes could be attributed to alterations in sensory-motor abilities of $CB1^{-/-}$ mice, as cannabinoids are known to influence pain perception, emotionality and locomotion[4,11,12]. However, mice of either genotype showed the same pain sensitivity to a rising electric foot-shock defined as the shock intensity at which mice showed first signs of discomfort, that is, jumping and/or vocalization (Fig. 1c). Moreover, if the same animals were repeatedly exposed to the tone, there were no significant differences in freezing behaviour between the genotypes (genotype: $F_{1,12} = 1.61$, $P = 0.228$; genotype × day interaction: $F_{3,36} = 0.225$, $P = 0.878$; Fig. 1d), indicating that CB1 deficiency does not affect foot-shock-induced behavioural sensitization or unconditioned freezing to the tone. Anxiety-related behaviour was analysed on an elevated plus maze. Animals of either genotype spent the same relative time on open arms of the maze ($P > 0.05$, *t*-test and *U*-test; Fig. 1e), and made the same relative number of entries into open arms ($CB1^{+/+}$: 22.0 ± 4.0%; $CB1^{-/-}$: 21.1 ± 7.6%, $P > 0.05$, *t*-test and *U*-test). In contrast, $CB1^{-/-}$ mice showed reduced exploratory activity (number of closed-arm entries: 11.6 ± 1.1 in $CB1^{+/+}$ mice compared with 6.5 ± 1.2 in $CB1^{-/-}$ mice, $P < 0.01$, *t*-test). However, in an open-field locomotor activity test, no significant differences were found, including horizontal (Fig. 1f) and vertical locomotion, resting time, and time spent close to the walls of the box (data not shown).

The failure of $CB1^{-/-}$ mice to diminish their freezing response during a limited number of 60-s tone presentations (Fig. 1a, b) raises the question as to whether $CB1^{-/-}$ mice are able to extinguish aversive memories at all. Thus, conditioned $CB1^{-/-}$ and $CB1^{+/+}$ mice were exposed to a stronger extinction protocol (3 min tone, six exposures; Fig. 1g). Both $CB1^{+/+}$ ($F_{17,119} = 15.01$, $P < 0.000001$) and $CB1^{-/-}$ mice ($F_{17,119} = 7.59$, $P < 0.000001$) extinguished their freezing response over the course of repeated tone presentations. Nevertheless, extinction was still more pronounced in $CB1^{+/+}$ as compared with $CB1^{-/-}$ mice (genotype: $F_{1,14} = 5.30$, $P < 0.05$). Notably, the most marked differences between $CB1^{-/-}$

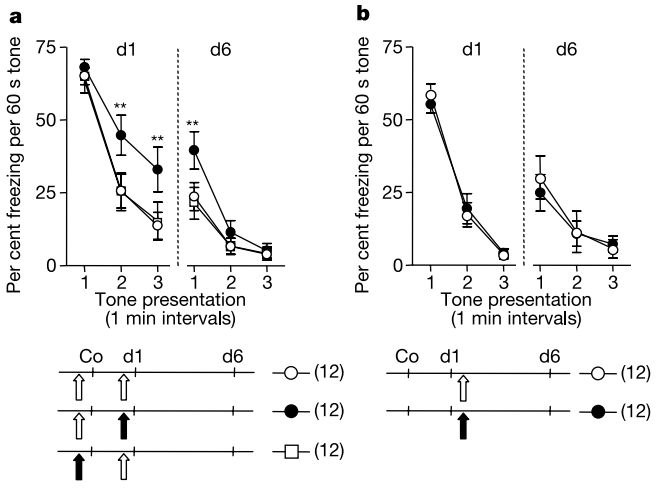


Figure 2 CB1 antagonist SR141716A impairs short-term and long-term extinction, but not acquisition and consolidation of aversive memories. **a**, Mice were treated with SR141716A (filled arrows) or vehicle (open arrows) 20 min before conditioning (Co) and the first extinction trial (d1; 3 min tone). **b**, Mice were treated with SR141716A or vehicle 10 min after the first extinction trial, as indicated. Freezing was analysed in 60-s intervals. Means ± s.e.m. are shown; number of animals are shown in parentheses. Double asterisk, $P < 0.01$ (compared with the two other groups).

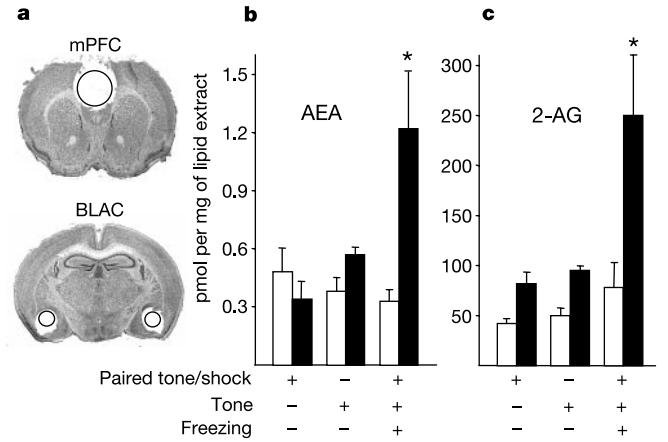


Figure 3 Re-exposure to the tone 24 h after conditioning causes increased endocannabinoid levels in the basolateral amygdala complex (BLAC) but not the medial prefrontal cortex (mPFC) of C57BL/6J mice. **a**, Micrographs of coronal brain sections showing representative examples of the dissected mPFC and BLAC. Circles indicate the size and positioning of tissue sampling. **b**, **c**, Anandamide (**b**, AEA) and 2-arachidonoylglycerol (**c**, 2-AG) levels of the three experimental groups (see text), which differed in conditioning procedure, re-exposure to the tone and resulting freezing response to the tone. Means ± s.e.m. are shown ($n = 4$ per group, 5 mice per n). Open bars, mPFC; filled bars, BLAC. Asterisk, $P < 0.05$ (compared with BLAC of the other groups).

and *CB1*$^{+/+}$ mice were observed during acute tone presentation (short-term extinction). Therefore, *CB1*$^{-/-}$ mice might be primarily impaired in short-term extinction, with a resulting impairment in long-term extinction, assessed in the course of the subsequent extinction trials. Accordingly, spontaneous recovery was not different between the genotypes (genotype: $F_{1,14} = 1.73$, $P = 0.208$; genotype × day interaction: $F_{4,56} = 1.19$, $P = 0.323$; Supplementary Information).

Our behavioural data clearly indicate an involvement of the endogenous cannabinoid system in extinction of aversive memories. However, the life-long absence of CB1 could result in developmental defects leading to the phenotype observed. It, furthermore, precludes any temporal dissection of the involvement of the endogenous cannabinoid system in different stages of memory formation. Thus, we treated wild-type C57BL/6J mice with the CB1 antagonist SR141716A (ref. 13), either before conditioning, or before the first extinction trial. Systemic application of SR141716A 20 min before the first extinction trial impaired both short-term and long-term extinction of the freezing response as compared with both vehicle-treated controls and animals treated with SR141716A before conditioning (treatment × time interaction: $F_{10,160} = 2.72$, $P < 0.005$), with no difference between the two latter treatments and with a similar performance of all three groups in the beginning of the first extinction trial (Fig. 2a). These data largely confirm the phenotype of *CB1*$^{-/-}$ mice (Fig. 1a, b, g), indicating that endocannabinoids have only a negligible function in memory acquisition, consolidation and recall (indicated by the similar performance at the beginning of the first extinction trial), but selectively interfere with extinction of the freezing response to the tone. Mice treated with SR141716A before the first extinction trial showed an attenuated extinction of freezing not only during the first tone presentation (short-term extinction) but also in the absence of pharmacological treatment during the first 60 s of tone presentation at day 6 (long-term extinction). Spontaneous recovery of the behavioural performance from the end of the first (day 1) to the beginning of the second tone presentation session (day 6) was not different among the three groups ($F_{2,34} = 0.29$, $P = 0.744$; Supplementary Information). Together, these findings support the idea that CB1 might be particularly important for the extinction of acute responses to the tone (short-term extinction), which, in turn, relates to behavioural extinction over repeated tone presentations (long-term extinction), without affecting spontaneous recovery of the behavioural performance. Accordingly, the CB1 antagonist had to be present at the time of tone presentation (that is, during aversive memory recall) in order to interfere with memory extinction, as SR141716A failed to affect extinction if administered immediately at the end of the extinction trial (data not shown) or 10 min later (Fig. 2b).

These observations, together with the pharmacokinetics of SR141716A (ref. 14), led us to assume that presentation of the tone during the extinction trial causes an instantaneous rise in endocannabinoid levels. To confirm this assumption, we measured in C57BL/6J mice levels of the two major endocannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG), in brain punches of the medial prefrontal cortex (mPFC) and the basolateral amygdala complex (BLAC), both of which are thought to have central roles in extinction of aversive memories[9,15]. In those animals forming an association between tone and foot-shock, levels of AEA and 2-AG were significantly higher in the BLAC at the end of tone presentation of the extinction trial on day 1, as compared with animals with unpaired tone and foot-shock presentation on the previous day and with animals with paired tone and foot-shock presentation but no re-exposure to the tone (Fig. 3). There were no significant differences in levels of AEA and 2-AG in the mPFC, suggesting a specific involvement of endocannabinoids in extinction processes within the BLAC. Data of the two control groups indicate that both a successful tone–foot-shock association and re-exposure to the tone are required to trigger the acute increase of endocannabinoid levels.

If the endogenous cannabinoid system is activated during tone presentation, how exactly does it facilitate memory extinction? To answer this question, we performed a series of electrophysiological experiments in the BLAC of brain slices from *CB1*$^{-/-}$ and *CB1*$^{+/+}$ mice. Basic electrical properties were similar in *CB1*$^{-/-}$ and *CB1*$^{+/+}$ littermates, including input resistance and resting membrane potential (data not shown). High-frequency stimulation (HFS) in the lateral amygdala close to the external capsule induced long-term potentiation (LTP) in the basolateral amygdala of both genotypes (Fig. 4a). This effect was significantly more pronounced in *CB1*$^{-/-}$ than in *CB1*$^{+/+}$ mice (potentiation of population spike amplitude to 147 ± 11% in *CB1*$^{-/-}$ compared with 117 ± 8% in *CB1*$^{+/+}$ mice, $n = 9$, $P < 0.05$). However, we failed to affect basal synaptic transmission and LTP induction in wild-type slices superfused with SR141716A (5 μM; data not shown). This indicates that the enhanced LTP in *CB1*$^{-/-}$ mice might reflect long-term develop-

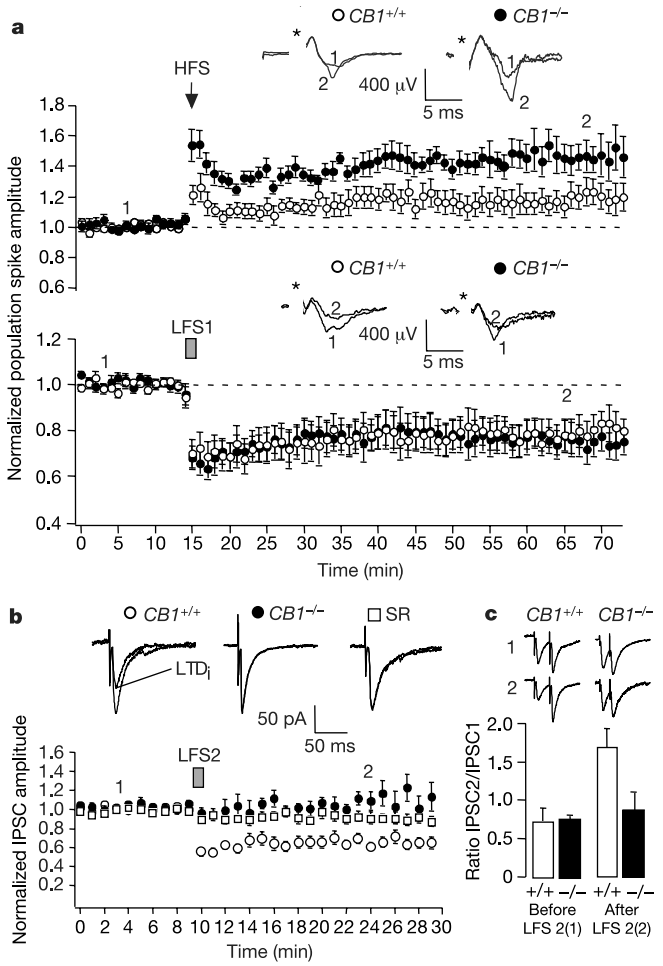


Figure 4 Endogenous cannabinoid system and synaptic plasticity in the basolateral amygdala. **a**, LTP (top) and LTD (bottom) in slices from *CB1*$^{+/+}$ and *CB1*$^{-/-}$ mice, induced by high-frequency stimulation (HFS) and low-frequency stimulation (LFS 1), respectively. Asterisks indicate stimulus artefacts. **b**, Long-term depression of IPSCs (LTD_i) requires CB1 activation. In principal neurons of slices of *CB1*$^{+/+}$ mice, low-frequency stimulation (LFS 2) induced a reduction of the amplitudes of isolated IPSCs. Slices of *CB1*$^{+/+}$ mice pre-incubated in SR141716A (SR) showed no LTD_i. LFS 2 had no effect in *CB1*$^{-/-}$ mice. **c**, LTD_i was accompanied by increased PPF, which was absent in *CB1*$^{-/-}$ mice. Insets show representative traces before and after HFS or LFS (1, 2, respectively). Means ± s.e.m. are shown.

mental adaptations to life-long absence of CB1, and cannot be easily attributed to the lack of CB1 during LTP induction. Low-frequency stimulation with 900 pulses at 1 Hz (LFS 1) of the same pathway induced a persistent decrease in excitatory synaptic transmission (long-term depression, LTD) in both $CB1^{-/-}$ and $CB1^{+/+}$ mice with no difference between genotypes (depression of population spike amplitude to 75 ± 7% in $CB1^{-/-}$ compared with 80 ± 7% in $CB1^{+/+}$ mice, $n = 9$, $P > 0.05$; Fig. 4a).

As several recent studies indicate an involvement of CB1 in GABA-mediated synaptic transmission in hippocampus[16,17] and amygdala[6], we next looked for possible differences in this process within the basolateral amygdala of $CB1^{-/-}$ and $CB1^{+/+}$ mice. Low-frequency stimulation with 100 pulses at 1 Hz (LFS 2) of the lateral amygdala close to the external capsule induced a significant suppression of isolated $GABA_A$ receptor-mediated inhibitory post-synaptic currents (IPSCs) in principal neurons of the basolateral amygdala of $CB1^{+/+}$ mice. This suppression lasted for more than 20 min (hereafter called long-term depression of IPSCs, LTD_i, to 66.7 ± 5.4%, $n = 8$, $P < 0.05$; Fig. 4b). Importantly, LTD_i was blocked in $CB1^{+/+}$ mice by SR141716A (5 μM; Fig. 4b), showing an acute involvement of the endocannabinoid system in the development of LTD_i. The involvement of CB1 in LTD_i was confirmed in $CB1^{-/-}$ mice in which LTD_i was completely abolished (to 110.1 ± 13.8%, $n = 8$, $P < 0.01$ compared with $CB1^{+/+}$; Fig. 4b). Consistent with previous reports[16,17], suppression of GABA-mediated synaptic transmission also increased paired-pulse facilitation (PPF) in $CB1^{+/+}$ ($P < 0.05$) but not in $CB1^{-/-}$ mice (Fig. 4c), indicating a local CB1-dependent decrease in GABA release from axon terminals in $CB1^{+/+}$ slices.

Extinction of aversive memories is thought to be an active mnemonic process[2]. As a new memory, it shares several attributes with other steps of memory formation[9,10,18]; however, there is increasing evidence that some cellular pathways are specifically involved in extinction, but not in acquisition or consolidation of fear memories[15,19,20]. We demonstrated a specific involvement of CB1-mediated neurotransmission in extinction of aversive memories. In principle, the enhanced excitatory synaptic plasticity in $CB1^{-/-}$ mice (LTP; Fig. 4a) might explain the prolonged maintenance of aversive memories observed in these animals (Fig. 1a, b, g). However, an enhanced LTP is expected to coincide with an increased initial freezing response in the first extinction trial[21], which was not observed in $CB1^{-/-}$ mice. Accordingly, acute blockade of CB1 by a selective antagonist failed to affect LTP induction as well as acquisition and consolidation of the aversive memory. In contrast, the same approach revealed a significant involvement of CB1 in extinction (Fig. 2a). Tone-induced recall of the aversive memory was accompanied by an activation of the endocannabinoid system within the BLAC (Fig. 3), which possibly leads to a decrease of GABA-mediated transmission in a CB1-dependent manner (LTD_i; Fig. 4b, c).

The role of GABA-mediated transmission for extinction is, however, controversial[22,23]. Within the amygdala, CB1 immunoreactivity was detected in a distinct subset of GABA-containing interneurons of the BLAC[6] (one of the sites where aversive memories might be formed and stored[24]), but not in the central nucleus of the amygdala[6] (the principal output site of the amygdala[1]). Taking into consideration that principal neurons of the BLAC and neurons of the central nucleus of the amygdala might be inversely correlated in their activities[25,26], we propose that the CB1-mediated decrease of activity of local inhibitory networks within the BLAC leads to a disinhibition of principal neurons and finally to extinction of the freezing response. The selective and locally restricted inhibition of GABA-mediated transmission might not be easily reproduced by systemic administration of GABA-interfering drugs[22,23]. Thus, future studies will have to confine such treatments to the BLAC to validate that CB1-mediated inhibition of GABA-mediated transmission is indeed crucially involved in the extinction of aversive memories mediated by CB1. It remains to be shown whether CB1 is not only involved in extinction of aversive memories but also in adaptation to aversive situations in general and/or in extinction of memories, independently from their emotional value.

Overall, our findings suggest that the endogenous cannabinoid system could represent a therapeutic target for the treatment of diseases associated with inappropriate retention of aversive memories or inadequate responses to aversive situations, such as post-traumatic stress disorders[2], phobias, and certain forms of chronic pain[11]. □

Methods

Animals

Adult male C57BL/6JOlaHsd mice (6–8 weeks; Harlan–Winkelmann) and male $CB1^{-/-}$ and $CB1^{+/+}$ littermates (10–16 weeks; see Supplementary Information) were housed individually with an inverse 12/12 h light/dark cycle (lights off at 8:00) for at least 2 weeks before starting the experiments.

Behavioural studies

Experimental procedures were approved by the Committee on Animal Health and Care of local Government. Experiments were performed between 9:00 and 14:00. Animal's behaviour was analysed in a blind fashion with regards to genotype and drug treatment. Data were analysed by analysis of variance (ANOVA) followed by Fisher's least significant difference test for planned comparisons, Mann-Whitney *U*-test or unpaired Student's *t*-test. A *P*-value of <0.05 was considered statistically significant. Experimental procedures for pain threshold and unconditioned freezing, elevated plus maze and open field are described in Supplementary Information.

Fear conditioning

For conditioning, animals were placed into conditioning chambers (MED Associates). After 3 min, a 20-s tone (9 kHz, 80 dB) was presented that co-terminated with a 2-s electric foot-shock (0.7 mA). In pharmacological experiments animals received a 1-s shock to avoid ceiling effects in the freezing response due to the combination of foot-shock and injection stress. Animals were returned to their home cages 60 s after shock application. At the given time points after conditioning, animals were placed into transparent plexiglas cylinders that differed from the conditioning context, and a 60-s or 180-s tone was presented 3 min later (extinction trials). Animals were returned to their home cages after another 60 s. Mice were experimentally naive except for the stronger extinction protocol, where they had been tested on the elevated plus maze 5 days before. Freezing behaviour (defined as the absence of all movements except for respiration) was quantified from videotapes by trained observers that were blind to genotype and drug treatment, and data were normalized to the respective observation periods.

Pharmacological treatment

SR141716A (NIMH Chemical Synthesis and Drug Supply Program) was dissolved in vehicle solution (1 drop of Tween-80 in 3 ml 2.5% dimethylsulphoxide in saline). SR141716A (3 mg per kg body weight) and vehicle were injected subcutaneously at 20 ml per kg body weight under light isofluran anaesthesia.

Measurement of endocannabinoids

C57BL/6JOlaHsd mice were randomly assigned to three groups ($n = 20$ each). On the conditioning day, two groups were conditioned as described before (paired). The remaining group received the foot-shock first and a 20 s tone 3 min later (unpaired). On the next day, all animals were placed into the cylinders, but only one of the paired groups and the unpaired group were exposed to a 3-min tone. Immediately after the end of the tone (or equivalent time in cylinder), animals were killed, brains were quickly removed and snap-frozen in isopentane/dry ice. mPFC and BLAC were punched from the frozen brain using a cryocut and cylindric brain punchers (Fine Science Tools, internal diameter 2.0 mm and 0.8 mm, respectively). Length of punches was approximately 1.6 mm for mPFC (start: bregma +2.8 mm[27]) and 1.2 mm for BLAC (start: bregma −1.0 mm[27]). Brain tissue of mPFC and bilateral BLAC, respectively, of 5 mice was pooled to obtain a single data point. Tissues (10–15 mg per data point) were dounce-homogenized with chloroform/methanol/Tris-HCl 50 mM, pH 7.4 (1/1/1 by volume) containing 5 pmol of octa-deuterated (d_8)-anandamide and 50 pmol of d_8-2-arachidonoylglycerol (Cayman Chemicals) as internal standards. Lipid-containing organic phase was dried down, weighed and pre-purified by open-bed chromatography on silica gel, and analysed by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) using a Shimadzu high-performance liquid chromatography (HPLC) apparatus (LC-10ADVP) coupled to a Shimadzu quadrupole mass spectrometer (LCMS-2010) via a Shimadzu APCI interface. Mass spectrometry analyses were carried out in the selected ion-monitoring (SIM) mode as described previously[28]. Temperature of the APCI source was 400 °C; HPLC column was a Phenomenex (5 μm, 150 × 4.5 mm) reverse phase column, eluted as described[28]. Anandamide (retention time of 14.5 min) and 2-AG (retention time of 17.0 min) quasi-molecular ions were quantified by isotope dilution with the above-mentioned deuterated standards[28] and their amounts in pmols normalized per mg of lipid extract. Data were statistically evaluated by ANOVA.

Electrophysiology

Brain slices were prepared essentially as described[29]. IPSCs and population spikes were evoked by square pulse stimuli (0.066 Hz, 5–12 mA, 200 μs) delivered by means of bipolar tungsten electrodes positioned within the lateral amygdala close to the external capsule. Population spikes were recorded in the basolateral amygdala close to lateral amygdala using glass microelectrodes (2–3 MΩ) filled with artificial cerebrospinal fluid (ACSF)[29]. HFS (five trains at 100 Hz for 1 s, 10-s interstimulus interval) was applied to induce LTP, and LFS1 (900 pulses at 1 Hz) was applied to induce LTD. Whole-cell GABA-mediated currents were isolated by adding NBQX (0.005 mM) and D-(-)-2-amino-5-phosphopentanoic acid (AP5; 0.05 mM) to ACSF (bubbled with 95% O_2/5% CO_2; pH 7.3), and were recorded from visually identified somata of principal neurons of the basolateral amygdala[30] by glass electrodes (4.5–5 MΩ)[16] containing (in mM): Mg-ATP 2, $CsCH_3SO_3$ 100, CsCl 60, EGTA 0.2, HEPES 10, $MgCl_2$ 1, QX314 5 and Na_3GTP 0.3 (pH 7.3). Patch clamp experiments were performed at 24 ± 1 °C at a holding potential of −70 mV. LTD_i was induced by 100 stimuli at 1 Hz (LFS 2). PPF was induced as described[30]. Data are expressed as means ± s.e.m. We tested significance using the Student's *t*-test.

Received 24 December 2001; accepted 16 April 2002; doi:10.1038/nature00839.

1. LeDoux, J. E. Emotion circuits in the brain. *Annu. Rev. Neurosci.* **23,** 155–184 (2000).
2. Davis, M., Falls, W. A. & Gewirtz, J. in *Contemporary Issues in Modeling Psychopathology* (eds Myslobodsky, M. S. & Weiner, I.) 113–141 (Kluwer Academic, Norwell, 2000).
3. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C. & Bonner, T. I. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature* **346,** 561–564 (1990).
4. Di Marzo, V., Melck, D., Bisogno, T. & De Petrocellis, L. Endocannabinoids: endogenous cannabinoid receptor ligands with neuromodulatory action. *Trends Neurosci.* **21,** 521–528 (1998).
5. Marsicano, G. & Lutz, B. Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. *Eur. J. Neurosci.* **11,** 4213–4225 (1999).
6. Katona, I. *et al.* Distribution of CB1 cannabinoid receptors in the amygdala and their role in the control of GABAergic transmission. *J. Neurosci.* **21,** 9506–9518 (2001).
7. Hampson, R. E. & Deadwyler, S. A. Role of cannabinoid receptors in memory storage. *Neurobiol. Dis.* **5,** 474–482 (1998).
8. Reibaud, M. *et al.* Enhancement of memory in cannabinoid CB1 receptor knock-out mice. *Eur. J. Pharmacol.* **379,** R1–R2 (1999).
9. Falls, W. A., Miserendino, M. J. & Davis, M. Extinction of fear-potentiated startle: blockade by infusion of an NMDA antagonist into the amygdala. *J. Neurosci.* **12,** 854–863 (1992).
10. Lu, K. T., Walker, D. L. & Davis, M. Mitogen-activated protein kinase cascade in the basolateral nucleus of amygdala is involved in extinction of fear-potentiated startle. *J. Neurosci.* **21,** RC162 (2001).
11. Pertwee, R. G. Cannabinoid receptors and pain. *Prog. Neurobiol.* **63,** 569–611 (2001).
12. Rodriguez de Fonseca, F., Del Arco, I., Martin-Calderon, J. L., Gorriti, M. A. & Navarro, M. Role of the endogenous cannabinoid system in the regulation of motor activity. *Neurobiol. Dis.* **5,** 483–501 (1998).
13. Rinaldi-Carmona, M. *et al.* SR141716A, a potent and selective antagonist of the brain cannabinoid receptor. *FEBS Lett.* **350,** 240–244 (1994).
14. Petitet, F., Jeantaud, B., Bertrand, P. & Imperato, A. Cannabinoid penetration into mouse brain as determined by *ex vivo* binding. *Eur. J. Pharmacol.* **374,** 417–421 (1999).
15. Morgan, M. A., Romanski, L. M. & LeDoux, J. E. Extinction of emotional learning: contribution of medial prefrontal cortex. *Neurosci. Lett.* **163,** 109–113 (1993).
16. Wilson, R. I. & Nicoll, R. A. Endogenous cannabinoids mediate retrograde signalling at hippocampal synapses. *Nature* **410,** 588–592 (2001).
17. Ohno-Shosaku, T., Maejima, T. & Kano, M. Endogenous cannabinoids mediate retrograde signals from depolarized postsynaptic neurons to presynaptic terminals. *Neuron* **29,** 729–738 (2001).
18. Santini, E., Muller, R. U. & Quirk, G. J. Consolidation of extinction learning involves transfer from NMDA-independent to NMDA-dependent memory. *J. Neurosci.* **21,** 9009–9017 (2001).
19. El Ghundi, M., O'Dowd, B. F. & George, S. R. Prolonged fear responses in mice lacking dopamine D1 receptor. *Brain Res.* **892,** 86–93 (2001).
20. Quirk, G. J., Russo, G. K., Barron, J. L. & Lebron, K. The role of ventromedial prefrontal cortex in the recovery of extinguished fear. *J. Neurosci.* **20,** 6225–6231 (2000).
21. Tang, Y. P. *et al.* Genetic enhancement of learning and memory in mice. *Nature* **401,** 63–69 (1999).
22. Harris, J. A. & Westbrook, R. F. Evidence that GABA transmission mediates context-specific extinction of learned fear. *Psychopharmacology (Berl.)* **140,** 105–115 (1998).
23. McGaugh, J. L., Castellano, C. & Brioni, J. Picrotoxin enhances latent extinction of conditioned fear. *Behav. Neurosci.* **104,** 264–267 (1990).
24. Medina, J. F., Repa, C. J., Mauk, M. D. & LeDoux, J. E. Parallels between cerebellum- and amygdala-dependent conditioning. *Nature Rev. Neurosci.* **3,** 122–131 (2002).
25. Collins, D. R. & Paré, D. Reciprocal changes in the firing probability of lateral and central medial amygdala neurons. *J. Neurosci.* **19,** 836–844 (1999).
26. Royer, S., Martina, M. & Paré, D. An inhibitory interface gates impulse traffic between the input and output stations of the amygdala. *J. Neurosci.* **19,** 10575–10583 (1999).
27. Franklin, K. B. J. & Paxinos, G. *The Mouse Brain* (Academic, San Diego, 1997).
28. Di Marzo, V. *et al.* Levels, metabolism, and pharmacological activity of anandamide in CB(1) cannabinoid receptor knockout mice: evidence for non-CB(1), non-CB(2) receptor-mediated actions of anandamide in mouse brain. *J. Neurochem.* **75,** 2434–2444 (2000).
29. Rammes, G. *et al.* Synaptic plasticity in the basolateral amygdala in transgenic mice expressing dominant-negative cAMP response element-binding protein (CREB) in forebrain. *Eur. J. Neurosci.* **12,** 2534–2546 (2000).
30. Rammes, G., Eder, M., Dodt, H.-U., Kochs, E. & Zieglgänsberger, W. Long-term depression in the basolateral amygdala of the mouse involves the activation of interneurons. *Neuroscience* **107,** 85–97 (2001).

Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com/nature).

Acknowledgements

S.C.A., T.B. and G.R. contributed equally to this work. We thank S. Bourier, B. Brachvogel and W. Wurst for feeder cells and technical support; K. Pfeffer for E14 embryonic stem cells; K. Rajewsky for Cre deleter mouse line; B. Lüscher for FRT-flanked PGK-neo cassette; M. Wiedemann, H. Dietrich, B. Wölfel, A. Daschner, F. Fezza and A. Rippl for technical assistance, mouse breeding and genotyping; A. Mederer for help with behavioural experiments; F. Holsboer for continuous support; E. Güll for secretarial work; and C. Behl for critically reading the manuscript.

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to B.L. (e-mail: lutz@mpipsykl.mpg.de).

A transcription factor response element for gene expression during circadian night

Hiroki R. Ueda*†, Wenbin Chen*, Akihito Adachi‡, Hisanori Wakamatsu*, Satoko Hayashi*, Tomohiro Takasugi*, Mamoru Nagano‡ Ken-ichi Nakahama‡, Yutaka Suzuki§, Sumio Sugano§, Masamitsu Iino†, Yasufumi Shigeyoshi‡ & Seiichi Hashimoto*

** Molecular Medicine Laboratories, Institute for Drug Discovery Research, Yamanouchi Pharmaceutical Company, Ltd, 21 Miyukigaoka, Tsukuba, Ibaraki 305-8585, Japan*
† Department of Pharmacology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Bunkyo-ku, Tokyo 113-0033, Japan
‡ Department of Anatomy and Neurobiology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osakasayama City, Osaka 589-8511, Japan
§ Human Genome Center, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokane-dai, Minato-ku, Tokyo 108-8639, Japan

Mammalian circadian clocks consist of complex integrated feedback loops[1–10] that cannot be elucidated without comprehensive measurement of system dynamics and determination of network structures[11]. To dissect such a complicated system, we took a systems-biological approach based on genomic, molecular and cell biological techniques. We profiled suprachiasmatic nuclei and liver genome-wide expression patterns under light/dark cycles and constant darkness. We determined transcription start sites of human orthologues for newly identified cycling genes and then performed bioinformatical searches for relationships between time-of-day specific expression and transcription factor response elements around transcription start sites. Here we demonstrate the role of the Rev-ErbA/ROR response element in gene expression during circadian night, which is in phase with *Bmal1* and in antiphase to *Per2* oscillations. This role was verified using an *in vitro* validation system, in which cultured fibroblasts transiently transfected with clock-controlled reporter vectors exhibited robust circadian bioluminescence[12].

To perform comprehensive measurement of mammalian circadian gene expression, we profiled genome-wide expression patterns of central (suprachiasmatic nuclei, SCN) and peripheral (liver) clocks every four hours during light/dark cycles (LD) or constant darkness (DD) over two days. We extracted total RNA from 50 pooled SCNs and four pooled livers at each time point, prepared biotinylated complementary RNA and used an Affymetrix mouse high-density oligonucleotide probe array (GeneChip) to determine SCN and liver gene expression.

The data obtained were analysed through two statistical cosine filters, one for LD and the other for DD time courses (see

Supplementary Information for the paper by Marsicano et al.

"The endogenous cannabinoid system controls extinction of aversive memories"

Generation and general behavioural characterisation of CB1-deficient mice.

Methods for the generation of CB1-null mutant mice. A lambda phage DASHII genomic library constructed from E14 embryonic stem cells was screened to isolate DNA fragments containing the CB1 locus. A construct containing the entire open reading frame of CB1 gene flanked by two loxP sites, two homology arms, and a FRT-flanked PGK-neo cassette, was generated and electroporated into E14 mouse embryonic stem cells to obtain the floxed-neo allele (Fig. **a**). Germ-line transmission was reached by standard procedures. Mice bearing the floxed-neo allele were then crossed with transgenic mice expressing Cre recombinase ubiquitously[1]. Cre-mediated recombination was proved by Southern blot hybridisation (Fig. **b**). Mice carrying a germ-line transmissible deletion of CB1 were backcrossed for five generations into C57BL/6N (Charles River). Experimental animals were genotyped by PCR, using primers G50 (5´-GCTGTCTCTGGTCCTCTTAAA), G51 (5´-GGTGTCACCTCTGAAAACAGA) and G54 (5´-CCTACCCGGTAGAATTAGCTT) (Fig. **c**). Homozygous CB1-deficient mice (called $CB1^{-/-}$) and wild-type littermates ($CB1^{+/+}$) from heterozygous breedings were used for the experiments. Lack of CB1 expression was shown by *in situ* hybridization[2] (Fig. **d**).

Consistent with previously reported CB1-deficient mouse lines[3,4], $CB1^{-/-}$ mice did not show any gross morphological defects and had normal neurological reflexes (righting, postural, eye blink, ear twitch and whisker orienting reflexes). Mendelian frequency reached values of 50.4%, 26.1% and 23.5% for heterozygous, $CB1^{+/+}$ and $CB1^{-/-}$ mice, respectively (n=1782).

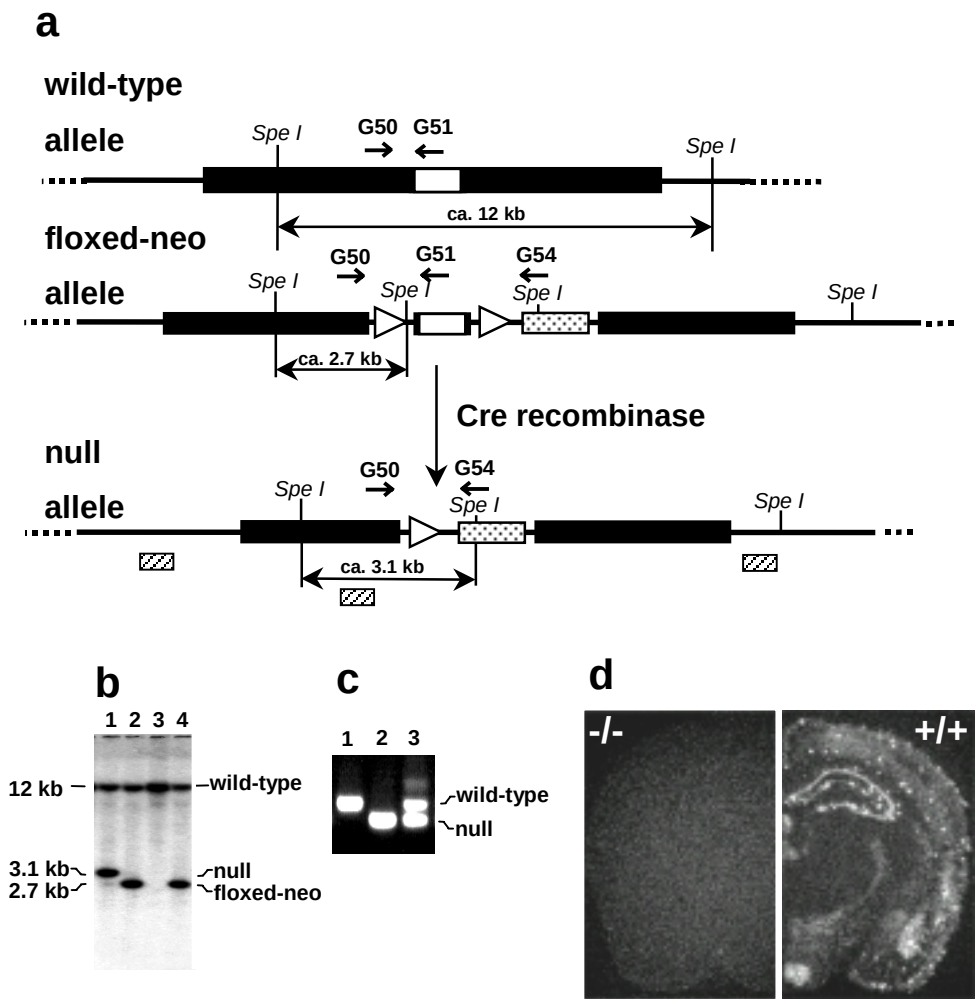


Figure Generation of CB1-deficient mice (homozygous $CB1^{null/null}$, called $CB1^{-/-}$). **a**, wild-type, floxed-neo and null allele of CB1, respectively. Black boxes, homology arms; white box, CB1 open reading frame; stippled box, PGK-neo cassette; white triangles, loxP sites; dashed bars, probes used for Southern blot experiments; small arrows, primers for PCR analyses. **b**, Southern analysis of wild-type (lane 3), heterozygous $CB1^{+/floxed\text{-}neo}$ (lanes 2 and 4) and heterozygous $CB1^{+/-}$ mice (lane 1). **c**, PCR analysis of experimental animals: $CB1^{+/+}$ (lane 1), $CB1^{-/-}$ (lane 2) and heterozygous $CB1^{+/-}$ (lane 3). **d**, *In situ* analysis of CB1 mRNA in $CB1^{-/-}$ and $CB1^{+/+}$ mice on coronal sections of adult forebrain.

Methods for general behavioural characterisation of CB1$^{-/-}$ mice.

Pain threshold and unconditioned freezing. Animals were placed into the conditioning chamber, and a scrambled electric footshock of rising intensity (starting from 0 mA) was applied. The shock was switched off as soon as the animals jumped or vocalised. The corresponding shock intensity was defined as pain threshold. After 6 days, animals were repeatedly exposed to 60-s tones and freezing behaviour was assessed as described in the main *Methods* part.

Elevated plus-maze. Elevated plus-maze was made of grey PVC with four opposing arms (5 x 25 cm, 72 cm elevated above the floor, 10 lux illumination), two of which were enclosed by walls of 14.5 cm height (closed arms) and the two others were open, except for a small 0.5 cm rim (open arms). A 5 x 5 cm neutral zone interconnected all four arms. Measured parameters (5-min exposure) were: number of closed and open arm entries, relative number of open arm entries (percentage of total number of open and closed arm entries) and relative time spent on open arms (percentage of total time on closed and open arms).

Open field. Animals were tested in an open field system (26 x 26 x 28 cm) equipped with two infrared sensor rings for measurement of horizontal and vertical locomotion (True Scan, Coulbourn Instruments) in complete darkness over 30 min with a sampling rate of 4 Hz.

Reference List for Supplementary Information

1. Schwenk,F., Baron,U. & Rajewsky,K. A cre-transgenic mouse strain for the ubiquitous deletion of loxP-flanked gene segments including deletion in germ cells. *Nucl. Acids Res.* **23**, 5080-5081 (1995).

2. Marsicano,G. & Lutz,B. Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. *Eur. J. Neurosci.* **11**, 4213-4225 (1999).

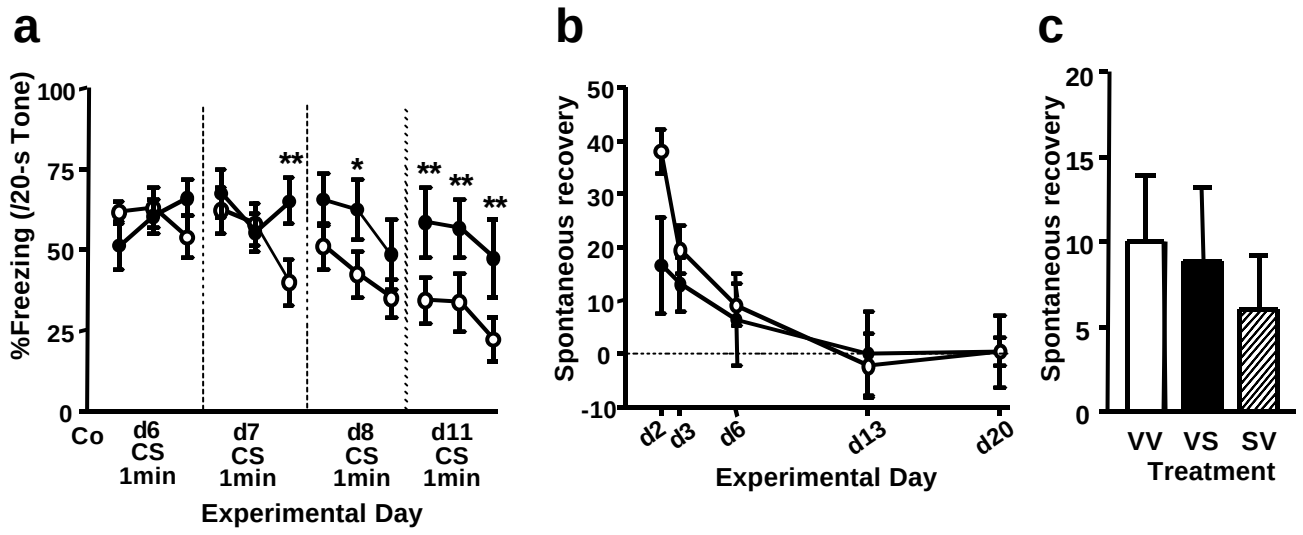
3. Zimmer,A., Zimmer,A.M., Hohmann,A.G., Herkenham,M. & Bonner,T.I. Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 5780-5785 (1999).

4. Ledent,C. *et al.* Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science* **283**, 401-404 (1999).

Supplementary Information for the paper by *Marsicano et al.*

"The endogenous cannabinoid system controls extinction of aversive memories"

Impaired long-term extinction following inactivation of CB1 relates to impaired short-term extinction but not enhanced spontaneous recovery



a, Re-analysis of the data of the experiment with the daily 60-s tone presentations in 20-s intervals (cf. Fig. 1b). At the beginning of the experiment (d6), both $CB1^{-/-}$ (black circles) and $CB1^{+/+}$ mice (open circles) showed little short-term extinction in the course of the 60-s tone presentation. With ongoing experiment, $CB1^{+/+}$ but not $CB1^{-/-}$ mice showed extinction of the freezing response due to differences in short-term (d7) and long-term extinction (d8, d11). Note that there was no significant difference in the spontaneous recovery of the freezing response, defined as the difference between the percentages of freezing at the beginning of tone presentation of a given day and at the end of tone presentation of the previous extinction trial (data not shown). * $p<0.05$, ** $p<0.01$ vs $CB1^{+/+}$. **b**, Re-analysis of the data of the experiment with the daily 180-s tone presentations (cf. Fig. 1g) revealed no significant differences in the spontaneous recovery between $CB1^{-/-}$ (black circles) and $CB1^{+/+}$ mice (open circles). Spontaneous recovery was calculated as the difference in freezing behaviour between the beginning (first 60s; cf. Fig. 1g) of the tone-presentation of a given extinction trial and the end (last 60s) of the preceding tone presentation (dy.1-dx.3). **c**, Re-analysis of the data of the pharmacological experiment with SR141716A administration before conditioning (SV) or before the first extinction trial (VS) or vehicle treatment only (VV; cf. Fig. 2a) revealed no difference in the spontaneous recovery (d6.1–d1.3) between the three experimental groups.

100 YEARS AGO

An interesting instance of that adaptability to changing tastes and conditions which is the mainspring of progress in industry as well as in science is afforded by a note in the *Journal* of the Society of Arts (July 18). For some years the demand for claret has greatly diminished in favour of the wines of Champagne, and has seriously affected the wine industry in the Bordeaux region. Several proprietors in the Médoc have, however, now commenced the production of sparkling wines by the same process as champagne is made, and their action has been the means of developing practically a new industry. It may at first seem strange that white wine should be able to be made in the Médoc, where only black grapes are grown, but as a matter of fact champagne is almost entirely made from black grapes, and the most celebrated vineyards in the Champagne district are all planted with them… It is stated that to the ordinary taster there is nothing but the label to distinguish the sparkling médoc from the best brands of champagne.
From *Nature* 31 July 1902.

50 YEARS AGO

***The Psychology of the Occult.* This stimulating and highly provocative book is an attempt to describe and analyse the part "played by various types of psychological anomaly in the creation and perpetuation of occult beliefs and practices"… Mr. Rawcliffe is completely unmoved by the flood of modern propaganda in favour of the reality of so-called psychic phenomena… As for the physical phenomena of the séance room, Mr. Rawcliffe finds the evidence scarcely worth considering. To him the whole of the studies of the psychical research worker are mere examples of magic and superstition dressed up in modern garb and often presented behind a façade of statistical jargon which is intended to disguise the faulty character of the original data. In support of his position he has skilfully put together a mass of material in which the incompetence and credulity of not a few workers in this field are cruelly displayed. Yet he has omitted much that would have strengthened his case and, it must be added, a good deal that would have weakened it… however, this book remains a useful handbook for those who suspect that much of what passes for psychical research and which is often unfortunately supported by leading parapsychologists is scarcely worth the paper on which it is recorded.**
From *Nature* 2 August 1952.

surface, see inside the structure and sample the rocks. But exposed impact structures are typically incomplete, lacking their uppermost parts through the vagaries of erosion and deformation processes. The only way of 'seeing' virgin morphology is when a structure has been rapidly buried after it formed, and so has been preserved. Geophysicists then need to use seismic techniques to reveal the three-dimensional structure, as has been done for Silverpit. The development of multiple concentric rings at such a small diameter may not be unusual because, until recently, we have been unable to obtain images with this degree of detail in such a well-preserved example.

Finally, the real test that Silverpit was created by an impact will be to look for shock effects in the rocks that form it. Shock-generated features such as unusual microscopic mineral deformations and shatter cones (conical fracture systems formed by shock waves in rock) would be compelling evidence of an impact origin. Two wells that were drilled in the search for oil and gas do penetrate the structure, but unfortunately, few samples of the drill cuttings from them were taken. Given that impact structures are among the most productive hydrocarbon sites on the planet, we may get more rock samples if Silverpit shows exploration potential. ■

John G. Spray is at the Planetary and Space Science Centre, University of New Brunswick, Fredericton, New Brunswick E3B 5A3, Canada.
e-mail: jgs@unb.ca

1. Stewart, S. A. & Allen, P. J. *Nature* **418,** 520–523 (2002).
2. Grieve, R. A. F., Therriault, A. & Rupert, A. *Annu. Rev. Planet. Sci.* **28,** 305–338 (2000).
3. Spudis, P. D. *The Geology of Multi-Ring Impact Basins* 263 (Cambridge Univ. Press, 1993).
4. Baldwin, R. B. *Phys. Earth Planet. Inter.* **6,** 327–339 (1972).
5. Melosh, H. J. *Nature* **373,** 104–105 (1995).

Neurobiology

Never fear, cannabinoids are here

Pankaj Sah

Although we understand how fearful memories are stored in the brain, how they are extinguished remains a mystery. The answers may lie with the cannabinoid compounds our bodies produce.

Cannabinoids such as marijuana and hashish have been used for over a thousand years for medicinal and recreational purposes. The active 'ingredient' of these drugs is Δ^9-tetrahydrocannabinol, which produces effects on nerve cells in the brain by binding to a protein on the neuronal surface, the CB1 receptor[1]. But of course the receptor is not there simply to detect this externally derived compound: it also binds to 'endogenous' cannabinoids, which are produced naturally by the body. On page 530 of this issue, Marsicano and colleagues[2] propose a new role for this 'endocannabinoid' system — extinguishing fear-related memories in mice. The finding might have implications for treating anxiety disorders in humans.

We can form memories in several different ways, one of which is Pavlovian conditioning — the classic example being that of Pavlov's dogs, which learned to expect food whenever they heard a ringing tone. We all form these types of associations; for instance, we may associate a particular piece of music with our first love affair. But the connection need not always be pleasant. Imagine you are having a quiet walk in a park when you are threatened by an armed person. During the attack you are terrified; your heart races and your palms are sweaty. You run and escape. Later, you may find that entering the same park brings back in detail the memory of the attack, right down to the sweaty palms.

In the lab, the neuronal and molecular mechanisms underlying fearful memories are often studied in animals by using 'fear conditioning'. Here, a neutral — or conditioned — stimulus, which is typically a tone or a light, is paired with an aversive (unconditioned) stimulus, typically a small electric shock to the foot. After the two stimuli are paired a few times, the conditioned stimulus alone evokes the stereotypical features of the fearful response to the unconditioned stimulus, including changes in heart rate and blood pressure and freezing of ongoing movements. Repeated presentation of the conditioned stimulus alone leads to extinction of the fearful response — the animal learns that it need no longer fear a shock from the tone or light.

A large body of work has established that a small, almond-shaped region in the brain, the amygdala, is crucial in acquiring and, possibly, storing the memory of conditioned fear[3,4]. It is thought that, at the cellular and molecular level, this learned behaviour requires neurons in the basolateral part of the amygdala, and changes in the strength of their connection with other neurons ('synaptic plasticity') that depend on the NMDA receptor[5], which responds to the neurotransmitter glutamate.

The extinction of aversive memories also involves the basolateral amygdala, but the cellular and molecular details are less clear. Infusing antagonists of the NMDA receptor into this region blocks extinction, implying that these receptors are important here, too[6]. Yet their exact role is not known. It has been proposed that synaptic plasticity is

again involved[6], but the possible sites of plasticity and the underlying physiology are not known, and NMDA-receptor-dependent plasticity has not yet been correlated with extinction. Moreover, it has been suggested that there are also NMDA-receptor-independent mechanisms of extinction[7].

Marsicano *et al.*[2] now propose just such a mechanism, which involves the endocannabinoids anandamide and 2-arachidonylglycerol, and their CB1 receptors. These receptors are some of the most abundant neuromodulatory receptors in the central nervous system and are expressed at high levels in the limbic system, cerebellum and basal ganglia[8]. The classical behavioural effects of exogenous cannabinoids — such as sedation and memory changes — have been correlated with the presence of CB1 receptors in the limbic system and striatum.

It has been difficult, however, to pin down the physiological role of endocannabinoids and how they are released in these regions. In studies that were the first to reveal such a role, the depolarization of neurons by repetitive activity led to the release of endocannabinoids[9], which diffused to the terminals of other neurons and inhibited neurotransmitter release. This effect was transient in the hippocampus and cerebellum[9] and long lasting in the striatum[10]. Yet these changes in neurotransmission have not been connected to any specific behavioural effects. So the study by Marsicano *et al.*[2] represents a leap forward in two areas of neurobiology, in that it clearly implicates the release of endocannabinoids in a well-known, simple learning task. It also links endocannabinoid release to synaptic plasticity.

After engineering mice to lack the CB1 receptor, Marsicano *et al.* first showed that although these animals could learn and later recall the association of a tone with a foot shock, they could not extinguish the memory. A drug that antagonizes the CB1 receptor similarly prevented extinction in wild-type mice. The authors then found that during the extinction protocol (exposure to the tone alone), the levels of both anandamide and 2-arachidonylglycerol were raised in the basolateral amygdala in mutant and normal mice. This implies that a process involving activation of the CB1 receptors by endocannabinoids is essential in the extinction of conditioned fear.

Next, in experiments with slices of normal mouse brains, the authors looked at neurons in the basolateral amygdala that can release GABA (an inhibitory neurotransmitter). They found that low-frequency stimulation of these neurons leads to a long-term reduction in the release of GABA, which in turn leads to less inhibition of the connecting 'pyramidal' neurons. This long-term 'depression' — a type of synaptic plasticity — was completely blocked by the CB1-receptor antagonist, and absent in CB1-deficient mice. These findings suggest that the endocannabinoids reduce GABA release in the basolateral amygdala, thereby helping to extinguish the fear-conditioned response. In mammals, the neurons that release GABA are largely interneurons, which can be divided into several populations on the basis of their expression of certain proteins and peptides (such as cholecystokinin). The role of endocannabinoids in reducing GABA release fits with the finding that CB1 receptors in the basolateral amygdala are present on the terminals of cholecystokinin-containing interneurons[11,12].

This is an entirely new cellular and molecular mechanism for extinction. But how does it tie in with the NMDA receptors? There seems little doubt that activation of these glutamate receptors in the basolateral amygdala is somehow required for extinction[6]. But Marsicano *et al.*'s brain-slice experiments were performed with blocked glutamate receptors, showing that the endocannabinoid-mediated synaptic plasticity they report does not need the NMDA receptors. So we have yet to find out how these receptors are involved in extinction.

It has been argued that the neuronal circuitry underlying fear conditioning has similarities to that responsible for fear-related clinical conditions, such as post-traumatic stress disorder[4]. Behavioural therapies for these conditions — including systematic desensitization and imagery therapies — share features with extinction. The finding that the endocannabinoids contribute to extinction raises the possibility that drugs that target these molecules and their receptors could be useful new treatments for anxiety disorders. Finally, there is much anecdotal evidence of patients using cannabis heavily in the early stages of psychiatric illness. This has often been thought to contribute to acute illness. But it seems possible that it may instead be a form of self-medication for the sometimes extreme anxiety that these people experience. ■

Pankaj Sah is in the Division of Neuroscience, John Curtin School of Medical Research, Australian National University, Canberra, ACT 2601, Australia.
e-mail: pankaj.sah@anu.edu.au

1. Matsuda, L. A. *et al. Nature* **346,** 561–564 (1990).
2. Marsicano, G. *et al. Nature* **418,** 530–534 (2002).
3. LeDoux, J. E. *Annu. Rev. Neurosci.* **23,** 155–184 (2000).
4. Davis, M. & Whalen, P. J. *Mol. Psychiatry* **6,** 13–34 (2001).
5. Blair, H. T., Schafe, G. E., Bauer, E. P., Rodrigues, S. M. & LeDoux, J. E. *Learn. Mem.* **8,** 229–242 (2001).
6. Walker, D. L. & Davis, M. *Pharmacol. Biochem. Behav.* **71,** 379–392 (2002).
7. Santini, E., Muller, R. U. & Quirk, G. J. *J. Neurosci.* **21,** 9009–9017 (2001).
8. Herkenham, M. *et al. J. Neurosci.* **11,** 563–583 (1991).
9. Wilson, R. I. & Nicoll, R. A. *Science* **296,** 678–682 (2002).
10. Gerdeman, G. L., Ronesi, J. & Lovinger, D. M. *Nature Neurosci.* **5,** 446–451 (2002).
11. Katona, I. *et al. J. Neurosci.* **21,** 9506–9518 (2001).
12. McDonald, A. J. & Mascagni, F. *Neuroscience* **107,** 641–652 (2001).

Earth science

Core values

John Brodholt and Francis Nimmo

Calculating the age of the Earth's solid inner core has proved to be a tricky business. But the suggestion that there is more potassium in the core than had been thought could help to reconcile differing estimates.

Potassium is a relatively insignificant element in the Earth, languishing in sixteenth place in the league table of chemical abundance. But, right now, radioactive decay of a potassium isotope, ^{40}K, is responsible for about 10% of the heat lost by the Earth. As ^{40}K has a half-life of 1.25 billion years, its decay would have produced much more heat in the past — just after the Earth formed, 4.6 billion years ago, daily decay of ^{40}K would have produced more heat than the present total daily heat loss of the Earth. Heat within the Earth drives processes such as convection in the mantle layer and the generation of the planet's magnetic field. So knowing how potassium and other radioactive elements are concentrated in different parts of the Earth is fundamental to understanding these processes.

There is a large concentration of potassium in the Earth's crust, and a significant proportion remains in the mantle below. What is not known is how much is in the Earth's core. Although experimental results have been ambiguous, it has generally been thought that, because of the relatively large radius of potassium ions, not much of this element could be absorbed in the core. But new results from Gessman and Wood[1], reported in *Earth and Planetary Science Letters*, show that the amount of potassium in the core depends on the core's sulphur and oxygen content, and on the structure of the coexisting silicate melt. Their findings help to explain some of the ambiguities in the earlier data, and also enable them to estimate the maximum concentration of potassium in the Earth's core — a number that has important bearing on the age of both the inner core and the Earth's magnetic field.

Although the magnetic field is generated by fluid flow in the liquid outer core, it is generally accepted that the solid inner core also plays a fundamental role. This is because