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ARTICLE

Peripheral CB1 receptor blockade acts as a memory enhancer through a noradrenergic mechanism

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Peripheral inputs continuously shape brain function and can influence memory acquisition, but the underlying mechanisms have not been fully understood. Cannabinoid type-1 receptor (CB1R) is a well-recognized player in memory performance, and its systemic modulation significantly influences memory function. By assessing low arousal/non-emotional recognition memory in mice, we found a relevant role of peripheral CB1R in memory persistence. Indeed, the peripherally-restricted CB1R specific antagonist AM6545 showed significant mnemonic effects that were occluded in adrenalectomized mice, and after peripheral adrenergic blockade. AM6545 also transiently impaired contextual fear memory extinction. Vagus nerve chemogenetic inhibition reduced AM6545-induced mnemonic effect. Genetic CB1R deletion in dopamine β-hydroxylase-expressing cells enhanced recognition memory persistence. These observations support a role of peripheral CB1R modulating adrenergic tone relevant for cognition. Furthermore, AM6545 acutely improved brain connectivity and enhanced extracellular hippocampal norepinephrine. In agreement, intra-hippocampal β-adrenergic blockade prevented AM6545 mnemonic effects. Altogether, we disclose a novel CB1R-dependent peripheral mechanism with implications relevant for lengthening the duration of non-emotional memory.

INTRODUCTION

Most everyday experiences create low arousal non-emotional memories. These are generally short-lived although they can be, in some instances, consolidated into long-term memories. Long-term memory persistence understood as the time a memory is accessible to retrieval, fluctuates in duration, even if long-lived memories were created from similar sensorial stimuli [1]. The spam memories persist is frequently modulated by those circumstances around the time of encoding or consolidation [2, 3]. Memory persistence is usually longer for those emotionally arousing experiences, where encoding is combined with the natural stress-coping response. This leads to a significant increase in memory persistence for the sensory information recorded at the time of a high arousal experience [4]. In contrast, the mechanisms involved in memory persistence modulation for low arousal experiences have not been well understood, although they are relevant for everyday life events.

The endocannabinoid system (ECS), highly expressed in the central nervous system (CNS) and peripheral tissues [5, 6], plays a

key role in learning and memory [5]. The cannabinoid type-1 receptor (CB1R) is strongly expressed in the brain [7], predominantly localized at presynaptic sites of different neuronal cell types, where it suppresses neurotransmitter release depending on local synaptic activity [8]. In animal models, exogenous compounds with agonist properties for CB1R contribute to memory impairment [9, 10] for low-arousal memory tasks, such as the novel object-recognition test (NORT), while pharmacological or genetic CB1R blockade increases memory persistence in these tests [11, 12]. Although the mechanisms involved are largely unknown, such regulation of memory by CB1R blockade was previously assumed to occur solely through centrally located receptors [13]. Peripheral CB1R activation was found relevant for the memory deficits associated to stress [14], but whether peripheral CB1R inhibition could enhance memory performance has not been previously evaluated.

The aim of this study was to assess the role of peripheral CB1R modulation in low arousal non-emotional memory and to identify the neurobiological mechanisms involved. For this purpose, we

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used a multidisciplinary approach including pharmacological and genetic interventions together with in vivo and ex vivo analyses. We found that peripheral blockade of CB1R, probably on adrenergic cells, enhanced low-arousal non-emotional memory persistence through an adrenergic mechanism that partly involves the vagus nerve. Under these conditions of peripheral CB1R inhibition, brain connectivity was enhanced, and hippocampal norepinephrine release was boosted as a plausible mechanism underlying this nootropic effects of peripheral CB1R inhibition.

MATERIALS AND METHODS
For a complete description of the experimental methods please refer to the Supplementary Information.

Animals
Animal experiments were approved by the local committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB) and conducted following "Animals in Research: Reporting Experiments" (ARRIVE) guidelines and standard ethical guidelines [15] (European Directive 2010/63/EU).

Experimental procedures
Supplementary Information contains all protocols for behavioral experiments, adrenalectomy, intra-hippocampal cannula implantation, vagus nerve surgery, c-Fos immunofluorescence, rsfMRI, chromatographic analysis and in vivo microdialysis and electrophysiological recordings.

Statistical analysis
All data are expressed as mean ± standard error of the mean (s.e.m). Data analyses were performed using GraphPad Prism software (GraphPad Software). Specific details are described in the supplemental methods and figure legends.

RESULTS
CB1R inhibition enhances memory persistence in the novel object-recognition memory test
Novel object-recognition memory is a labile low-arousal non-emotional type of memory usually evaluated 3 h or 24 h after the training session, when mice readily discriminate novel and familiar objects (Fig. 1A). Notably, discrimination index values significantly decrease when, instead, novel object-recognition memory is assessed 48 h after the training phase (one-way ANOVA, interaction: $F_{(2,19)} = 8.55$, $p = 0.002$; post hoc Tukey, 3 h vs 48 h $p = 0.003$; 24 h vs 48 h $p = 0.007$) (Fig. 1A), a sign that the memory trace for familiar objects, or object-recognition memory persistence, diminishes over time. We used this low-arousal memory paradigm assayed 48 h after the training phase to evaluate the role of CB1R inhibition in memory persistence. We found that mice that received an acute post-training treatment with a low dose of the systemic CB1R selective antagonist rimonabant (1 mg/kg, i.p.) showed higher memory persistence than vehicle-treated mice (Student's t-test: $p = 0.02$) (Fig. 1B). In addition, partial genetic inhibition of CB1R, studied in mice heterozygous for the Cnr1 gene (CB1HZ) also showed enhanced recognition memory persistence (Student's t-test: $p = 0.004$) compared to wild-type littermates (Fig. 1C), indicating that such a modulation in memory persistence is CB1R dependent. Post-training administration of the peripherally-restricted CB1R antagonist AM6545 also enhanced recognition memory at 48 h in a dose-related manner with a maximum effect at 1 mg/kg (one-way ANOVA: $F_{(5,50)} = 4.535$, $p = 0.0017$; post hoc Tukey, VEH vs AM1, $p = 0.014$) (Supplementary Fig. 2A, B), observed both in outbreed CD-1 mice (Student's t-test: $p = 0.002$) (Fig. 1D) and inbreed C57BL/6 J mice (Student's t-test: $p = 0.0003$) (Fig. 1E). We also performed the novel object-recognition memory test under challenging conditions by reducing the length of the training phase to 3 min. We then tested

the memory at 24 h and observed that AM6545 enhanced recognition memory (Student's t-test: $p = 0.0003$) (Fig. 1F), indicating not only a better memory consolidation but a facilitation in memory acquisition. Notably, another peripherally-restricted CB1R antagonist such as TM38837 (1 mg/kg, i.p.) also enhanced recognition memory persistence at 48 h (Supplementary Fig. 2C, D) further supporting a relevant peripheral mechanism independent of the specific drug used.

No differences in total exploration time were detected between genotypes or pharmacological treatments in any of the experimental groups above (Supplementary Figs. 1 and 2) discarding a possible bias due to differences between groups in exploratory behavior.

Next, we studied whether peripheral CB1R inhibition would modify the extinction of a fear memory in the context fear conditioning paradigm by administering an acute dose of AM6545 (1 mg/kg, i.p.) after the first extinction trial. We observed that AM6545-treated mice prevented fear extinction as assessed in the second extinction trial, and that fear memory extinction continued for both groups in the following third and fourth extinction trials (two-way repeated measures ANOVA, treatment effect: $F_{(1,44)} = 9.363$, $p = 0.0038$; post hoc Bonferroni, Ext2-VEH vs Ext2-AM6545, $p = 0.04$) (Fig. 1G).

Furthermore, AM6545 administration did not affect locomotor activity analyzed for 120 min post-administration (Supplementary Fig. 3) excluding a major unspecific effect of the treatment on behavioral responses.

Enhanced memory persistence of peripheral CB1R inhibition involves a peripheral adrenergic mechanism
We hypothesized that a peripherally located tissue, such as the adrenal glands which express CB1R [16], could be a relevant player modulating memory consolidation [17]. Therefore, we evaluated the effect of post-training AM6545 administration in bilaterally adrenalectomized mice. Memory persistence enhancement by AM6545 was significantly reduced in mice without adrenal glands (two-way ANOVA, interaction: $F_{(1,22)} = 4.89$, $p = 0.037$; post hoc Tukey, naive-VEH vs naive-AM6545, $p = 0.022$; naive-AM6545 vs ADX-AM6545, $p = 0.026$) (Fig. 2A), supporting the role of CB1R blockade in this particular peripheral tissue. Adrenal glands release glucocorticoids and catecholamines into the blood, both relevant for memory [17]. To figure out which hormones are responsible for the mnemonic effects produced by peripheral CB1R blockade, mice were pre-treated after the training phase with the glucocorticoid receptor antagonist mifepristone (50 mg/kg, i.p.) or the peripherally-restricted β-adrenergic receptor antagonist sotalol (10 mg/kg, i.p.) 20 min before AM6545 injection (Fig. 2B). Mifepristone pre-treatment did not prevent enhancement of memory persistence by AM6545 (two-way ANOVA, interaction: $F_{(1,21)} = 0.038$, $p = 0.845$; mifepristone/vehicle effect: $F_{(1,21)} = 0.707$, $p = 0.409$; AM6545/vehicle effect: $F_{(1,21)} = 25.11$, $p < 0.001$) (Fig. 2C). In contrast, mice pre-treated with sotalol did not show the memory improvement observed in AM6545-treated mice (two-way ANOVA, interaction: $F_{(1,31)} = 7.58$, $p = 0.009$; post hoc Tukey, Saline-VEH vs Saline-AM6545 $p = 0.01$; Saline-AM6545 vs Sotalol-AM6545 $p = 0.001$) (Fig. 2D).

In the light of these data, we assessed whether inhibition of CB1R exclusively in dopamine β-hydroxylase cells (DBH + cells), the main cells responsible for circulating levels of epinephrine/norepinephrine, could mimic the mnemonic effect of systemic and peripheral CB1R antagonists. We used a combination of genetic and pharmacological approaches to first show that conditional knock-out mice lacking the CB1R in DBH + cells (DBH-CB1KO mice) displayed enhanced novel object-recognition memory persistence compared to wild-type controls (Student's t-test: $p = 0.04$) (Fig. 2E). In addition, enhanced memory persistence in DBH-CB1KO mice was abolished by sotalol administration (two-way ANOVA, interaction: $F_{(1,22)} = 10.47$, $p = 0.003$; post hoc
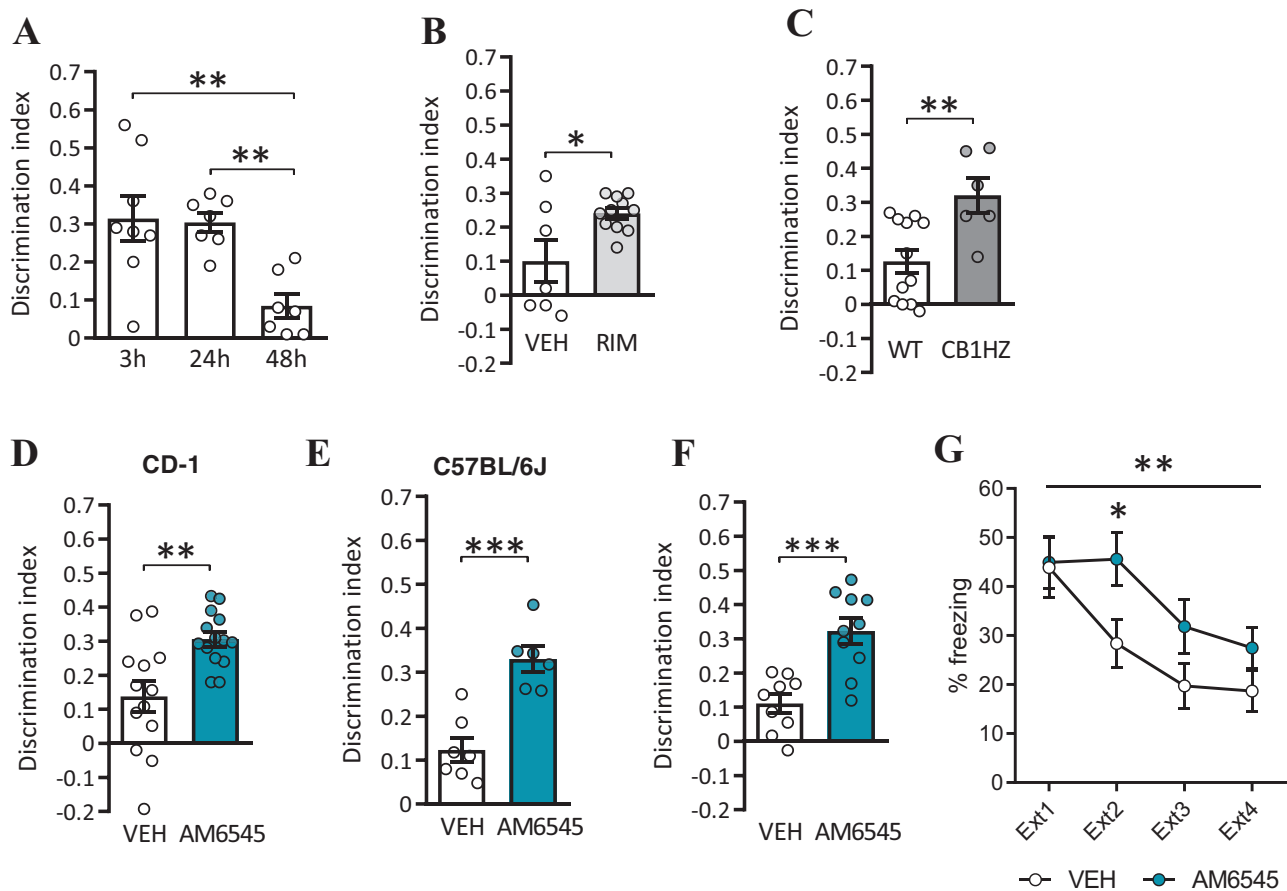
Fig. 1 Pharmacological or genetic inhibition of CB1R improves memory persistence in the novel object-recognition test (NORT).
A Discrimination index values obtained at 3 h, 24 h, and 48 h after the training phase ($n = 5$–8). Discrimination index values in NORT at 48 h (**B**) after acute post-training treatment with vehicle (VEH) or rimonabant (RIM) (1 mg/kg) ($n = 7$–11), **C** in CB1HZ and WT mice ($n = 6$–8), after acute post-training treatment with vehicle (VEH) or AM6545 (1 mg/kg) in **D** CD-1 ($n = 13 - 14$) and **E** C57BL/6 J ($n = 6$, 7). **F** Discrimination index values in NORT at 24 h after acute post-training treatment with vehicle (VEH) or AM6545 (1 mg/kg) with a 3 min training period ($n = 9$–10). **G** Percentage of freezing in the context fear conditioning across extinction sessions (Ext1-Ext4) after acute post-extinction 1 treatment with vehicle (VEH) or AM6545 (1 mg/kg) ($n = 12$). Data are expressed as mean ± s.e.m. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one-way ANOVA or two-way repeated measures ANOVA test followed by Bonferroni post hoc or Student's t-test.

Tukey, Saline-WT vs Saline-DBH-CB1KO $p = 0.01$; Saline-DBH-CB1KO vs Sotalol-DBH-CB1KO $p = 0.04$) (Fig. 2F), pointing to a relevant role of CB1R-modulated peripheral adrenergic/noradrenergic tone in memory persistence. Sotalol pre-treatment totally prevented the cognitive improvement elicited by systemically acting rimonabant supporting the relevance of peripheral CB1R blockade in this cognitive effect of rimonabant (two-way ANOVA, interaction: F (1,37) = 9.408, $p = 0.004$; post hoc Tukey, Saline-VEH vs Saline-rimonabant $p = 0.01$; Saline-rimonabant vs Sotalol-rimonabant $p = 0.001$) (Fig. 2G). No differences in total exploration time were detected in the memory test between genotypes or pharmacological treatments in any of the experimental groups above (Supplementary Fig. 4).

Chemogenetic vagus nerve inhibition reduces memory improvement produced by peripheral CB1R blockade
Circulating epinephrine, whether endogenous or systemically administered, does not cross the blood brain barrier while it enhances hippocampal-dependent memory in rodents by the activation of peripheral β-adrenergic receptors [18]. Such mnemonic effects have been hypothesized to occur either by the activation of β-adrenergic receptors in the liver and the subsequent increase of blood glucose levels, or by the activation of β-adrenergic receptors on the afferent fibers of the vagus nerve [19].

To further elucidate whether any of these two mechanisms could be involved in the mnemonic effects produced by acute AM6545 administration, we first measured blood glucose levels after AM6545 administration in mice. No differences in blood glucose levels were observed in mice treated with AM6545 in comparison to vehicle-treated group (Fig. 3A).

To assess the involvement of vagus nerve fibers as a major link between peripheral AM6545 effects and memory performance, we used a chemogenetic approach to selectively reduce the neural activity of vagus nerve. We first confirmed that surgery and vagus nerve infection with adenoviral vectors were compatible with the assessment of memory persistence. Indeed, AM6545 effect on memory persistence was maintained in control mice receiving bilateral vagus nerve injection of AAV5-mCherry and treated with clozapine N-oxide (CNO, 3 mg/kg, i.p.) (Student's t test: $p = 0.03$) (Fig. 3B). Chemogenetic inhibition with bilateral vagus nerve injection of AAV5-hM4Di showed AM6545 memory enhancement in animals pre-treated with vehicle (two-way ANOVA, AM6545 effect: F (1,35) = 5.25, $p = 0.028$). Instead, animals pre-treated with CNO showed a reduced memory enhancement by AM6545 (Fig. 3C), indicating a possible participation of vagus nerve afferents in the mnemonic effect of AM6545. As expected, no differences were observed neither when memory was evaluated 24 h after
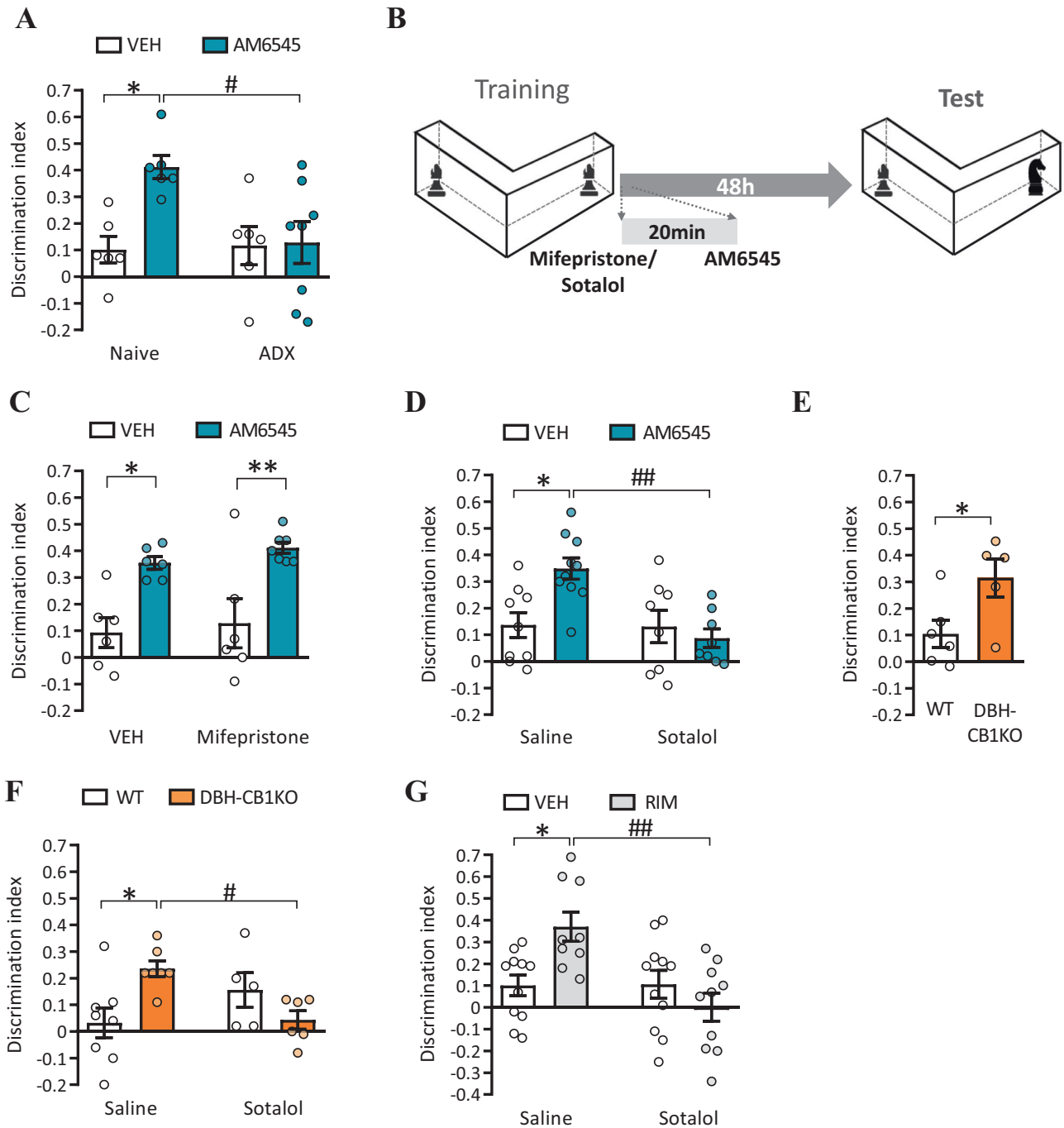
Fig. 2 AM6545 enhances novel object-recognition memory through a peripheral β-adrenergic mechanism. A Discrimination index values obtained in the NORT performed at 48 h of adrenalectomized (ADX) or naive mice treated with vehicle (VEH) or AM6545 (1 mg/kg) ($n = 6$–8). **B** Schematic representation of pre-treatment and treatment after the training phase. Discrimination index values obtained in the NORT performed at 48 h of mice treated with vehicle (VEH) or AM6545 (1 mg/kg) after pre-treatment with **C** vehicle (VEH) or mifepristone (50 mg/kg) ($n = 6$–7) and **D** saline or sotalol (10 mg/kg) ($n = 8$–10). **E** Discrimination index values in WT or DBH-CB1KO mice in NORT at 48 h **F** and after saline or sotalol (10 mg/kg) treatment ($n = 6$–8). **G** Discrimination index values for mice pre-treated with saline or sotalol (10 mg/kg) prior to rimonabant (RIM) (1 mg/kg) or vehicle (VEH) in the NORT at 48 h ($n = 9$–11). Data are expressed as mean ± s.e.m. *$p < 0.05$ (treatment effect) #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$ (pre-treatment effect) by two-way ANOVA test followed by Tukey post hoc or Student's *t*-test.

training in AAV5-hM4Di mice (Supplementary Fig. 5A) nor in exploratory behavior during the memory test (Supplementary Fig. 5B–D). mCherry expression was assessed in nodose ganglion samples of infected mice (Supplementary Fig. 5E) as control of transgene expression. Thus, AM6545 administration improves memory consolidation through an adrenergic mechanism involving the vagus nerve afferents.

Central consequences of AM6545 administration

We first analyzed the pattern of c-Fos expression, as a marker of neuronal activity, focusing on brain regions relevant for object-recognition memory performance. Samples were obtained 90 min after receiving AM6545 or vehicle once mice came out of the training phase in the NORT. Brain areas under study included CA1 and CA3 hippocampal regions, dentate gyrus, prelimbic and
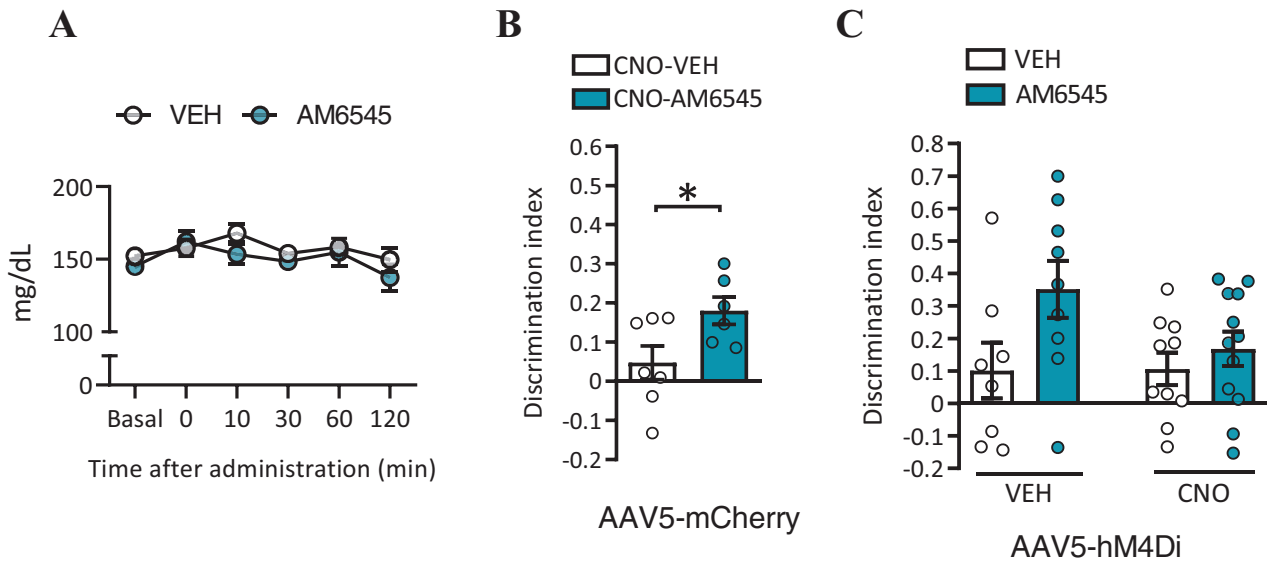
Fig. 3 AM6545 memory improvement depends on vagus nerve activation. A Glucose blood levels for 120 min after acute vehicle (VEH) or AM6545 (1 mg/kg) administration in mice ($n = 6$–7). **B** Discrimination index values in NORT at 48 h after acute vehicle (VEH) or AM6545 (1 mg/ kg) and CNO (3 mg/kg) administration in mice infected with AAV5-mCherry-control in the vagus nerve ($n = 6$–7). **C** Discrimination index values in NORT at 48 h after acute vehicle (VEH) or AM6545 (1 mg/kg) and VEH or CNO (3 mg/kg) administration in mice infected with AAV5-mCherry-hM4Di in the vagus nerve ($n = 8$–12). Data are expressed as mean ± s.e.m. For the blood glucose measures statistical significance was calculated by two-way repeated measures ANOVA test. For NORT *$p < 0.05$ by Student's t-test or two-way ANOVA test.

infralimbic prefrontal cortex, the *locus coeruleus*, and basal, lateral, and central regions of the amygdala. No significant differences in c-Fos density were observed between AM6545- and vehicle-treated mice in the areas analyzed (Supplementary Fig. 6). However, network analysis using the Pearson correlation coefficient for each pair of regions revealed that AM6545 treatment produced a significant alteration over the functional connectivity of these brain areas compared to the vehicle condition (Fig. 4A, B and Supplementary Fig. 7). We calculated Z-scores starting from the Pearson r correlation values to compare positive and negative connectivity between groups. AM6545 treatment did not significantly modified neither positive nor negative correlations between the regions studied (Fig. 4C, D).

In the past, CB1R antagonists were shown to contain important central effects, which have represented a limitation for the therapeutic use [20]. To reveal if AM6545 treatment induces important central effects in an unbiased fashion, we evaluated both the overall or global brain connectivity with resting state functional magnetic resonance imaging (rsfMRI), and the brain regions known to be relevant for object recognition performance. Analysis was performed using Graph Theory tools to reveal the functional efficiency of brain networks [21]. Local efficiency analysis provided an indication of how effectively information is transmitted between the immediate neighbors of a given network node (region). If averaged for all brain nodes we can obtain the whole brain local efficiency. After 1 h of AM6545 administration, whole brain local efficiency was significantly increased (Fig. 4E) (uncorrected $p < 0.05$) indicating an enhanced ability of parallel transmission of information. Regional connectivity analysis also showed significant increases of nodal efficiency values in AM6545-treated mice compared to vehicle in left frontal cortex (Kruskal-Wallis: $p < 0.01$), right hippocampus (Kruskal-Wallis: $p = 0.023$) and right globus pallidus (Kruskal-Wallis: $p = 0.023$) (Fig. 4F–H). We hypothesized that the nucleus tractus solitarius (NTS), a region receiving vagal afferents, may exhibit a different pattern of connectivity in AM6545-treated animals compared to vehicle. Thus, seed-based analysis of rsfMRI with seed in the posterior brainstem, which contains the NTS, showed differences in the connectivity with different areas corresponding to hippocampus,

frontal cortex, cingulate cortex, brainstem nuclei such as pontine reticular nucleus, different preolivary and spinal trigeminal nuclei and cerebellar nuclei (Fig. 4I). In these regions AM6545-treated animals showed strong negative correlation values with the seed BOLD signal, especially in hippocampus, that is not observed in vehicle-treated animals (Supplementary Fig. 8). In these regions AM6545-treated animals showed strong negative correlation values with the seed BOLD signal, especially in hippocampus, that is not observed in vehicle-treated animals (Supplementary Fig. 8). Thus, AM6545 administration did not result in widespread central disturbances, but rather in specific brain connectivity alterations which under those conditions might contribute to improved recognition memory persistence.

Increased hippocampal norepinephrine mobilization by peripheral CB1R blockade induces memory improvement
Next, we measured the firing rate at *locus coeruleus* neurons after CB1R peripheral modulation with AM6545. Systemic AM6545 significantly altered basal firing rates as compared to vehicle (two-way repeated measures ANOVA, interaction: F (2,12) = 9.037, $p = 0.004$; post hoc Bonferroni, 70 min VEH vs 70 min AM6545 $p = 0.023$) (Fig. 5A). To assess the functional significance of the effect on basal firing rate, we performed extracellular microdialysis analysis in the hippocampus after systemic AM6545 treatment. Analysis of norepinephrine (NE), dopamine (DA) and serotonin (5-HT) extracellular levels after AM6545 administration revealed a specific transient increase in NE in comparison to the vehicle-treated mice (two-way repeated measures ANOVA, interaction: F (1,14) = 2.19, $p = 0.009$) (Fig. 5B and Supplementary Fig. 9). Moreover, we analyzed the area under the curve (AUC) for the noradrenaline levels between vehicle and AM6545 since the time of the administration. AUC for AM6545-treated animals was significantly increased as compared to wild-type (Student's t-test: $p = 0.0077$) (Fig. 5C). We then tested whether β-adrenergic receptors in the hippocampal region were involved in the increased memory persistence mediated by AM6545. We found that local intra-hippocampal microinjection of β-adrenergic receptor antagonist propranolol at a dose that did not affect memory performance on its own (1 μg per 0.5 μL per
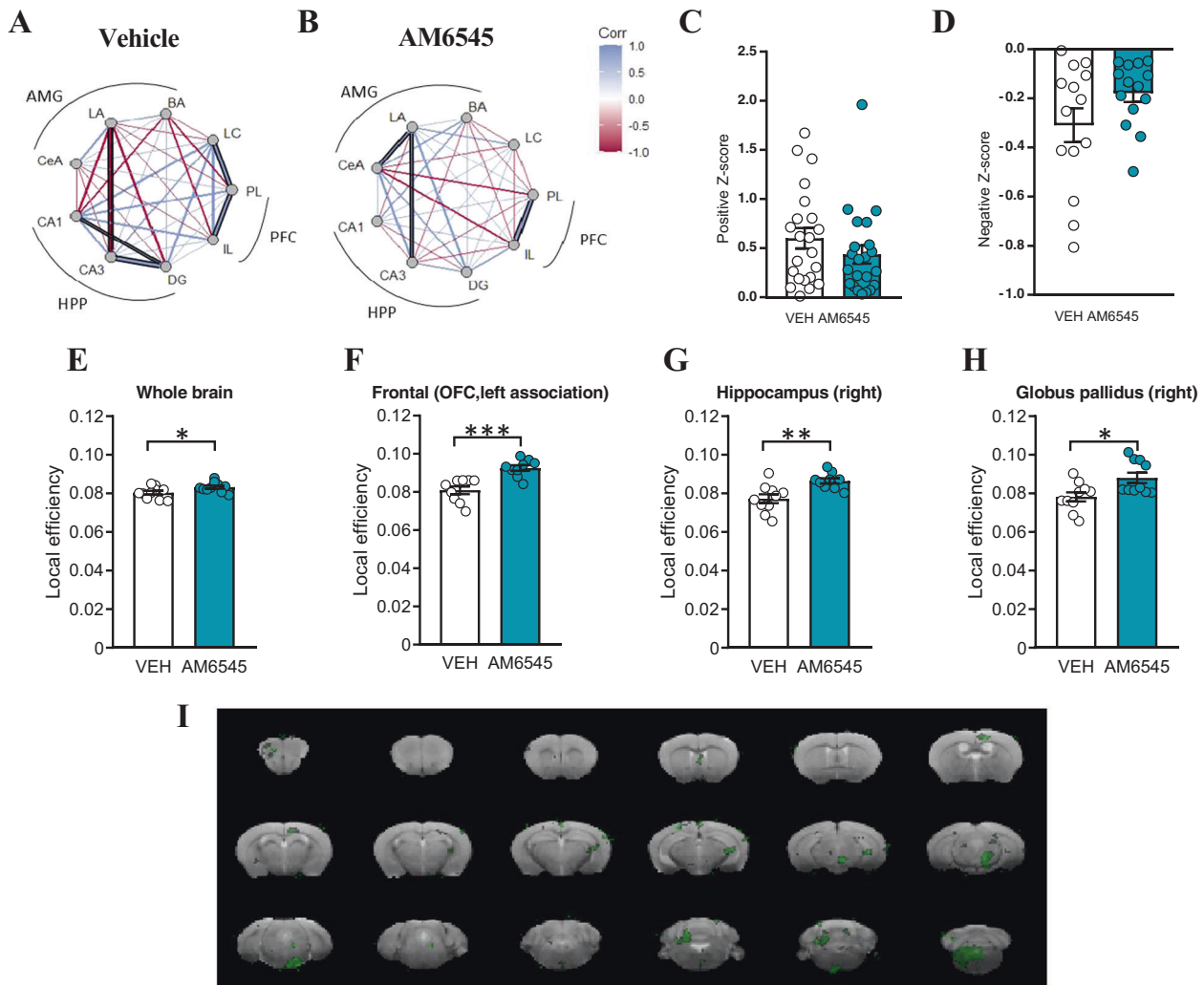
Fig. 4 Central effects of AM6545 administration on c-Fos expression and brain functional connectivity by resting state functional magnetic resonance imaging (rsfMRI). Network graphs of c-Fos expression based on the Pearson coefficient in vehicle (**A**) and AM6545 (1 mg/kg) (**B**) between different brain areas, including prelimbic cortex (PL), infralimbic cortex (IL), cornu ammonis 1 (CA1), dentate gyrus (DG), cornu ammonis 3 (CA3), locus coeruleus (LC), basal amygdala (BA), central amygdala (CeA) and lateral amygdala (LA) ($n = 5$–9). Significant correlations are depicted with black lines. Z-score values of the positive (**C**) and negative (**D**) Pearson r correlation coefficients between the different brain regions analysed. **E** Whole brain local efficiency and nodal efficiencies of left frontal (**F**) cortex, right hippocampus (**G**) and right globus pallidus (**H**) ($n = 10$). **I** Statistical map showing significant differences between connectivity of posterior brainstem with the rest of the brain in vehicle and AM6545-treated animals (comparison vehicle > AM6545; $p < 0.01$) ($n = 10$). Data are expressed as mean ± s.e.m. *$p < 0.05$, **$p < 0.01$ by Kruskal-Wallis test.

side, see Supplementary Fig. 10) blocked the mnemonic effects produced by systemic AM6545 administration (two-way ANOVA, interaction: F $(1,119) = 5.03$, $p = 0.03$; post hoc Tukey, Saline-VEH vs Saline-AM6545 $p = 0.004$; Saline-AM6545 vs Propranolol-AM6545 $p = 0.01$) (Fig. 5D), without affecting the exploratory behavior on the memory test (Fig. 5E). These data indicate the functional relevance of noradrenergic hippocampal activation in the effect of peripheral CB1R blockade on recognition memory persistence.

DISCUSSION
Our study identifies a relevant role of the peripheral ECS in modulating memory persistence through central and peripheral adrenergic/noradrenergic mechanisms. We chose to study novel object-recognition memory, a low arousal hippocampal-dependent test [22] where memory persistence might be modulated by post-training manipulation during the consolidation phase [23, 24]. In this regard, we and others have previously

observed deficits in novel object-recognition memory by post-training systemic administration of CB1R agonists [24, 25], endocannabinoid build-up [26], and stress [14, 24], all revealed when the recall test was performed 24 h after training. In the present study, we focused our analysis in novel object-recognition memory recall 48 h after training since at this time, mice naturally show reduced signs of novel object discrimination compared to shorter intervals such as 3 h or 24 h. In addition, we reasoned that the natural performance at 24 h shows a ceiling effect that does not allow measuring further cognitive improvements. Thus, we found the 48 h interval between training and test to be a suitable timing to measure memory potentiation in the NORT. Using this approach, we found that overall CB1R attenuation, through systemic pharmacological inhibition with a low dose of rimonabant, or by genetic inhibition, as detected using constitutive CB1HZ mice, significantly increased memory signs denoted by enhanced discrimination indexes 48 h after training. These results are in agreement with previous studies targeting overall (including both central and peripheral) CB1R through pharmacological and
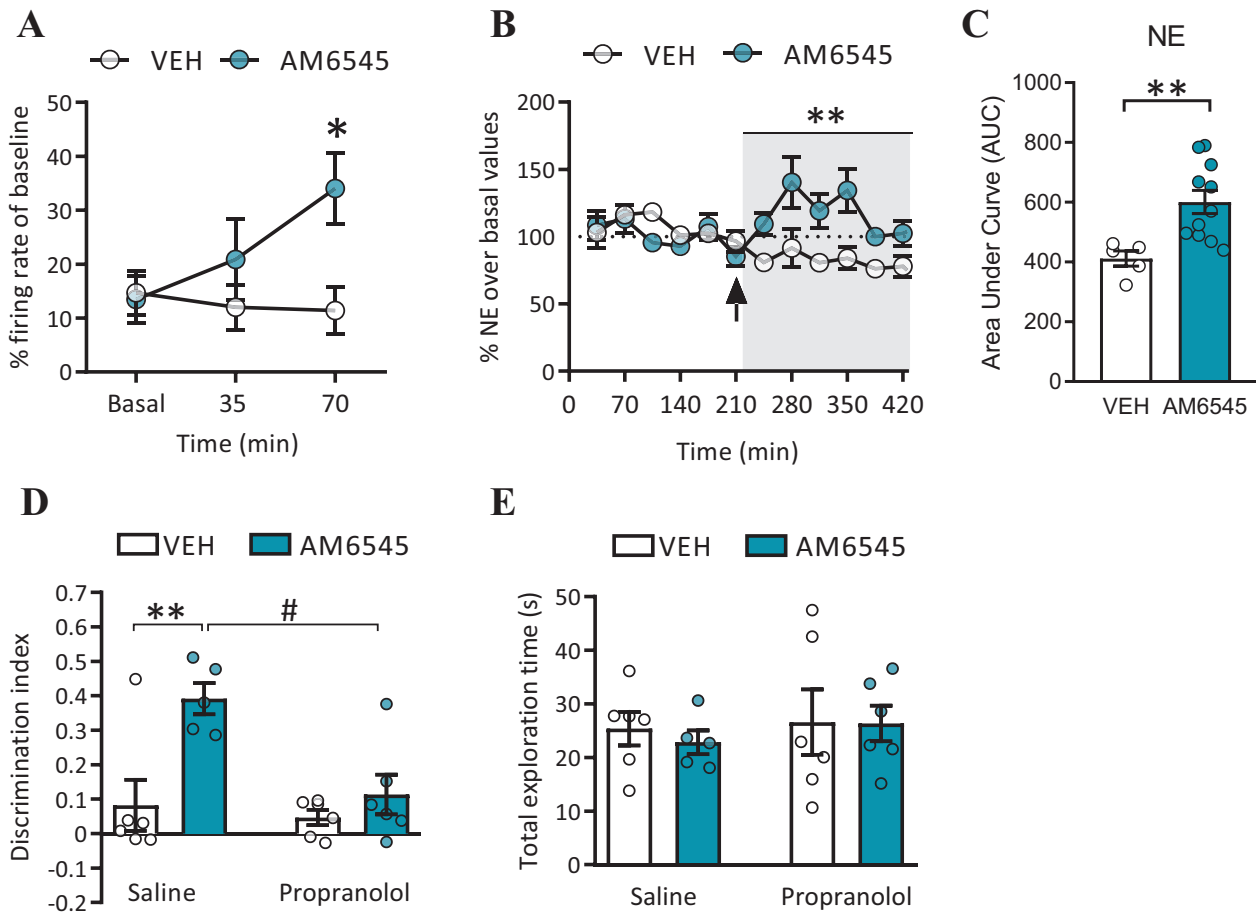
Fig. 5 Acute AM6545 treatment increases central noradrenergic activity. A Percentage of mean firing rate in the LC after acute vehicle (VEH) or AM6545 (1 mg/kg) administration respect to baseline values ($n = 4$). **B** Percentage of extracellular norepinephrine (NE) levels in the hippocampus after acute vehicle (VEH) or AM6545 (1 mg/kg) administration respect to baseline values ($n = 5$–11). The arrow indicates the time of administration. **C** Area under the curve for norepinephrine (NE) levels since the time of vehicle (VEH) or AM6545 (1 mg/kg) administration. **D** Discrimination index values and **E** total exploration time obtained in the NORT performed at 48 h mice treated with AM6545 (1 mg/kg) or vehicle (VEH) after bilateral intrahippocampal injection of saline or propranolol (1 µg/µl 0.5 µl per side) ($n = 5$–6). Data are expressed as mean ± s.e.m. For the firing rate at *locus coeruleus* *$p < 0.05$ by two-way repeated measures ANOVA test followed by Bonferroni post hoc. For microdialysis **$p < 0.01$ by two-way repeated measures ANOVA test and Student's *t*-test. For NORT **$p < 0.01$ (treatment effect) #$p < 0.05$ (pre-treatment effect) by two-way ANOVA test followed by Tukey post hoc.

genetic approaches that found enhanced memory in different hippocampal-dependent tasks [11, 12, 27–30]. Notably, AM6545 delivered after context fear conditioning training also demonstrated freezing enhancement when this type of emotional memory was assessed on the next day, further supporting the nootropic effect of this peripheral intervention. However, many contradictory results have been reported, especially when drugs were administered before the event to be studied, therefore addressing memory acquisition, or by local microinjection. For example, systemic administration in mice of the CB1R inverse agonist/antagonist AM251 30 min before training impaired spatial learning in mice [31], and when it was administered directly in the basolateral amygdala of rats, AM251 prevented fear memory reconsolidation, only when administered after memory reactivation [32]. On the contrary, infusions of CB1R agonists in the basolateral amygdala prevented fear memory by preventing reconsolidation [33], and the administration of CB1R agonists directly into infralimbic cortex and CA1 hippocampus region improved fear memory reconsolidation [34]. These discrepancies highlight the complexity of CB1R mediated effects on learning and memory. One possible explanation of this complexity is the differential expression of CB1Rs in different cell types and organs. However, in the present study we restricted CB1R effects on the

periphery. We observed that both peripherally restricted CB1R antagonists, AM6545 [35] and TM38837 [36], administered intraperitoneally after the training phase were also effective at enhancing object-recognition memory persistence in mice. AM6545 does not show significant blood-brain barrier permeability at doses even ten times higher than the one used in the present study [35], suggesting that AM6545 effects on memory derive in our study from a peripheral mechanism. Notably, adrenalectomized mice treated with AM6545 did not present an enhancement in memory, pointing to a relevant role of adrenal glands in this response, and discarding other potential mediators for memory enhancing such as cholecystokinin release, found to enhance memory performance through activation of the vagus nerve [37]. In this regard, AM6545 was recently found to promote the gut-brain satiation signal mediated by cholecystokinin in diet-induced obesity [38], but according to the study, these mechanisms would not be relevant in regular diet conditions as those in our setting. Epinephrine/norepinephrine and corticosteroids secreted by the adrenal glands have a significant impact in memory consolidation [17, 39, 40]. In addition, both rimonabant [41] and high doses of AM6545 have been observed to increase circulating corticosteroids [42]. We then gathered pharmacological evidence that pointed AM6545-memory improvement was

dependent on adrenergic transmission but not corticosteroids, given that AM6545 memory enhancement was prevented by the peripherally restricted β-adrenergic receptor antagonist sotalol, but not by the corticosteroid receptor antagonist mifepristone.

Remarkably, mice lacking CB1R in DBH+ cells [14] (DBH-CB1KO), similar to CB1HZ mice, showed enhanced recognition memory pinpointing the adrenergic/noradrenergic modulation by CB1R as a key factor in such mnemonic effects. Although previous studies have shown that AM6545 can reduce food intake in rodents and potentiate stress-induced hypothalamic–pituitary–adrenal axis via a non-CB1R mechanism [42, 43], our present findings where sotalol prevented the mnemonic phenotype in DBH-CB1KO mice and the memory effect of systemic rimonabant treatment, point to an overall significant role of CB1R inhibition that can be targeted in the periphery to attain acute central effects.

The study of c-Fos expression after AM6545 administration allowed to confirm no major differences in neuronal stimulation due to the treatment, but significant alterations in functional connectivity between areas analyzed. Although c-Fos may not reveal the real activity of brain regions given its limited expression and the time constraints of the analysis [44], this approach has been successfully performed to acquire a glimpse of brain functional connectivity between discrete areas [45, 46]. The subtle alterations in brain connectivity revealed by our biased c-Fos inter-regional correlation analysis were further confirmed by rsfMRI. The brain's intrinsic functional organization associated to the resting state has been proposed to determine the ability to create flexible and suitable behavioral outcomes to cognitive demands [47]. Indeed, enhancements in nodal efficiency, a representation for the ability of information propagation in the subnetwork of regions connected to a node, were observed specifically in the frontal cortex, hippocampus, and *globus pallidus*, a result in agreement with the engagement of these regions in long-term memory consolidation [46, 48] that was promoted by AM6545 treatment. Moreover, previous studies agree with our results showing the effect of the peripheral treatment in functional connectivity. For instance, transcutaneous vagus nerve stimulation in healthy adult humans produced significant BOLD activation in cortical, subcortical and cerebellar brain regions, associated with the afferent vagal pathway [49]. Another fMRI investigation in humans also reported that vagus nerve stimulation activates NTS and other central vagal projections [50]. Cervical vagus nerve stimulation in rats also showed strengthened correlations between the hippocampal formation and the retrosplenial cortex, areas related to memory, learning, and monitoring sensory inputs [51].

AM6545 central effects should be secondary to engaging peripheral mechanisms. In this regard, a growing body of evidence has demonstrated that systemic epinephrine administration, which does not cross the blood-brain barrier, enhances hippocampal-dependent memory in rodents [52, 53] by increasing central noradrenergic signaling, a way to encode event salience [54]. In addition, epinephrine administration results in increased vagal firing that was sensitive to sotalol administration [55]. Our findings show AM6545 triggering a relevant noradrenergic mechanism with mnemonic effects. Indeed, AM6545 enhanced norepinephrine extracellular levels observed in the hippocampus, sotalol administration was sufficient to blunt the memory improvement caused by peripheral CB1R inhibition and vagus nerve chemogenetic inhibition ameliorated AM6545 memory impact.

Notably, vagus nerve stimulation has been found to enhance memory retention in mice [56] as well as in humans [57]. In addition, LC activity, partially determined by vagal nerve afferents, is well stablished to be physiologically engaged on novelty and to prime the persistence of hippocampal-based long-term memories [58, 59]. Indeed, LC stimulation modulates hippocampal synaptic strength [60–62], spike coupling of CA1 pyramidal neurons [54],

and improves memory through a β-adrenergic-dependent mechanism [60, 63]. Moreover, norepinephrine release in the hippocampus facilitates the storage of new memories through the regulation of neural excitability and synaptic plasticity [63]. Although norepinephrine can bind to both α- and β-adrenergic receptors, synaptic information, and plasticity in the hippocampus is proposed to depend largely on the activation of β-adrenergic receptors [62]. In agreement, we observed that the intra-hippocampal blockade of β-adrenergic receptors with propranolol prevented the increase of novel object-recognition memory persistence produced by AM6545.

Altogether, our study identifies peripheral CB1R as a relevant target to enhance non-emotional memory persistence through central and peripheral adrenergic mechanisms that may engage vagal nerve afferents. This novel peripheral mechanism involving CB1R could underlie previously described effects of systemic CB1R antagonists formerly assumed to be associated to direct interaction with central targets.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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