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Competing interests statement

The authors declare that they have no competing financial interests.

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The endogenous cannabinoid system controls extinction of aversive memories

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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

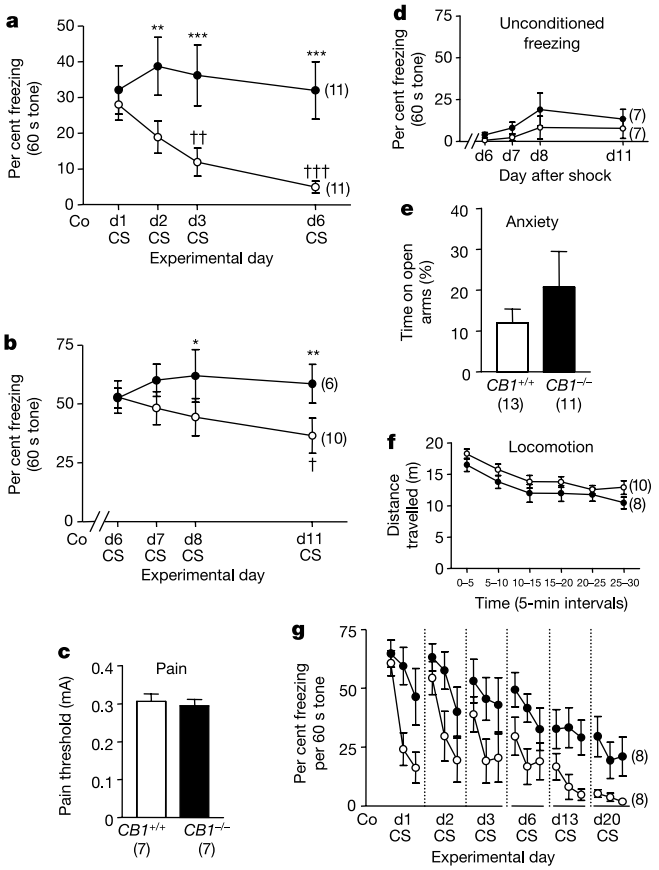

Figure 1 Impaired extinction of aversive memory in an auditory fear-conditioning task of *CB1* $^{-/-}$ mice (filled circles) as compared to their *CB1* $^{+/+}$ littermates (open circles). **a**, **b**, After conditioning (Co) animals were repeatedly exposed to 60 s tones (conditioned stimulus, CS) 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).

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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 $CB1^{-/-}$

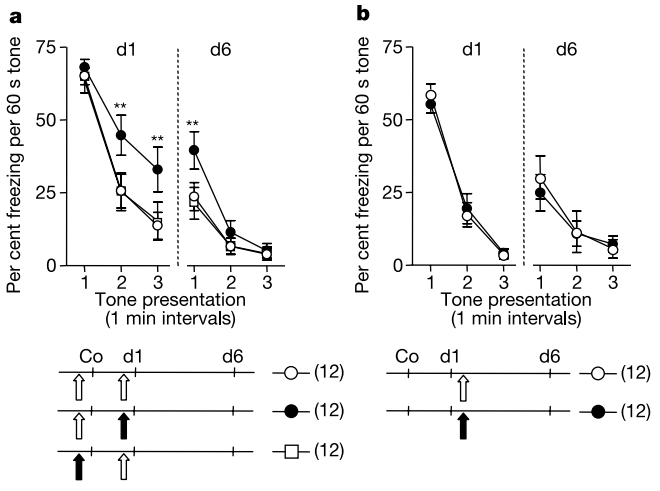

Figure 2 CB1 antagonist SR141716A impairs short-term and long-term extinction, but not acquisition and consolidation of aversive memories. **a**, Mice were treated with SR141716A (filled arrows) or vehicle (open arrows) 20 min before conditioning (Co) and the first extinction trial (d1; 3 min tone). **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).

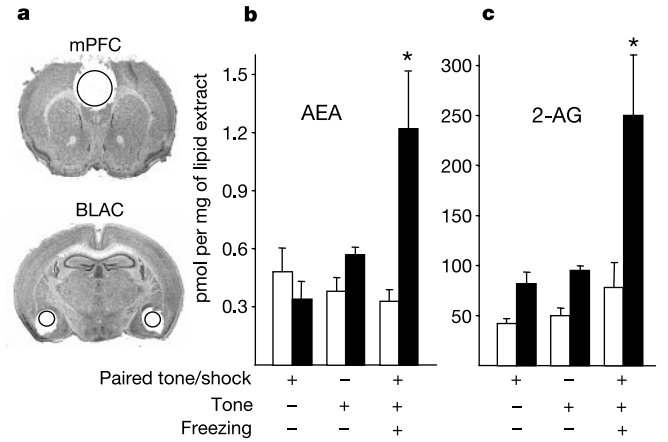

Figure 3 Re-exposure to the tone 24 h after conditioning causes increased endocannabinoid levels in the basolateral amygdala complex (BLAC) but not the medial prefrontal cortex (mPFC) of C57BL/6J mice. **a**, Micrographs of coronal brain sections showing representative examples of the dissected mPFC 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-

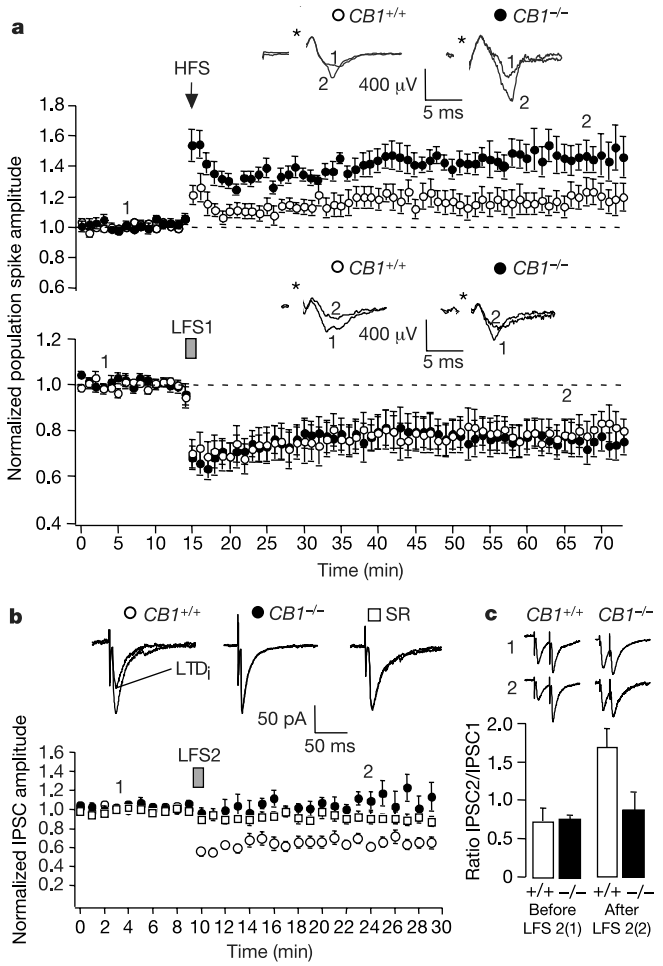

Figure 4 Endogenous cannabinoid system and synaptic plasticity in the basolateral amygdala. **a**, LTP (top) and LTD (bottom) in slices from $CB1^{+/+}$ and $CB1^{-/-}$ mice, induced by high-frequency stimulation (HFS) and low-frequency stimulation (LFS 1), respectively. Asterisks indicate stimulus artefacts. **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.

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A transcription factor response element for gene expression during circadian night

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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