Endogenous cannabinoid release within prefrontal-limbic pathways affects memory consolidation of emotional training

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Previous studies have provided extensive evidence that administration of cannabinoid drugs after training modulates the consolidation of memory for an aversive experience. The present experiments investigated whether the memory consolidation is regulated by endogenously released cannabinoids. The experiments first examined whether the endocannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG) are released by aversive training. Inhibitory avoidance training with higher footshock intensity produced increased levels of AEA in the amygdala, hippocampus, and medial prefrontal cortex (mPFC) shortly after training in comparison with levels assessed in rats trained with lower footshock intensity or unshocked controls exposed only to the training apparatus. In contrast, 2-AG levels were not significantly elevated. The additional finding that posttraining infusions of the fatty acid amide hydrolase (FAAH) inhibitor URB597, which selectively increases AEA levels at active synapses, administered into the basolateral complex of the amygdala (BLA), hippocampus, or mPFC enhanced memory strongly suggests that the endogenously released AEA modulates memory consolidation. Moreover, in support of the view that this emotional training-associated increase in endocannabinoid neurotransmission, and its effects on memory enhancement, depends on the integrity of functional interactions between these different brain regions, we found that disruption of BLA activity blocked the training-induced increases in AEA levels as well as the memory enhancement produced by URB597 administered into the hippocampus or mPFC. Thus, the findings provide evidence that emotionally arousing training increases AEA levels within prefrontal-limbic circuits and strongly suggest that this cannabinoid activation regulates emotional arousal effects on memory consolidation.

anandamide | endocannabinoids | cannabinoid receptors | inhibitory avoidance | emotional arousal

It is well-established that stressful or emotionally arousing experiences are well-remembered (1). In addition, there is extensive evidence that the basolateral complex of the amygdala (BLA), hippocampus, and medial prefrontal cortex (mPFC) are all crucially involved in mediating emotional arousal effects on memory consolidation (2, 3). The consolidation of memories of arousing experiences requires an orchestration of neural activity in these brain systems, and the cannabinoid system has emerged as a key modulator of such function (1, 4–6). Endogenous cannabinoid ligands [termed endocannabinoids, mainly *N*-arachidonoyl ethanolamine (anandamide; AEA) and 2-arachidonoyl glycerol (2-AG)] are released from postsynaptic membranes and feedback in a retrograde manner onto either excitatory or inhibitory presynaptic terminals, thus suppressing both excitatory and inhibitory signaling within specific neuronal circuits (7).

Extensive evidence indicates that exogenous cannabinoids administered into this neural circuitry modulate memory processing of emotionally arousing training (8–12). We previously showed that the synthetic cannabinoid agonist WIN55,212-2, administered into the BLA immediately after inhibitory avoidance training, enhances the consolidation of long-term memory (8). Conversely, inhibition of endogenous cannabinoid signaling within the BLA with posttraining infusions of the cannabinoid type 1 (CB1) receptor antagonist AM251 impairs inhibitory avoidance memory (8). We recently reported that the level of emotional arousal at the time of training is an important factor in determining cannabinoid effects on memory (13): WIN55,212-2 administration enhanced long-term object recognition memory when rats were trained under a high arousal condition but was ineffective with low-arousing training (13). Although the findings of our prior studies, as well as those of other investigators (6, 8, 11–13), indicate that administration of cannabinoids can modulate memory consolidation, studies have not yet investigated whether AEA or 2-AG are released physiologically in response to emotionally arousing training and normally play a role in creating strong memories for these experiences. The present experiments investigated this issue. Rats were trained on an inhibitory avoidance task under different arousal conditions and were killed after training for determination of AEA and 2-AG levels in the amygdala, hippocampus, and mPFC. To investigate whether training-induced endocannabinoid release contributes to memory consolidation, we pharmacologically

Significance

An involvement of exogenous cannabinoids in the regulation of memory for emotional events has emerged over the past decade. The present findings demonstrate that after an aversive training experience the endogenous cannabinoid anandamide is released into the amygdala, hippocampus, and medial prefrontal cortex (mPFC) and normally plays a role in the formation of a strong memory trace. Furthermore, an intact basolateral amygdala is required to coordinate both hippocampal and mPFC endocannabinoid activity during aversive memory consolidation. Thus, our findings provide the first evidence, to our knowledge, that a dynamic network involving anandamide signaling within this prefrontal-limbic circuit modulates the consolidation of memory for emotionally arousing training.

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blocked AEA degradation in the BLA, hippocampus, or mPFC by locally infusing the fatty acid amide hydrolase (FAAH) inhibitor URB597 immediately after inhibitory avoidance training. In contrast to direct cannabinoid receptor agonists, URB597 selectively augments AEA signaling at active synapses, thus mimicking the physiological condition normally occurring after an emotionally arousing training experience.

The experiments also investigated whether training-associated endocannabinoid neurotransmission within this neurocircuitry depends on functional interactions between the BLA, hippocampus, and mPFC. Extensive evidence indicates that the BLA contributes to enhancement of memory for emotional events primarily by integrating neuromodulatory influences and modulating neural activity and synaptic plasticity in other brain regions (1). The BLA is known to project both directly and indirectly to the hippocampus (14, 15) and mPFC (16, 17), and it has been shown that a disruption of BLA activity blocks the memory-enhancing effects of drugs administered directly into either the hippocampus or mPFC (18, 19). Therefore, in this last series of experiments, we investigated whether an intact BLA activity is required to allow for a normal endocannabinoid response in the hippocampus and mPFC after an aversive training experience.

Results

Aversive Inhibitory Avoidance Training Increases AEA Levels Within the Amygdala, Hippocampus, and mPFC. This experiment investigated whether inhibitory avoidance training elevates AEA and 2-AG levels within the amygdala, hippocampus, and mPFC, and whether the magnitude of the effects depends on the aversiveness of the training procedure. Rats were trained on an inhibitory avoidance task with either a lower (0.35-mA; lower FS) or higher footshock intensity (0.45-mA; higher FS) or were exposed to the experimental apparatus without any footshock (no FS). Fig. 1 shows that these two FS intensities resulted in different 48-h retention latencies ($F_{2,17} = 20.41$; $P < 0.0001$). For endocannabinoid measurements, rats were killed 10, 30, or 60 min after training. Our findings indicate that the more aversive training condition elevated AEA, but not 2-AG, levels in all three brain regions. As shown in Fig. 2*A*, two-way ANOVA for amygdala AEA levels revealed a significant FS condition effect ($F_{2,50} = 16.92$; $P < 0.0001$) but no time effect ($F_{2,50} = 0.003$; $P = 0.99$) or interaction between both factors ($F_{4,50} = 0.40$; $P = 0.81$). Post hoc tests indicated that the higher FS significantly increased amygdala AEA levels at 10, 30, and 60 min after the training trial compared with rats given the lower FS (30 min: $P < 0.05$; 10 and 60 min: $P < 0.01$) or those unshocked (10 and 30 min: $P < 0.05$; 60 min: $P < 0.01$). As shown in Fig. 2*B*, two-way ANOVA for AEA levels in the hippocampus revealed a significant FS condition effect ($F_{2,44} = 14.25$; $P < 0.0001$), no time effect ($F_{2,44} = 1.27$; $P = 0.29$), and a significant interaction between both factors ($F_{4,44} = 2.73$; $P = 0.04$). Post hoc tests indicated that the higher FS significantly increased hippocampal AEA levels 10 min after the training trial compared with the unshocked controls ($P < 0.01$). Two-way ANOVA for AEA levels in the mPFC revealed a significant FS condition effect ($F_{2,49} = 5.47$; $P < 0.007$) but no time effect ($F_{2,49} = 2.94$; $P = 0.06$) or interaction between both factors ($F_{4,49} = 1.54$; $P = 0.20$; Fig. 2*C*). Post hoc tests indicated that the higher FS significantly increased AEA levels in the mPFC 60 min after the training trial compared with unshocked rats ($P < 0.05$; Fig. 2*C*). Rats exposed to the lower FS did not differ in AEA levels compared with unshocked rats in the three brain areas at any time point. Inhibitory avoidance training did not affect 2-AG levels in any of the brain regions at any time point (*SI Results* and Table S1). In plasma, AEA levels were significantly elevated 60 min after inhibitory avoidance training with the higher, but not lower, FS (*SI Results* and Table S2).

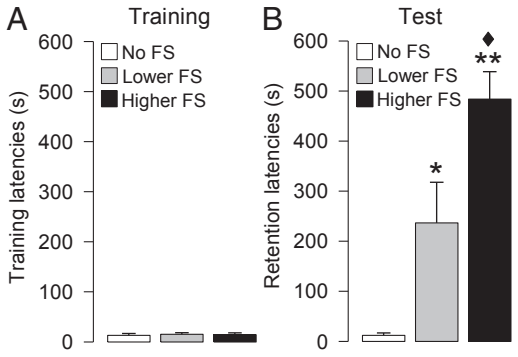

Fig. 1. Effect of different levels of emotional arousal on inhibitory avoidance memory. (*A*) Step-through latencies during inhibitory avoidance training of rats trained under three different conditions (only exposed to the context without receiving any FS, no FS; with a lower FS intensity, lower FS; with a higher FS intensity, higher FS). (*B*) Step-through latencies on a 48-h retention test. The increase of FS intensity during the training trial enhanced memory consolidation of the inhibitory avoidance task. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. the no FS group; $^{\blacklozenge}P < 0.05$ vs. the lower FS group. Results represent mean ± SEM (n = 6–7 per group).

Posttraining Infusions of the FAAH Inhibitor URB597 into the BLA, Hippocampus, and mPFC Enhance Inhibitory Avoidance Memory via Indirect Activation of CB1 Receptors. To determine whether the elevated AEA levels induced by the higher FS contribute to the enhancement of memory of this more aversive training experience, we next investigated whether exogenous augmentation of training-induced AEA levels with posttraining infusions of the FAAH inhibitor URB597 into the BLA, hippocampus, or mPFC modulates memory consolidation. Our findings indicate that URB597 administered into all three brain regions enhances memory consolidation.

As shown in Fig. 3*A*, 48-h retention latencies of rats administered vehicle into the BLA immediately after training were significantly longer than their entrance latencies during the training trial ($t_{11} = -3.63$; $P = 0.004$), indicating that the rats retained memory of the shock experience. Posttraining URB597 into the BLA enhanced 48-h retention latencies in an inverted U-shape relationship ($F_{3,46} = 3.63$; $P = 0.02$). Post hoc tests indicated that the 10-ng dose of URB597 produced retention latencies that were significantly longer than those of rats given vehicle ($P < 0.05$). Lower or higher doses (3 or 30 ng) were ineffective. Fig. 3*B* shows that URB597 infused posttraining into the hippocampus also enhanced 48-h retention latencies ($F_{3,32} = 3.30$; $P = 0.03$). Post hoc tests indicated that the 10-ng dose of URB597 significantly enhanced retention performance ($P < 0.05$), with lower or higher doses (3 or 30 ng) being ineffective. Fig. 3*C* shows that URB597 infusions into the mPFC also enhanced 48-h retention latencies ($F_{3,48} = 3.97$; $P = 0.01$). Post hoc comparisons indicated that the 30-ng dose of URB597 significantly enhanced retention performance ($P < 0.05$). Lower doses (3 or 10 ng) were ineffective.

Next, we examined whether the elevated AEA levels, induced by URB597 administration, enhance memory consolidation via an activation of CB1 receptors. Rats were administered the effective dose of URB597 together with a nonimpairing dose of the CB1 receptor antagonist AM251 immediately after the training trial. Our findings indicate that the URB597 effect on memory consolidation depends on CB1 receptor activity in all three brain regions investigated. Fig. 3*D* shows 48-h retention test latencies of rats administered URB597 together with AM251 into the BLA. Two-way ANOVA for retention latencies revealed a significant URB597 effect ($F_{1,34} = 4.27$; $P = 0.04$), a significant AM251 effect ($F_{1,34} = 5.47$; $P = 0.03$), and a significant interaction between both factors ($F_{1,34} = 5.45$; $P = 0.03$). Post hoc tests indicated that

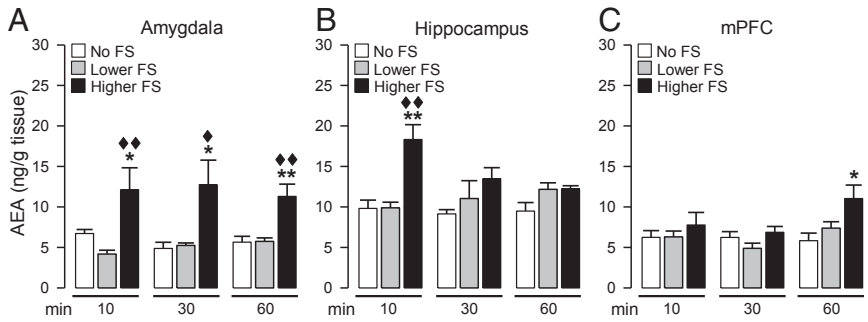

Fig. 2. Effect of the level of emotional arousal of an inhibitory avoidance training trial on AEA levels in the amygdala, hippocampus, and mPFC, measured at different time points after the training. The higher FS condition induced an increase in AEA levels in the amygdala (*A*) at 10, 30, and 60 min after the training, in the hippocampus (*B*) at 10 min after the training, and in the mPFC (*C*) at 60 min after the training. *$P < 0.05$, **$P < 0.01$ vs. the corresponding no FS group; $^{\blacklozenge}P < 0.05$, $^{\blacklozenge\blacklozenge}P < 0.01$ vs. the corresponding lower FS group. Results represent mean ± SEM (n = 4–8 per group).

retention latencies of rats given URB597 (10 ng) were significantly longer than those of rats given vehicle ($P < 0.05$). Retention latencies of rats given a nonimpairing dose of AM251 (0.14 ng) together with URB597 were significantly shorter than those of rats treated with URB597 alone ($P < 0.05$). Fig. 3*E* shows 48-h retention latencies of rats given URB597 and AM251 into the hippocampus. Two-way ANOVA for retention latencies revealed a significant URB597 effect ($F_{1,47} = 8.42$; $P = 0.006$), a significant AM251 effect ($F_{1,47} = 11.13$; $P = 0.002$), and a significant interaction between both factors ($F_{1,47} = 10.74$; $P = 0.002$). Post hoc comparisons indicated that retention latencies of rats given URB597 (10 ng) were significantly longer than those of vehicle-treated rats ($P < 0.01$). Retention latencies of rats given a nonimpairing dose of AM251 (0.28 ng) together with URB597 were significantly shorter than those of rats treated with URB597 alone ($P < 0.01$). Fig. 3*F* shows 48-h retention latencies of rats administered URB597 and AM251 into the mPFC. Two-way ANOVA revealed a significant URB597 effect ($F_{1,38} = 5.58$; $P = 0.03$), no AM251 effect ($F_{1,38} = 3.87$; $P = 0.06$), and a significant interaction between both factors ($F_{1,38} = 4.41$; $P = 0.04$). Post hoc comparisons indicated that retention latencies of rats given URB597 (30 ng) were significantly longer than those of rats given vehicle ($P < 0.05$). Retention latencies of rats given a nonimpairing dose of AM251 (0.28 ng) together with URB597 were significantly shorter than those of rats treated with URB597 alone ($P < 0.05$).

An Intact BLA Is Required for Enabling the Training-Induced Increase of AEA Levels Within the Hippocampus and mPFC. It is well-established that prefrontal-limbic circuits regulate emotional arousal effects on memory consolidation. In this experiment, we investigated whether functional interactions between the BLA, hippocampus, and mPFC are necessary for modulating endocannabinoid activity in response to an aversive training experience. We made bilateral excitotoxic lesions of the BLA and subsequently measured AEA and 2-AG levels in the hippocampus and mPFC after inhibitory avoidance training. Rats were trained with the higher FS and killed 10 or 60 min later for hippocampus and mPFC dissection (in the previous experiments, we found that AEA levels in the hippocampus and mPFC were elevated 10 and 60 min after training, respectively; Fig. 2 *B* and *C*). As shown in Fig. 4 *A* and *B*, bilateral BLA lesions abolished the training-induced increases in AEA levels in both the hippocampus and mPFC (hippocampus: $t_9 = 7.11$; $P < 0.0001$; mPFC: $t_{11} = 2.79$; $P = 0.02$). Bilateral BLA lesions did not affect 2-AG levels in either the hippocampus or mPFC after inhibitory avoidance training ($t_9 = 0.41$; $P = 0.69$; $t_{11} = -0.12$; $P = 0.91$, respectively; Table S3). These findings indicate that an intact BLA is necessary for inducing a training-associated increase of AEA levels within the hippocampus and mPFC, and provide important support for the view that functional interactions between these brain regions are required to coordinate endocannabinoid responses after emotionally arousing training.

An Intact BLA Is Required for Enabling the Memory-Enhancing Effect of Posttraining URB597 Infusions into the Hippocampus and mPFC. To further investigate the significance of functional interactions between these brain regions in regulating endocannabinoid effects on memory consolidation, we examined whether an intact BLA is also required for enabling the memory-enhancing effect

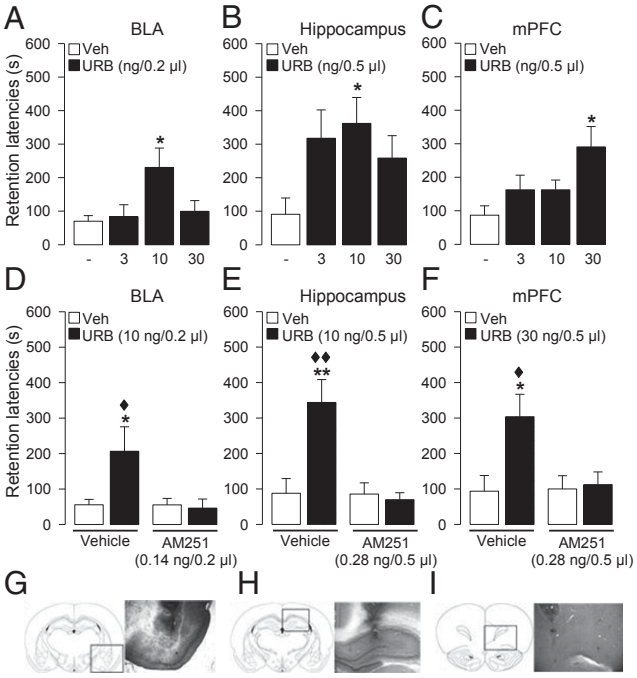

Fig. 3. Effects of intra-BLA, intrahippocampus, or intra-mPFC infusions of the FAAH inhibitor URB597, either alone or together with the CB1 receptor antagonist AM251, on 48-h retention. Immediate posttraining bilateral infusions of URB597 (URB; 3, 10, or 30 ng) into the BLA (*A*), hippocampus (*B*), or mPFC (*C*) enhanced memory consolidation. Concurrent administration of a nonimpairing dose of AM251 (0.14–0.28 ng per side) blocked the memory-enhancing effects of URB597 (10 or 30 ng) in the BLA (*D*), hippocampus (*E*), or mPFC (*F*). Representative photomicrographs (Nikon 801 microscope; original magnification 2×) illustrating the placement of the cannula and needle tip in the BLA (*G*), hippocampus (*H*), and mPFC (*I*). *$P < 0.05$, **$P < 0.01$ vs. the corresponding vehicle (Veh) group; $^{\blacklozenge}P < 0.05$, $^{\blacklozenge\blacklozenge}P < 0.01$ vs. the corresponding AM251 group. Results represent mean ± SEM (n = 8–13 per group).

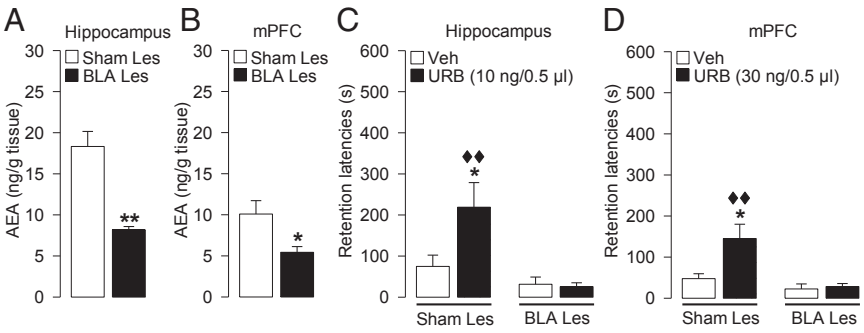

Fig. 4. Role of the BLA in modulating the endocannabinoid response to emotionally arousing inhibitory avoidance training in the hippocampus and mPFC. Bilateral permanent lesions of the BLA blocked the training-induced increase of AEA levels in the hippocampus (*A*) and mPFC (*B*), 10 and 60 min after the training trial, respectively. $*P < 0.05$, $**P < 0.01$ vs. the corresponding sham lesion group (Sham Les). Results represent mean ± SEM (n = 4–7 per group). Bilateral lesions of the BLA blocked the memory-enhancing effects induced by URB597 infused into the hippocampus (10 ng; *C*) or mPFC (30 ng; *D*). $*P < 0.05$, $**P < 0.01$ vs. the corresponding vehicle group; $\blacklozenge\blacklozenge P < 0.01$ vs. the corresponding BLA lesion group. Results represent mean ± SEM (n = 10–14 per group).

of URB597 when infused into either the hippocampus or mPFC. Concerning the hippocampus, two-way ANOVA for training latencies revealed no BLA lesion effect ($F_{1,46} = 1.46$; $P = 0.23$) and no difference between later drug treatment groups ($F_{1,46} = 0.28$; $P = 0.60$) or interaction between both factors ($F_{1,46} = 0.68$; $P = 0.41$). Two-way ANOVA for 48-h retention latencies revealed a significant BLA lesion effect ($F_{1,46} = 12.10$; $P = 0.001$), a significant URB597 effect ($F_{1,46} = 4.12$; $P = 0.048$), and a significant interaction between these two factors ($F_{1,46} = 4.84$; $P = 0.03$). As shown in Fig. 4*C*, URB597 (10 ng) infused into the hippocampus of sham-lesioned rats enhanced 48-h retention relative to their corresponding vehicle group ($P < 0.05$). Furthermore, and importantly, BLA lesions blocked the memory-enhancing effect of URB597 infused into the hippocampus ($P < 0.01$).

Next, we examined URB597 effects in the mPFC. Two-way ANOVA for training latencies revealed no BLA lesion effect ($F_{1,41} = 1.99$; $P = 0.17$) and no difference between later drug treatment groups ($F_{1,41} = 1.04$; $P = 0.31$) or interaction between both factors ($F_{1,41} = 0.46$; $P = 0.50$). Two-way ANOVA for 48-h retention test latencies revealed a significant BLA lesion effect ($F_{1,41} = 11.80$; $P = 0.001$), a significant URB597 effect ($F_{1,41} = 6.26$; $P = 0.02$), and a significant interaction between these two factors ($F_{1,41} = 4.91$; $P = 0.03$). As shown in Fig. 4*D*, post hoc tests indicated that URB597 (30 ng) infused into the mPFC of sham-lesioned rats enhanced 48-h retention compared with the vehicle group ($P < 0.05$). BLA lesions blocked the memory-enhancing effect of URB597 infused into the mPFC ($P < 0.01$). Thus, our findings indicate that the BLA is critically involved in regulating the memory-enhancing effects of endocannabinoids in the hippocampus and mPFC.

Discussion

The present findings indicate that a dynamic network involving AEA signaling within the BLA, hippocampus, and mPFC modulates the consolidation of memory for emotionally arousing training. Moreover, our results suggest that the BLA plays a crucial role in coordinating hippocampal and mPFC endocannabinoid activity within this neurocircuitry during the consolidation of memories for aversive experiences.

Rats trained under a higher arousal condition retained better memory of the aversive event than did rats trained under a lower arousal condition, and this effect was paralleled by an increase in AEA levels within the amygdala, hippocampus, and mPFC. In contrast, 2-AG levels were unaffected. The increase in AEA content showed a temporal-dependent response during the early phases of memory consolidation, with a sustained increase of AEA levels in the amygdala (from 10 to 60 min after training) and an increase only at 10 and 60 min after training in the hippocampus and mPFC, respectively. The increase of AEA signaling induced by posttraining administration of the FAAH inhibitor URB597 enhanced 48-h inhibitory avoidance retention performance through indirect activation of CB1 receptors. Additionally, we found that this training-induced increase in endocannabinoid neurotransmission depends on the integrity of functional interactions between these brain regions, because disruption of BLA activity resulted in a loss of the training-induced AEA response as well as the URB597 effect in the hippocampus and mPFC.

It is well-known that CB1 receptors, highly expressed throughout the limbic system (20), modulate neuronal signaling and synaptic plasticity (21, 22), thereby regulating emotional behavior and memory processes activated by emotionally arousing events (8, 9, 23–26). However, studies have reported conflicting findings concerning cannabinoid effects on memory consolidation (for a review, see ref. 6). Cannabinoid effects on memory are highly dependent on the level of emotional arousal induced by the stressfulness of the experimental condition used in the different studies (6, 13, 27). Our findings are consistent with prior evidence in indicating that inhibitory avoidance training induces the release of the endocannabinoid AEA in the amygdala, hippocampus, and mPFC only under highly aversive conditions. This endocannabinoid response was not detectable when animals received a lower FS.

To investigate whether the endogenous release of AEA after inhibitory avoidance training is linked to the stabilization of the memory trace, we tested whether pharmacological amplification of AEA signaling would affect memory consolidation for inhibitory avoidance training. Our findings indicate that the FAAH inhibitor URB597, which increases endogenous AEA levels in the synaptic cleft (28), enhanced 48-h retention when infused into the BLA, hippocampus, or mPFC after training. URB597 also affects, to a certain extent, the hydrolysis of other fatty-acid ethanolamides that do not bind to CB1 receptors. Our result that the URB597 effect was blocked by concurrent administration of the CB1 receptor antagonist AM251 indicates that the URB597-induced memory enhancement involves an increase in AEA signaling and activation of CB1 receptors. Our findings are, thus, consistent with previous evidence indicating that a blockade of endocannabinoid neurotransmission in the hippocampus (29), amygdala (30), and mPFC (12) impairs the consolidation of aversively motivated training experiences.

We previously reported that endocannabinoid effects on memory consolidation depend on the level of emotional arousal at the time of encoding (6, 13, 27). Such findings suggest that an interaction with stress hormones is important in determining the modulatory effects of cannabinoids on memory processes. The BLA is known to influence the association of environmental

information with emotional significance by engaging stress-related hormones and neurotransmitters to modulate memory processes (31, 32). We have previously shown that intra-BLA administration of the CB1 receptor antagonist AM251 blocks the ability of systemically administered corticosterone to facilitate the consolidation of memory of inhibitory avoidance training (8). Such findings indicate that glucocorticoids recruit endocannabinoid signaling in the BLA to modulate memory consolidation (33, 34). Our current results add to this evidence the findings that, after a stressful event, the endogenous release of AEA in prefrontal-limbic areas enhances memory consolidation for aversive experiences via an activation of CB1 receptors. Findings in the literature indicate a direct correlation between FS intensity and norepinephrine release in the amygdala (35). Therefore, it is likely that a certain degree of emotional arousal, induced by the higher FS intensity, leads to an optimal activation of stress mediators that is necessary to recruit the endocannabinoid system and, ultimately, mediate the formation of a strong memory trace.

Considerable evidence indicates that the BLA modulates memory consolidation by coordinating the activation of other brain regions and by regulating memory processes that take place elsewhere in the brain (1, 31, 36–38). This modulation might involve either direct or indirect neural connections to various limbic structures, including the hippocampus and mPFC (14, 15, 39, 40). Our findings provide additional evidence supporting this implication in indicating that a functional BLA is required to enable the modulatory effect of AEA within the hippocampus and mPFC on memory consolidation. These findings are also consistent with evidence that interactions of the BLA with the hippocampus (18, 41, 42) and mPFC (19) regulate emotional arousal effects on memory consolidation. Our findings further indicate that, depending on the brain region, AEA increases with distinct temporal windows during early phases of memory consolidation. We show that activation of the BLA regulates the endocannabinoid response in the hippocampus and mPFC, as permanent lesions of the BLA blocked the training-induced increase of AEA levels and the memory-enhancing effects of URB597 in both the hippocampus and mPFC.

Previous findings suggest that the initiation of a strong emotional experience activates memory-related neuroplasticity in the amygdala and hippocampus whereas suppressing PFC functioning (43). Such a model suggests a rapid activation of the BLA and hippocampus after a stressful experience, followed by an inhibitory phase. Moreover, there is evidence that corticosterone rapidly but transiently enhances the frequency of miniature postsynaptic currents (mEPSCs) in the hippocampal CA1 area (44), whereas it also rapidly increases mEPSCs in the BLA, but over a longer time window (45). Accordingly, we found a rapid and transient increase of AEA levels in the hippocampus and a rapid but enduring increase within the amygdala. It is well-accepted that CB1 receptor activation mediates a decrease in GABA release in the BLA (46). Thus, the rapid increase in amygdala AEA content observed in our study may represent one of the most rapid actions of BLA activation after experiencing an aversive event. In particular, the AEA-mediated activation of CB1 receptors in the BLA may decrease feedforward inhibition via inhibitory interneurons, thereby increasing the activity of BLA projection neurons (47). On the other hand, basal mPFC activity is known to provide an inhibitory influence on BLA activity (16, 48), whereas stress and glucocorticoids are known to suppress mPFC functions (49). It has previously been reported that glucocorticoid infusions into the mPFC enhance memory consolidation of an inhibitory avoidance training via functional and bidirectional interactions with the BLA (19). Thus, the present findings provide additional evidence that increased BLA activation during emotionally arousing conditions (38, 49, 50) enhances the consolidation of different kinds of information via its projections to other brain regions.

Considered together, our findings indicate that, as a response to emotional events, endocannabinoids are released within the prefrontal-limbic neurocircuitry, where they enhance the consolidation of memory for emotionally arousing events. These findings are of crucial importance, helping to understand the neural underpinnings of the temporal interactions between limbic regions after experiencing a stressful event and shedding light on the neural mechanism involved in the process of aversive memory formation.

Materials and Methods

Animals. Male Sprague–Dawley rats (320–370 g at the time of behavioral experiments; Charles River) were housed individually in a temperature-controlled (20 ± 1 °C) vivarium room and maintained under a 12-h/12-h light/dark cycle (7:00 AM to 7:00 PM lights on). Food and water were available ad libitum. All procedures were in compliance with Italian law (D.L. 26/14), a European Union directive (2010/63/EU), the Declaration of Helsinki, and *Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research* (51).

Surgery. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), given atropine sulfate (0.4 mg/kg, i.p.) to maintain respiration, and subsequently injected with 3 mL of saline (s.c.) to facilitate clearance of drugs and prevent dehydration. The rats were then placed in a stereotaxic frame (Kopf Instruments), and two stainless-steel guide cannulae (23-gauge) were implanted bilaterally, with the cannula tips 2 mm above the BLA [15 mm; coordinates: anteroposterior (AP), −2.8 mm from Bregma; mediolateral (ML), ±5.0 mm from midline; dorsoventral (DV), −6.5 mm from skull surface] or 1.5 mm above the CA1 region of the dorsal hippocampus [11 mm; coordinates: AP, −3.4 mm; ML, ±1.8 mm; DV, −2.7 mm] or the prelimbic region of the mPFC [11 mm; coordinates: AP, +3.7 mm; ML, ±0.7 mm; DV, −2.4 mm] (19, 52, 53). Stylets (15- or 11-mm-long 00 insect dissection pins) were inserted into each cannula to maintain patency.

The NMDA solution (Sigma-Aldrich; 2.5 μg in 0.2 μL of phosphate buffer) was backfilled into a 10-μL Hamilton microsyringe (30-gauge) driven by a stereotaxic minipump (Stoelting) and infused into the BLA over a 30-s period (54). The needle was retained in place for an additional 3 min to optimize diffusion.

Rats were allowed to recover from surgery for 10 d before training and handled three times for 1 min before training.

Inhibitory Avoidance Apparatus and Experimental Procedures. Rats were trained in an inhibitory avoidance apparatus (for details, see *SI Materials and Methods* and ref. 8). Nonoperated rats were placed in the starting compartment and allowed to freely explore the apparatus. After the rat stepped into the dark (shock) compartment, the sliding door was closed and a single inescapable FS with an intensity of either 0.35 mA (lower FS) or 0.45 mA (higher FS) was delivered for 1 s. Other rats were unshocked (no FS) upon entering the dark compartment. Cannulated rats received higher FS intensities (0.45–0.85 mA) to ensure memory in all experimental groups. Rats were removed from the shock compartment 15 s later and returned to their home cages. Some groups of rats were killed at 10, 30, or 60 min after the training trial for endocannabinoid detection. Others were left undisturbed and tested for retention 48 h later. For retention testing, the rat was placed into the starting compartment and the latency to reenter the shock compartment with all four paws was measured (maximum latency of 600 s). Longer latencies indicate better retention.

Drug Treatment. URB597 (Tocris; 3, 10, or 30 ng) was infused posttraining into the BLA, hippocampus, or mPFC. To examine whether the URB597 effect was mediated by activation of CB1 receptors, other groups of rats received the effective dose of URB597 (10 ng BLA and hippocampus, 30 ng mPFC) concurrently with a nonimpairing dose of the CB1 receptor antagonist AM251 [Tocris; 0.14 ng for BLA (8), 0.28 ng for hippocampus and mPFC]. Doses were based on pilot experiments performed in our laboratory. Bilateral sham- or BLA-lesioned rats were administered the effective dose of URB597 into either the hippocampus or mPFC. Drugs were dissolved in 5% (vol/vol) PEG, 5% (vol/vol) Tween 80, and 90% (vol/vol) saline. The infusion procedure is detailed in *SI Materials and Methods*.

Histology. Rats were anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with 0.9% saline.

The brains were removed to verify cannula placement and the size and location of lesions (for details, see *SI Materials and Methods*).

Endocannabinoid Measurement. At 10, 30, or 60 min after inhibitory avoidance training (no FS, lower FS, or higher FS), rats were decapitated and the amygdala, hippocampus, and mPFC were rapidly dissected for AEA and 2-AG measurement (55).

Statistics. Data are expressed as mean ± SEM. Inhibitory avoidance latencies as well as endocannabinoid levels were analyzed with one- or two-way ANOVAs. The source of the detected significances was determined by Tukey–Kramer post hoc tests. *P* values of less than 0.05 were considered statistically significant. The number of rats per group is indicated in the figure legends.

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