Peripheral and central CB1 cannabinoid receptors control stress-induced impairment of memory consolidation

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Edited by Jean-Pierre Changeux, CNRS, Institut Pasteur, Paris, France, and approved July 6, 2016 (received for review January 5, 2016)

Stressful events can generate emotional memories linked to the traumatic incident, but they also can impair the formation of nonemotional memories. Although the impact of stress on emotional memories is well studied, much less is known about the influence of the emotional state on the formation of nonemotional memories. We used the novel object-recognition task as a model of nonemotional memory in mice to investigate the underlying mechanism of the deleterious effect of stress on memory consolidation. Systemic, hippocampal, and peripheral blockade of cannabinoid type-1 (CB1) receptors abolished the stress-induced memory impairment. Genetic deletion and rescue of CB1 receptors in specific cell types revealed that the CB1 receptor population specifically in dopamine β-hydroxylase (DBH)-expressing cells is both necessary and sufficient for stress-induced impairment of memory consolidation, but CB1 receptors present in other neuronal populations are not involved. Strikingly, pharmacological manipulations in mice expressing CB1 receptors exclusively in DBH$^+$ cells revealed that both hippocampal and peripheral receptors mediate the impact of stress on memory consolidation. Thus, CB1 receptors on adrenergic and noradrenergic cells provide previously unrecognized cross-talk between central and peripheral mechanisms in the stress-dependent regulation of nonemotional memory consolidation, suggesting new potential avenues for the treatment of cognitive aspects on stress-related disorders.

memory consolidation | stress response | cannabinoid receptor | endocannabinoid system | noradrenergic signaling

Memory consolidation is sensitive to emotion-related manipulations after acquisition (1). However, the underlying neurobiological mechanisms are only partly understood. Emotions can contribute to memorizing important life events (1, 2); they can also impair memory consolidation (2). Specifically, emotional arousal caused by stress has been studied extensively in animal models and in humans, and it has been reported to produce both facilitation and impairment of memory (3–5). Most studies that investigated the neural mechanisms mediating the effects of stress have focused on emotional memories; the mechanisms underlying the effects of acute stress on nonemotional memories are less understood.

Acute stressful stimuli activate the sympathetic–adrenal system and the hypothalamic–pituitary–adrenal (HPA) axis (6, 7). Increased activity in the sympathetic–adrenal system involves a rapid release of adrenaline and noradrenaline from adrenal chromaffin cells and sympathetic nerve terminals, respectively (7). Moreover, stress-induced activation of the HPA axis involves the synthesis and secretion of glucocorticoids (cortisol in humans and corticosterone in most rodents) from the adrenal cortex (8). Both animal and human studies have shown that these stress hormones have profound effects on cognition by acting on specific brain regions involved in the processing of emotional stimuli (1, 9–12).

The endocannabinoid system is an endogenous neuromodulatory system playing a relevant role in the regulation of the stress response (13–18). Endocannabinoids, such as 2-arachidonoylglycerol (2-AG) and *N*-arachidonoyl ethanolamine (anandamide; AEA), act mainly at two types of cannabinoid receptors, cannabinoid type-1 (CB1) and type-2 (CB2) receptors. The predominant localization of the CB1 receptor at presynaptic sites has been associated with its role in suppressing neurotransmitter release upon synaptic activity (19). Accordingly, activation of the CB1 receptor in adrenergic and noradrenergic cells is expected to decrease the release of adrenaline and noradrenaline (20–22). Moreover, the endocannabinoid system also participates in the negative feedback regulation of the HPA axis after stress (13, 23). Thus, glucocorticoids enhance the production of endocannabinoids to counteract the activity of the HPA axis in many brain regions, including the hippocampus, the prefrontal cortex, and the hypothalamus (16, 24). Overall, endocannabinoid production is induced by acute stress and acts by buffering stress-induced behavioral and endocrine stress effects (16, 17, 23).

In this study we reveal a mechanism mediating stress-induced impairment of object-recognition memory consolidation. Using a combination of acute systemic and local pharmacological approaches and newly generated mouse lines, we found that peripheral and hippocampal CB1 receptors in dopamine β-hydroxylase (DBH)-expressing cells (i.e., adrenergic/noradrenergic cells) are both necessary and sufficient to impair object-recognition memory consolidation produced by acute stress.

Significance

This study discloses specific central and peripheral mechanisms involving cannabinoid type-1 (CB1) receptors in impairing the effect of acute stress on the consolidation of nonemotional memory in rodents. Both hippocampal and peripheral CB1 receptors in dopamine β-hydroxylase–expressing cells (i.e., in adrenergic/noradrenergic cells) are identified as necessary and sufficient for stress-induced memory impairment. Our study provides the foundation for the development of novel potential approaches to tackle cognitive alterations in stress-related disorders.

Author contributions: A.B.-G., M.G.-G., R.K.S., L.C., F.R., L. Bellocchio, G.M., B.L., R.M., and A.O. designed research; A.B.-G., M.G.-G., R.K.S., L.C., A.O.-A., F.R., L. Bindila, L. Bellocchio, and A.O. performed research; R.K.S., S.R., F.R., G.M., B.L., and R.M. contributed new reagents/analytic tools; A.B.-G., M.G.-G., R.K.S., L.C., F.R., L. Bindila, and A.O. analyzed data; and A.B.-G., M.G.-G., R.K.S., L.C., F.R., L. Bindila, G.M., B.L., R.M., and A.O. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1525066113/-/DCSupplemental.

Results

CB1 Receptors Control Stress-Induced Impairment of Memory Consolidation. We evaluated the effects of acute stress on the consolidation of long-term (24 h) nonemotional memory by using the novel object-recognition memory paradigm (Fig. S1*A*). This memory was impaired when mice were exposed to different acutely stressful events [footshock (Fig. 1*A*) and tail suspension (Fig. S1*B*)] 20 min after the training session without reducing the overall exploratory behavior during the memory test (Fig. S1 *C* and *D*). Under these conditions, c-Fos expression was enhanced in the CA1 region of the hippocampus after mice were exposed to the footshock (Fig. S1*E*). When the footshock was administered at different time points after memory training, we found that long-term object-recognition memory was progressively less sensitive to footshock exposure (Fig. 1*B*), indicating that this kind of stress affected the consolidation of object-recognition memory in a specific critical time window after acquisition. Notably, the effects of footshock on object-recognition memory were not observed when short-term memory was tested (60 min after stress exposure) (Fig. 1*C*), indicating a specific disrupting effect of acute stress on memory consolidation. Consistently, the protein synthesis inhibitor anisomycin (15 mg/kg, i.p.) blocked the long-term memory impairment induced by the footshock (Fig. S2*A*).

Next, we evaluated whether the endocannabinoid system was involved in the selective modulation of memory consolidation by acute stress in our experimental conditions. The CB1 receptor antagonist rimonabant (1 mg/kg) administered immediately after training prevented the memory impairment produced by both stressors, footshock (Fig. 1*D*) and tail suspension (Fig. S3), but the CB2 receptor antagonist AM630 (1 mg/kg) had no effect (Fig. S4). Consistently, stress-induced memory impairment was not observed in mutant animals lacking the CB1 receptor (CB1-KO mice) (Fig. 1*E*).

Hippocampal CB1 Receptors in Stress-Induced Impairment of Memory Consolidation. Under our experimental conditions, footshock stress transiently enhanced endocannabinoid levels in the hippocampus (Fig. 2*A*), in agreement with previous reports (23). For this reason we then focused on the hippocampus to ask whether this brain area is involved in the effects of acute stress on the consolidation of object-recognition memory. Notably, intrahippocampal injections of rimonabant after the acquisition of object-recognition memory prevented the stress-induced memory deficit in WT mice without affecting memory performance in nonstressed mice (Fig. 2*B*). These

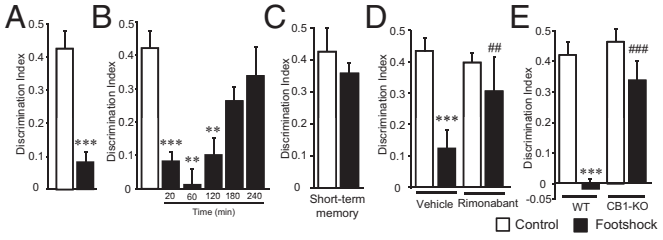

Fig. 1. Stress-induced memory impairment is mediated by CB1 receptors. (*A*) Effect of stress in object-recognition memory consolidation [n = 9 or 10; t (17) = 5.492]. (*B*) Object-recognition memory impairment was reduced when footshock was applied at longer times after training [n = 4–10; F(5, 32) = 15.836]. (*C*) Object-recognition short-term memory was not affected by the footshock [n = 6; t (10) = 0.86]. (*D*) DI values obtained on mice treated with the CB1 receptor antagonist rimonabant immediately after the training session (i.e., 20 min before the footshock) [n = 9–15; treatment: F(1, 42) = 3.90, P = 0.054; stress condition: F(1, 42) = 20.61, P = 0.00005; interaction: F(1, 42) = 6.85, P = 0.012]. (*E*) DI values were obtained from mice lacking the CB1 receptor (CB1-KO) and littermate control (WT) mice. Loss of CB1 receptor obliterated stress-induced memory impairment [n = 6–12; genotype: F(1, 30) = 11.65, P = 0.002; stress condition: F(1, 30) = 24.23, P = 0.00003; interaction: F(1, 30) = 7.94, P = 0.008]. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with nonstress condition; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with vehicle or WT.

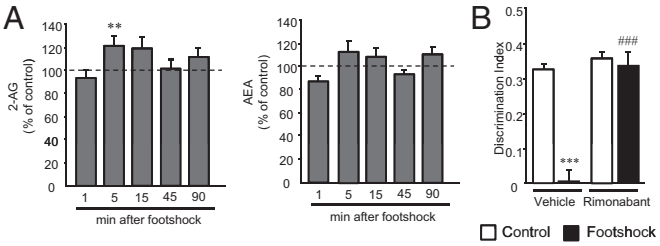

Fig. 2. Hippocampal CB1 receptors mediate stress-induced memory impairment. (*A*) Measurements of endocannabinoid levels in the hippocampus at different time points showed a stress-induced rise in 2-AG levels [n = 7–8; 5 min: t (12) = –1.77]. At other time points, footshocked animals did not show significant alterations with respect to controls. (*B*) Intrahippocampal injection of rimonabant immediately after the training session prevented stress-induced memory impairment in WT mice [n = 7–10; treatment: F(1, 28) = 37.15, P = 0.00001; stress condition: F(1, 28) = 34.22, P = 0.000003; interaction: F(1, 28) = 25.26, P = 0.00003]. Injection sites were confirmed by postmortem histological analysis. $^{**}P < 0.01$ compared with control; $^{***}P < 0.001$ compared with nonstressful condition; $^{\#\#\#}P < 0.001$ compared with vehicle.

data indicated the functional relevance of hippocampal CB1 receptor activation by the elevation of endocannabinoids, specifically 2-AG, in the memory impairment produced by acute stress.

Peripheral CB1 Receptors also Contribute to Stress-Induced Memory Impairment. We next investigated the relevance of the peripheral stress response in stress-induced memory impairment using adrenalectomized mice. These animals did not show the memory deficit induced by the footshock compared with sham-operated control mice (Fig. 3*A*), suggesting that adrenal gland hormones have a crucial role in stress-induced memory impairment. Indeed, under our stress conditions, circulating stress hormones increased transiently above naive-handling conditions (Fig. S5 *A–C*). Also, in accordance with previous findings (25), the dose of rimonabant that blocked acute stress-induced memory impairment enhanced corticosterone levels in nonstressed mice 90 min after the treatment without affecting adrenaline and noradrenaline levels (Fig. S5 *D–F*). However, rimonabant administration did not alter memory performance in nonstressed mice (Fig. 2*B*), suggesting that corticosterone enhancement does not play a major role in the inhibition of stress-induced memory consolidation by CB1 receptor blockade.

Based on these observations, we consequently focused on the involvement of peripheral adrenergic and noradrenergic transmission in our behavioral paradigm. Mice were trained in the object-recognition test and received the peripherally restricted β-adrenergic receptor antagonist sotalol (10 mg/kg) (26) before rimonabant administration and footshock stress. Under these conditions, sotalol did not affect memory consolidation per se but did prevent the blockade of stress-induced memory impairment by rimonabant (Fig. 3*B*), suggesting that peripheral β-adrenergic receptor signaling has a role downstream of the systemic blockade of CB1 receptor function.

To assess the involvement of peripheral CB1 receptors in the stress-induced memory impairment, we used AM6545, a CB1 receptor antagonist with limited brain penetrance (27). AM6545 (1 mg/kg) administration before footshock (Fig. 3*C*) completely prevented the stress-induced memory deficit. AM6545 pretreatment under stress conditions (Fig. S5 *G–L*) maintained an enhancement of circulating adrenaline and noradrenaline as detected 90 min after footshock (Fig. S5 *J–L*). These data revealed a crucial role of peripheral CB1 receptors in the stress-induced memory impairment by controlling the adrenergic tone after acute stress.

CB1 Receptors in DBH$^+$ Cells Are Key in the Stress-Induced Memory Deficits. Different CB1 receptor conditional-KO mouse lines were assessed to investigate which specific CB1 receptor populations modulate these processes (Fig. 4). Stress-induced

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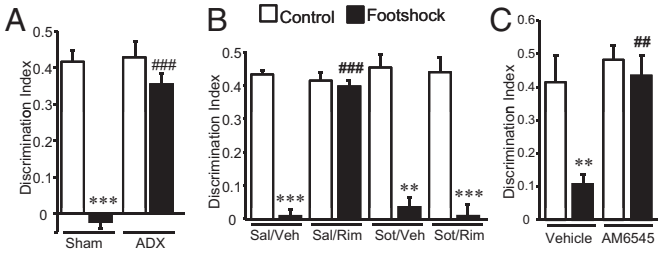

Fig. 3. Peripheral CB1 receptors also participate in the stress-induced memory impairment. (*A*) Stress-induced memory impairment was not observed in adrenalectomized mice [n = 5–10; surgery: $F(1, 26) = 23.56$, $P = 0.00005$; stress condition: $F(1, 26) = 41.01$, $P = 0.000001$; interaction: $F(1, 26) = 20.79$, $P = 0.0001$]. (*B*) The β-adrenergic receptor antagonist sotalol (sot) alleviated the beneficial effect of rimonabant (Rim) in stress-induced memory impairment [n = 4–6; treatment 1 (sotalol): $F(1, 30) = 16.94$, $P = 0.0003$; treatment 2 (rimonabant): $F(1, 30) = 19.61$, $P = 0.0001$; stress condition: $F(1, 30) = 273.23$, $P = 0.00001$; interaction treatments: $F(1, 30) = 27.50$, $P = 0.00001$; interaction treatment 1 × stress condition: $F(1, 30) = 25.64$, $P = 0.000019$; interaction treatment 2 × stress condition: $F(1, 30) = 25.40$, $P = 0.00002$; three-way interaction: $F(1, 30) = 30.97$, $P = 0.000005$]. (*C*) The peripherally restricted CB1 receptor antagonist AM6545 prevented the stress-induced memory deficit in the object-recognition memory test [n = 4–6; treatment: $F(1, 16) = 11.64$, $P = 0.0035$; stress condition: $F(1, 16) = 9.47$, $P = 0.007$; interaction: $F(1, 16) = 5.05$, $P = 0.039$]. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with nonstress condition; $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.01$ compared with vehicle.

memory impairment was present in mice lacking the CB1 receptor in forebrain GABAergic neurons (GABA-CB1–KO mice) (Fig. 4*A*) (28), in dorsal telencephalic glutamatergic neurons (Glu-CB1–KO mice) (Fig. 4*A*) (28), in both GABAergic and glutamatergic neurons (GABA/Glu-CB1–KO mice) (Fig. 4*A*) (29), and in central serotonergic neurons, i.e., in tryptophan hydroxylase 2^+ cells (TPH2-CB1–KO mice) (Fig. 4*B*) (30, 31). Strikingly, mice lacking the CB1 receptor in DBH cells (DBH-CB1–KO mice) (Fig. S6*A*) were insensitive to stress-induced nonemotional memory impairment (Fig. 4*C*), although no genotype differences were observed in contextual and cued fear-conditioning tasks (Fig. S7).

To evaluate whether the expression of CB1 receptor in DBH^+ cells is not only necessary but also sufficient (32) to produce stress-induced memory deficits, specific mouse lines were developed to rescue CB1 receptor expression globally (CB1-RS mice) (32) or specifically in DBH^+ cells (DBH-CB1–RS mice) (Fig. S6 *B* and *C*). Analysis of memory performance after footshock showed that both CB1-RS and DBH-CB1–RS mice exhibited stress-induced memory impairment (Fig. 4*D*). In contrast, Stop-CB1 mice [mice without CB1 receptor expression, similar to constitutive CB1-KO mice (28)] were not sensitive to the cognitive deficit induced by footshock. Importantly, there were no differences between mouse lines in general activity as measured in the modified Irwin test, in nociceptive sensitivity, or in context conditioning memory (Fig. S8); these findings remove the possibility that confounding factors were induced by developmental alterations resulting from genetic manipulations. Moreover, these data highlight the labile characteristics of a nonemotional memory in comparison with an emotional one, confirm the pivotal and diverse roles of CB1 receptors in memory processing, and demonstrate the crucial role of the CB1 receptors specifically expressed in adrenergic/noradrenergic cells in stress-induced memory impairment.

Peripheral and Hippocampal CB1 Receptors in DBH^+ Cells Control Stress-Induced Memory Impairment. DBH^+ cells are localized in the central nervous system (33) and also at peripheral locations, the adrenal gland in particular (33). CB1 receptor mRNA and protein were specifically detected in adrenal medulla (Fig. 5*A* and Fig. S9*B*). As expected, CB1 receptor mRNA was reduced in the DBH-CB1–KO mice compared with WT control animals (Fig. S9 *A* and *B*). In the hippocampus, CB1 receptor protein was present to different degrees in the CA1 region (Fig. S9*C*). Notably, the expression of CB1 receptor protein was detected at low levels in the hippocampus of DBH-CB1–RS mice colocalizing to DBH^+ fibers (Fig. 5*B*). This low level of expression contrasted with the strong CB1 receptor expression detected in WT, CB1-RS, and DBH-CB1–KO mice (Fig. S9*C*). Taken together, these data indicate that DBH^+ fibers contain low but functionally important levels of CB1 receptor protein.

Remarkably, the peripheral CB1 receptor antagonist AM6545 prevented the stress-induced memory impairment in DBH-CB1–RS and control CB1-RS mice, strongly suggesting that peripheral CB1 receptors have a crucial role in the effect of stress (Fig. 5*C*). Similarly, intrahippocampal injections of rimonabant in DBH-CB1–RS mice and in control Stop-CB1 mice fully prevented stress-induced memory impairment (Fig. 5*D*). Together these data reveal that both peripheral and hippocampal CB1 receptors present in DBH^+ cells have a crucial role in stress-induced memory impairment.

Discussion

Our study demonstrates the crucial role of CB1 receptors in the impairment of nonemotional memory consolidation induced by acute stress. We identified the hippocampal and peripheral CB1 receptors present in DBH^+ cells as necessary and sufficient determinants for the deficit observed in the object-recognition memory task triggered by stress.

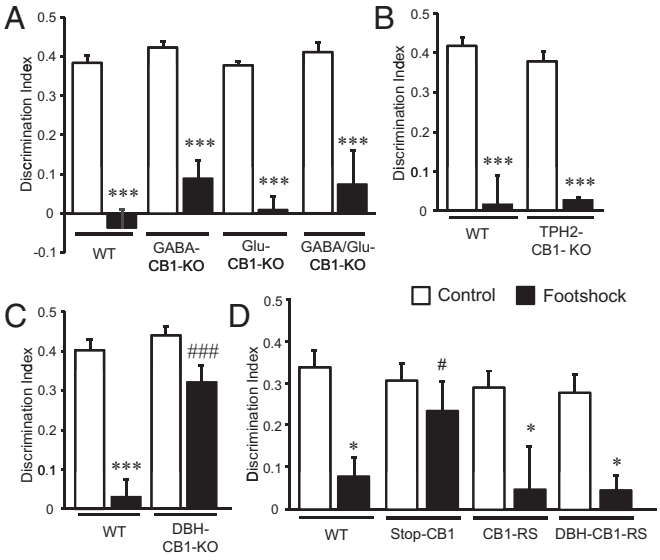

Fig. 4. CB1 receptors in adrenergic/noradrenergic cells are necessary and sufficient to mediate stress-induced memory impairment. (*A*) Mice lacking CB1 receptors in forebrain GABAergic neurons (GABA-CB1–KO mice), in dorsal telencephalic/cortical glutamatergic neurons (Glu-CB1–KO mice), or in both GABAergic and glutamatergic neurons (GABA/Glu-CB1–KO mice) [n = 5; genotype: $F(3, 32) = 1.84$, $P = 0.16$; stress condition: $F(1, 32) = 161.68$, $P = 0.0000$; interaction: $F(3, 32) = 0.49$, $P = 0.69$] and (*B*) in central serotonergic neurons (TPH2-CB1–KO) show the memory impairment caused by stress [n = 5; genotype: $F(1, 16) = 0.14$, $P = 0.712$; stress condition: $F(1, 16) = 97.12$, $P = 0.0000$; interaction: $F(1, 16) = 0.44$, $P = 0.514$]. (*C*) Object-recognition memory is not affect by stress in mice lacking CB1 receptors in DBH cells (DBH-CB1–KO mice) [n = 9–11; genotype: $F(1, 36) = 9.23$, $P = 0.00009$; stress condition: $F(1, 36) = 43.23$, $P = 0.0000$; interaction: $F(1, 36) = 11.48$, $P = 0.002$]. (*D*) Effect of footshock stress on novel object-recognition memory in WT CB1 receptor-deficient mice (Stop-CB1 mice), control mice reexpressing CB1 receptors in all cells (CB1-RS mice), and mice reexpressing CB1 receptors exclusively in DBH cells (DBH-CB1–RS mice) [n = 4–6; genotype: $F(3, 34) = 2.58$, $P = 0.26$; stress condition: $F(1, 34) = 23.25$, $P = 0.00003$; interaction: $F(3, 34) = 2.12$, $P = 0.38$]. $^{*}P < 0.05$, $^{***}P < 0.001$ compared with nonstress conditions; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with WT group.

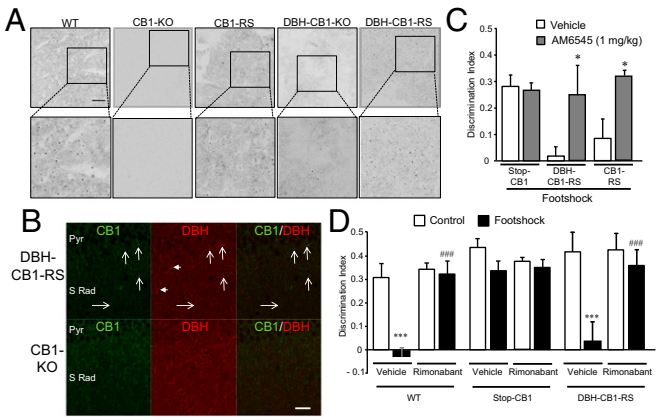

Fig. 5. Peripheral and central CB1 receptors in DBH$^+$ cells are crucial to mediate stress-induced memory impairment. (*A*) Detection of CB1 receptor protein in the adrenal medulla in the different mouse lines studied. CB1 receptor protein was detected in DBH-CB1–RS mice. (Scale bar, 100 μm.) (*B*) Detection of CB1 receptors (arrows) in DBH$^+$ fibers in the stratum radiatum (S Rad) and pyramidal layer (Pyr) of the hippocampus in DBH-CB1–RS mice. Arrowheads indicate DBH fibers where CB1 receptors were not detected. CB1-KO mice were used as negative controls. (Scale bar, 25 μm.) (*C*) The peripherally restricted CB1 receptor antagonist AM6545 prevented the stress-induced memory deficit in DBH-CB1–RS mice [n = 5–8; genotype: $F(1, 33) = 2.57$, $P = 0.09$; treatment: $F(1, 33) = 11.30$, $P = 0.002$; interaction: $F(1, 33) = 3.609$, $P = 0.038$]. (*D*) Intrahippocampal injection of rimonabant prevented stress-induced memory impairment in DBH-CB1–RS mice, substantiating the specific role of this CB1 receptor population in mediating stress-induced memory impairment [n = 3–6; genotype: $F(2, 44) = 9.16$, $P = 0.0005$; treatment: $F(1, 44) = 16.08$, $P = 0.0002$; stress condition: $F(1, 44) = 30.70$, $P = 0.000002$; interaction genotype × treatment: $F(2, 44) = 6.61$, $P = 0.003$; interaction genotype × stress condition: $F(2, 44) = 3.09$, $P = 0.05$; interaction treatment × stress condition: $F(1, 44) = 17.39$, $P = 0.0001$; three-way interaction: $F(2, 44) = 2.29$, $P = 0.1$]. $^*P < 0.05$ compared with vehicle; $^{***}P < 0.001$ compared with nonstress condition; $^{\#\#\#}P < 0.001$ compared with vehicle.

Stress can modulate cognitive performance in opposite ways, depending on its intensity and the type of memory evaluated (1, 34). To understand better the effects of acute stress on a nonemotional memory, we used an object-recognition paradigm, a memory task that allows accurate definition of the memory consolidation period. Interestingly, although different types of acute stress produced a clear long-term memory deficit, short-term object-recognition memory was not affected by our stress paradigm, in agreement with observations in human declarative short-term memory, which is not influenced by emotional arousal (35).

The endocannabinoid system plays a pivotal role both in the modulation of the stress response (13, 36) and in the control of cognitive functions (14, 18). CB1 receptors modulate HPA feedback inhibition secondary to glucocorticoid receptor activation (15, 37), and endocannabinoids can alter both emotional (38) and nonemotional (39) memories. Our data demonstrate that systemic and local pharmacological blockade or complete genetic inactivation of the CB1 receptor prevents the memory deficit triggered by different stressors. According to previous findings (25), pharmacological blockade of CB1 receptors by rimonabant is able to enhance circulating corticosterone levels, although it does not modify circulating amounts of adrenaline or noradrenaline. However, that rimonabant treatment alone did not affect the object-recognition memory and completely blocked the memory-impairing effects of the acute stress suggesting that this corticosterone enhancement does not play a prominent role in the effects of CB1 receptor blockade. Notably, we identified a specific population of CB1 receptors expressed in DBH$^+$ cells as being both necessary and sufficient for stress-induced impairment of object-recognition memory, but other, much more abundant, CB1 receptor populations were not involved in this process. Although developmental changes produced by noninducible genetic manipulations cannot be fully discarded, the observation that mutant mice behaved similarly to controls in other tests, together with our genetic and pharmacological data, indicates that acute activation of CB1 receptors in DBH$^+$ cells is responsible for stress-induced memory impairment.

Importantly, adrenalectomy also blocked stress-induced memory impairment, indicating that the adrenal gland is a key peripheral tissue controlling the memory impairment induced by acute stress. Interestingly, the involvement of the HPA axis in cognitive performance has been described (40), but the mechanisms involving the sympathetic system, in which adrenal glands are also involved, are less well characterized. In this study we propose that CB1 receptors expressed in DBH$^+$ cells of the adrenal medulla, which release adrenaline and noradrenaline upon sympathetic activation (41), are relevant for the object-recognition memory deficit produced by acute footshock. Our proposal is based on the following observations: (*i*) circulating adrenaline and noradrenaline levels after stress are sustained by AM6545 pretreatment; (*ii*) stress-induced memory impairment is blocked by AM6545 in the DBH-CB1–RS mice; (*iii*) CB1 receptor expression in the adrenal medulla was reduced in DBH-CB1–KO animals and was re-expressed in the DBH-CB1–RS mice; (*iv*) the peripherally restricted β-adrenergic receptor antagonist sotalol prevented the effect of rimonabant in rescuing the consequences of stress on object-recognition memory consolidation, pointing to key peripheral β-adrenergic receptor signaling downstream of the CB1 receptor blockade.

Interestingly, recent data revealed that the anorectic and anxiogenic effects of rimonabant require peripheral activation of sympathetic activity (26). Consistently, the present data indicate that control of adrenergic/noradrenergic transmission by CB1 receptors is involved in the central–peripheral control of behavior. Thus, in agreement with previous findings (23, 36, 42), acute stress enhanced endocannabinoid levels in the hippocampus during the consolidation window of the object-recognition task. Notably, the intrahippocampal administration of rimonabant completely blocked the stress-induced memory impairment in WT mice and, importantly, in DBH-CB1–RS mice also. Although the role of other brain circuits such as the prefrontal cortex (43) or the perirhinal cortex (44) in the object recognition task cannot be discarded, our data strongly suggest that the hippocampus plays a prominent role in mediating the complex impact of endocannabinoid signaling on stress-induced memory impairment.

Our data demonstrate a transient increase in endocannabinoid levels in the hippocampus after acute stress. We hypothesize that endocannabinoids are enhanced at specific synapses, because the 2-AG–synthesizing enzyme diacylglycerol lipase α (DGLα) is heavily expressed in the hippocampus (45). The synthesis of endocannabinoids triggered by stress could be mediated through glucocorticoid receptors in the hippocampus (36) or by the engagement of hippocampal α1-adrenergic receptors and $G_{q/11}$-mediated signaling activated by local noradrenaline release (46). Mobilized endocannabinoids, in turn, may act on CB1 receptors expressed at low levels in noradrenergic fibers, as shown by immunohistochemical experiments. At these fibers, which project from the locus coeruleus or the nucleus tractus solitarius (47), CB1 receptors would control noradrenaline transmission, as has been proposed in other brain regions (22, 48). These findings lead us to propose that the endocannabinoid-mediated decrease in noradrenaline release at hippocampal noradrenergic terminals is a key step in modulating nonemotional memory consolidation studied in the object-recognition task. In this regard, it has been shown that noradrenaline is able to modulate synchronized hippocampal activity that can interfere with nonemotional memory consolidation (49, 50).

In summary, our multidisciplinary study revealed the involvement of central and peripheral mechanisms in stress-induced object-recognition memory impairment in which CB1 receptors in adrenergic and noradrenergic cells are key players. The discovery of this mechanism warrants the study of new approaches in the treatment of those cognitive aspects associated to stress-related conditions.

Materials and Methods

Animals. Male Swiss albino (CD-1) mice (Charles River) and CB1 receptor constitutive-KO mice (8–10 wk of age) and their WT controls in the CD-1 background (51) weighing 29–33 g were used. Conditional-KO mice lacking CB1 receptors in DBH-expressing cells were generated as detailed in *SI Materials and Methods*. Rescued mice expressing CB1 receptor exclusively in DBH-expressing cells were generated as detailed in *SI Materials and Methods*. Conditional-KO mice lacking CB1 receptors in forebrain GABAergic neurons (GABA-CB1–KO mice), in dorsal telencephalic glutamatergic neurons (Glu-CB1–KO mice), in both GABAergic and glutamatergic neurons (GABA/Glu-CB1–KO mice), in central serotonergic (tryptophan hydroxylase 2-positive) neurons (TPH2-CB1–KO mice), and their WT (floxed/floxed) littermates (25–30 g) were in a mixed genetic background, with a predominant C57BL/6N contribution (at least seven generations of backcrossing) (28, 29, 31).

Mice were housed in cages holding a maximum of four mice per cage and were maintained at a controlled temperature (21 ± 1 °C) and humidity (55 ± 10%). Food and water were available ad libitum. Lighting was maintained in 12-h light/dark cycles (light on at 8:00 AM and off at 8:00 PM). All experiments were performed during the light phase of the dark/light cycle. Animals were habituated to the experimental room and were handled daily for 1 wk before the experiments began. All animal procedures were conducted in accordance with the standard ethical guidelines (European Communities Directive 2010/63/UE) and were approved by the local ethical committees [Comitè Ètic d'Experimentació Animal, Parc de Recerca Biomedica de Barcelona (PRBB), Spain; Ethical Committee on Animal Care and Use of Rhineland-Palatinate, Germany; and the Committee on Animal Health and Care of INSERM and Ministry of Agriculture and Forestry, France]. The PRBB has an authenticated Public Health Service approved Animal Welfare Assurance (no. A5388-01) granted by the Office of Laboratory Animal Welfare of the National Institutes of Health. All behavioral data were obtained by experimental observers blinded to the experimental conditions.

Drugs and Treatments. Cremophor-EL, AM6545, and anisomycin were purchased from Sigma. Rimonabant was kindly provided by Sanofi-Aventis Recherche. AM630 was purchased from Tocris Bioscience. Rimonabant and AM630 were dissolved in 5% (vol/vol) ethanol: 5% (vol/vol) cremophor-EL: 90% (vol/vol) saline. Anisomycin was dissolved in saline. AM6545 was dissolved in 0.26% DMSO [4.74% (vol/vol) ethanol: 5% (vol/vol) cremophor-EL: 90% (vol/vol) saline]. All drugs were administered intraperitoneally in a volume of 10 mL/kg. Rimonabant (1 mg/kg), AM6545 (1 mg/kg), and anisomycin (15 mg/kg) were administered 20 min before the exposure to the stressful stimuli. The doses of rimonabant, AM6545, and anisomycin used did not affect nociceptive responses measured in the hot-plate test or anxiety-like behavior measured in the elevated plus-maze test (Fig. S10).

Surgical Procedures. Surgical procedures for local hippocampal administration and bilateral adrenalectomy are detailed in *SI Materials and Methods*.

Object-Recognition Task. Object-recognition memory was assayed in the V-maze (Fig. S1) under dim light conditions as described previously (39). On day 1, mice were habituated to the empty maze for 9 min. On the second day mice were introduced in the maze, in which two identical objects were presented, for 9 min. For the memory test, mice were placed again in the V-maze, in which one of the familiar objects had been replaced by a novel object, at the indicated time points for a period of 9 min, and the total time spent exploring each of the two objects (novel and familiar) was recorded by an experimenter blind to the experimental conditions. Object exploration was defined as orientation of the nose toward the object at a distance of less than 2 cm. A discrimination index (DI) was calculated as the difference between the time spent exploring either the novel (Tn) or familiar (Tf) object divided by the total time spent exploring both objects: (Tn + Tf): DI = (Tn − Tf)/(Tn + Tf). A DI of 0 indicates no preference for either object, and a DI higher than 0.3 was considered to reflect memory retention for the familiar object. Long-term memory was assessed 24 h after the training session or as indicated. Drug administration and stress exposure were always performed after the training session to avoid possible intrinsic effects during the acquisition phase.

Stress Paradigms. Footshock or tail suspension was applied in different groups of mice 20 min after the training session in the object-recognition memory task. For footshock stress, mice were placed for 150 s in a conditioning chamber (Panlab) with a stainless steel grid floor through which a single electric footshock was delivered (0.5 mA; 2 s). Mice were removed from the chamber 150 s after footshock. Control mice were handled exactly as the footshock-stressed mice but did not receive the footshock in the conditioning chamber. For tail-suspension stress, mice were individually suspended by the tail for 5 min at a height of 35 cm above a cushioned surface. The control/unstressed mice for this stress condition remained undisturbed in their home cages.

Behavioral Characterization of Mouse Lines. Fear-conditioning paradigms for emotional memory, nociceptive responses to hot and cold stimuli, and details on the modified Irwin test and anxiety-like behavior are given in *SI Materials and Methods*.

Corticosterone and Adrenaline/Noradrenaline Measurement. Plasma fractions were obtained from trunk blood samples recovered with EDTA (1 mM) and sodium metabisulphite (4 mM). Plasma corticosterone was measured by ELISA following the manufacturer's instructions (IBL International). Plasma adrenaline and noradrenaline were measured by ELISA kits following the manufacturer's instructions (Labor Diagnostika Nord).

Endocannabinoid Extraction and LC/Multiple Reaction Monitoring Quantification. Hippocampal tissues were rapidly isolated at different time points after footshock stress, weighed, and frozen. Endocannabinoid quantification was then performed as detailed in *SI Materials and Methods*.

Real-Time Quantitative PCR. Adrenal glands were removed, cleaned from adhered fat tissues, and rapidly frozen on dry ice for further processing as described in *SI Materials and Methods*.

Tissue Preparation for Immunofluorescence. Mice were deeply anesthetized 90 min after stress exposure by intraperitoneal injection (0.2 mL/10 g of body weight) of a mixture of ketamine (100 mg/kg) and xylazine (20 mg/kg) before intracardiac perfusion with 4% (wt/vol) paraformaldehyde in 0.1 M Na_2HPO_4/NaH_2PO_4 buffer (PB), pH 7.5, delivered with a peristaltic pump at 19 mL/min for 3 min. Subsequently, brains and adrenal glands were extracted and postfixed in the same fixative solution for 4 h and were cryoprotected overnight at 4 °C in 30% (wt/vol) sucrose in PB. Coronal frozen sections (30 μm) of the dorsal hippocampus (coordinates relative to bregma: −1.22 mm to −1.82 mm) were obtained on a freezing microtome and were stored until used. Adrenal glands were processed in a cryostat (Leica) to obtain 7-μm-thick slices mounted on gelatin-coated glass slides.

Immunofluorescence. Free-floating brain slices or glass slide-mounted adrenal gland slices were rinsed in PB. Brain slices were washed three times with PB; then coronal brain sections were incubated with 10 mM citrate buffer, pH 6.0, at 95 °C for 30 min. Afterwards, sections were blocked with 3% (vol/vol) normal goat serum and 0.3% Triton X-100 in PB (NGS-T-PB) at room temperature for 2 h and were incubated overnight in the same solution with the primary antibody to CB1 receptor (1:500 rabbit or 1:1,000 guinea pig; both from Frontier Science), DBH (1:500 rabbit; Merck-Millipore), or c-Fos (1:750 rabbit; Calbiochem) at 4 °C. The next day, after three rinses in PB, sections were incubated at room temperature with the secondary antibody to rabbit conjugated to Cy2 or Cy3 (1:500; Jackson ImmunoResearch) in NGS-T-PB for 2 h. After incubation, brain sections were rinsed and mounted immediately onto glass slides coated with gelatin. Mowiol was used as mounting medium.

Image Analysis. Confocal images were obtained using a Leica TCS SP2 confocal microscope adapted to an inverted Leica DM IRBE microscope. Cy2 and Cy3 were excited with the 488-nm line of an argon laser and the 543-nm line of a green neon laser, respectively. Tissue sections were examined with a 40× or 63× oil-immersion objective. The images (eight-bit color; 1,024 × 1,024 pixels) were analyzed using ImageJ software.

Statistical Analysis. Comparisons between groups were performed by Student's *t* tests only when assessing two-group comparisons and by one-way, two-way, three-way, or repeated-measurement ANOVA, as appropriate, for multiple-group comparisons. Post hoc comparisons were performed by Student–Newman–Keuls test or Bonferroni test only when a significant main effect of one-way ANOVA or a significant interaction between the factors of two- and three-way ANOVA was revealed. All results are expressed as mean ± SEM. Differences were considered significant at $P < 0.05$. The statistical analysis was performed using the Statistical Package for Social Science program SPSS 19.0 (SPSS Inc.).

ACKNOWLEDGMENTS. We thank Gal Raz for critical reading of the manuscript; Cristina Fernández-Avilés and Dulce Real for expert technical assistance; and the Laboratori de Neurofarmacologia-NeuroPhar for helpful

discussions. We acknowledge a predoctoral fellowship from the Spanish Ministry of Education and a postdoctoral fellowship from the Investments for the Future Programme, Initiative of Excellence Bordeaux, ANR-10-IDEX-03-02 (to A.B.-G.); a postdoctoral fellowship from the Marie Curie action Seventh Framework Programme for Research and Technological Development, FP7-PEOPLE-2013 (to A.B.-G.); the partial support by FRAXA Research Foundation (M.G.-G.); a European Molecular Biology Organization postdoctoral fellowship (to L. Bellocchio); grants from Ministerio de Ciencia e Innovación [BFU2012-33500 and BFU2015-68568P (to A.O.), and SAF2014-59648-P (to R.M.) (MINECO/FEDER, UE)]; Instituto de Salud Carlos III Grant RD06/0001/0001 (to R.M.); Generalitat de Catalunya Grant SGR-2014-1547 (to R.M.); ICREA (Institució Catalana de Recerca i Estudis Avançats) Academia (R.M.); the German Research Council DFG Grants FOR926 (Core Unit Endocannabinoid Measurements) (to B.L.), CRC-TRR58 (to B.L.), and CRC1080 (to B.L.); EU-FP7 Grant REPROBESITY, HEALTH-F2-2008-223713 (to B.L. and G.M.); PainCage Grant HEALTH-2013-INNOVATION-1-603191 (to G.M.); INSERM (G.M.); European Research Council Grant ENDOFOOD, ERC–2010–StG–260515 (to G.M.); Fondation pour la Recherche Medicale (G.M.); Region Aquitaine (G.M.); and LABEX BRAIN Grant ANR-10-LABX-43 (to G.M.).

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