Astroglial CB₁ Receptors Determine Synaptic D-Serine Availability to Enable Recognition Memory

Highlights

- Astrocytes are important for long-term object recognition memory
- Astroglial CB₁ receptors are coupled to D-serine availability at synapses
- Appropriate D-serine levels are required for NMDAR activity and LTP induction

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In Brief

Robin et al. show that astroglial CB₁ receptors in the hippocampus regulate D-serine supply to NMDA receptors, a process necessary for LTP induction and object recognition memory.
Astroglial CB₁ Receptors Determine Synaptic D-Serine Availability to Enable Recognition Memory

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https://doi.org/10.1016/j.neuron.2018.04.034

SUMMARY

Bidirectional communication between neurons and astrocytes shapes synaptic plasticity and behavior. D-serine is a necessary co-agonist of synaptic N-methyl-D-aspartate receptors (NMDARs), but the physiological factors regulating its impact on memory processes are scanty known. We show that astroglial CB₁ receptors are key determinants of object recognition memory by determining the availability of D-serine at hippocampal synapses. Mutant mice lacking CB₁ receptors from astroglial cells (GFAP-CB₁-KO) displayed impaired object recognition memory and decreased in vivo and in vitro long-term potentiation (LTP) at CA3-CA1 hippocampal synapses. Activation of CB₁ receptors increased intracellular astroglial Ca²⁺ levels and extracellular levels of D-serine in hippocampal slices. Accordingly, GFAP-CB₁-KO displayed lower occupancy of the co-agonist binding site of synaptic hippocampal NMDARs. Finally, elevation of D-serine levels fully rescued LTP and memory impairments of GFAP-CB₁-KO mice. These data reveal a novel mechanism of in vivo astroglial control of memory and synaptic plasticity via the D-serine-dependent control of NMDARs.

INTRODUCTION

The endocannabinoid system is an important modulator of physiological functions. It is composed of cannabinoid receptors, their endogenous ligands (i.e., endocannabinoids, eCB), and the enzymatic machinery responsible for their synthesis and degradation (Busquets-Garcia et al., 2018; Piomelli, 2003). The presence of type-1 cannabinoid receptors (CB₁) and the activity-dependent mobilization of endocannabinoids in different brain regions, including the hippocampus, are particularly involved in the modulation of several types of memory and associated cellular processes (Kano et al., 2009; Marsicano and Laﬀe`tre, 2009). Moreover, brain CB₁ receptors are expressed in different neuronal types, including inhibitory gamma-aminobutyric acid (GABA)ergic and excitatory glutamatergic neurons, where their stimulation negatively regulates the release of neurotransmitters (Kano et al., 2009).

CB₁ receptors are also expressed in glial cells, particularly astrocytes (Andrade-Talavera et al., 2016; Han et al., 2012; Min and Nevian, 2012; Navarrete and Araque, 2008; Rasooli-Nejad et al., 2014). For more than a century, astrocytes were thought to play an important supportive and nutritive role for neurons without actively participating in brain information processing (Allaman et al., 2011; Araque et al., 2014). However, it is now known that peri-synaptic astroglial processes surrounding pre- and postsynaptic neuronal elements form the so-called “tripartite synapse,” where astrocytes actively contribute to information processing (Araque et al., 2014; Perea et al., 2009).
**In vivo and in vitro studies** showed that astroglial CB1 receptor signaling indirectly modulates glutamatergic transmission onto hippocampal pyramidal neurons (Han et al., 2012; Metna-Laurent and Marsicano, 2015; Navarrete and Araque, 2010; Oliveira da Cruz et al., 2016). For instance, the disruptive effect of exogenous cannabinoids on short-term spatial working memory is mediated by astroglial CB1 receptors through an N-methyl-D-aspartate receptor (NMDAR)-dependent mechanism in the hippocampus (Han et al., 2012). Yet, the role of astroglial CB1 receptors in physiological long-term memory processes and the precise mechanisms involved are still unknown (Metna-Laurent and Marsicano, 2015).

D-serine is the co-agonist of synaptic NMDARs and its action is required to induce different forms of synaptic plasticity (Henneberger et al., 2010; Panatier and Oliet, 2006; Panatier et al., 2006; Papouin et al., 2012, 2017b; Shigetomi et al., 2013; Sultan et al., 2015). Although the direct source of the amino acid is still under debate (Araque et al., 2014; Papouin et al., 2017c; Wolosker et al., 2016), there is convergent consensus that its supply to synapses requires Ca2+-dependent astrocyte activity (Araque et al., 2014). However, whether astroglial CB1 receptors control the synaptic availability of D-serine during memory processing is not known.

Using genetic, behavioral, electrophysiological, imaging, and biochemical experimental approaches, in this study we asked whether the physiological activity of astroglial CB1 receptors is involved in long-term object recognition memory and whether the mechanisms involved imply the regulation of glial-neuronal interactions. The results show that physiological activation of astroglial CB1 receptors in the hippocampus is necessary for long-term object recognition memory consolidation via a mechanism involving the supply of D-serine to synaptic NMDARs and, consequently, the regulation of hippocampal synaptic plasticity. Thus, astroglial CB1 receptors contribute to the time- and space-specific synaptic actions of astrocytes to promote memory formation.

**RESULTS**

**Deletion of Hippocampal Astroglial CB1 Receptors Impairs Object Recognition Memory and In Vivo NMDAR-Dependent LTP**

To study the physiological role of astroglial CB1 receptors in memory, we tested conditional mutant mice lacking CB1 receptors in glial fibribrillary acidic protein (GFAP)-positive cells (GFAP-CB1-KO mice) (Han et al., 2012) in a long-term novel object recognition memory task in an L-maze (NOR) (Busquets-Garcia et al., 2011; Puighermanal et al., 2009, 2013). GFAP-CB1-KO mice displayed a significant memory deficit as compared to their control littermates (Figure 1A; see also Figure S1A), with no alteration in total object exploration time (Figure S1B). Hippocampal NMDAR-dependent transmission is involved in many forms of memory (Kandel et al., 2002; Puighermanal et al., 2009; Warburton et al., 2013), yet the involvement of hippocampal NMDARs on NOR memory is still under debate, as it seems to depend on specific experimental conditions (Balderas et al., 2015; Warburton and Brown, 2015). To clarify this issue, we set to investigate where these receptors are required for NOR memory formation in our behavioral paradigm. Intra-hippocampal administration of the NMDARs antagonist D-AP5 (15 μg/side; Figure S1C) fully abolished memory performance in wild-type (WT) mice when injected immediately after acquisition (Figure 1B; see also Figure S1D), but not 6 hr later (Figures S1F–S1H), with no alteration in total exploration time (Figure S1E). Thus, consolidation of long-term object recognition memory in the NOR task specifically requires astroglial CB1 receptors and hippocampal NMDARs signaling.

Activity-dependent plastic changes of synaptic strength, such as NMDAR-dependent long-term potentiation (LTP), are considered cellular correlates of memory formation (Kandel et al., 2002; Whitlock et al., 2006). To study astroglial CB1 receptor involvement in LTP, we recorded in vivo evoked field excitatory postsynaptic potentials (fEPSPs) in the hippocampal CA3-CA1 pathway of anesthetized WT and mutant mice. High-frequency stimulation (HFS) induced LTP in C57BL/6N mice (Figures 1C and 1D). The systemic administration of the NMDAR antagonist MK-801 (3 mg/kg, i.p.), which did not alter basal evoked fEPSPs (Figures S1I–S1K), fully blocked the induction of LTP (Figures 1C and 1D; Figures S1I–S1K), confirming its NMDAR dependency. Notably, this form of plasticity was abolished in GFAP-CB1-KO mice as compared to their WT littermates (Figures 1E and 1F), showing that CB1 receptors expressed in GFAP-positive cells are necessary for in vivo hippocampal NMDAR-dependent LTP induction. Altogether, these data demonstrate that astroglial CB1 receptors are essential for hippocampal NMDAR-dependent object recognition memory and LTP.

**Activation of CB1 Receptors Increases Astroglial Ca2+ Levels and Extracellular D-Serine**

Increase of astroglial intracellular Ca2+ modulates synaptic glutamatergic activity and plasticity via the release of gliotransmitters, whose identity likely depend on the brain region and the type of plasticity involved (Araque et al., 2014; Sherwood et al., 2017). Because activation of CB1 receptors generate Ca2+ signals in astrocytes (Araque et al., 2014; Metna-Laurent and Marsicano, 2015; Oliveira da Cruz et al., 2016), the impaired object recognition memory and synaptic plasticity in GFAP-CB1-KO mice might result from alterations of astroglial Ca2+ regulation of specific hippocampal gliotransmitters.

First, we tested whether the CB1 receptor-dependent modulation of intracellular Ca2+ levels (Gómez-Gonzalo et al., 2015; Min and Nevian, 2012; Navarrete and Araque, 2008, 2010) depends on direct activation of astroglial CB1 receptors. Local pressure application of the CB1 receptor agonist WIN55,212-2 (WIN) induced a reliable increase of Ca2+ levels in somas and principal processes of hippocampal astrocytes in slices from GFAP-CB1-KO WT mice (Figures 2A–2E). As expected (Gómez-Gonzalo et al., 2015; Min and Nevian, 2012; Navarrete and Araque, 2008, 2010), this effect was fully blocked by the CB1 receptor antagonist AM251 (2 μM; Figures 2B–2E). Notably, WIN had no effect in slices from GFAP-CB1-KO littermates (Figures 2B–2E), clearly indicating the direct impact of astroglial CB1 receptor activation on intracellular Ca2+ levels.

Via Ca2+-dependent mechanisms, astrocytes can promote the synaptic release of several signaling molecules known as gliotransmitters (Araque et al., 2014). One of them is D-serine, which
plays a key role in NMDAR signaling (Araque et al., 2014). Therefore, we asked whether activation of CB1 receptors might modulate the release of this amino acid. Application of WIN (5 μM) to hippocampal slices did not alter the tissue levels of several amino acids (Figures S2A–S2D). However, the same treatment slightly but specifically increased the extracellular levels of D-serine (Figures 2F–2I), indicating that activation of astroglial CB1 receptors can control the release of this signaling amino acid, a process that depends on intracellular Ca2+ signaling (Bohmbach et al., 2018; Henneberger et al., 2010).

Astroglial CB1 Receptor-Mediated D-Serine Supply Is Required for Hippocampal LTP
D-serine is the co-agonist of hippocampal synaptic NMDARs and its presence is necessary for LTP induction (Bohmbach et al., 2018; Henneberger et al., 2010; Papouin et al., 2012).

Figure 1. Hippocampal Astroglial CB1 Receptors Are Necessary for NMDAR-Dependent Object Recognition Memory and In Vivo LTP
(A) Memory performance of GFAP-CB1-WT mice (n = 10) and GFAP-CB1-KO littermates (n = 11) in the NOR task.
(B) Effects of intra-hippocampal infusions of vehicle (n = 10) or D-AP5 (15 μg/side; n = 8) on NOR performance.
(C and D) High-frequency stimulation in the CA3 area of hippocampus induces NMDAR-dependent LTP in CA1 stratum radiatum. (C) Summary plots of normalized iEPSPs in anesthetized mice under vehicle (n = 6) or MK-801 treatment (3 mg/kg; i.p.; n = 5). (D) Bar histograms of normalized iEPSPs from experiment in (C), 40 min after HFS.
(E and F) In vivo LTP is absent in GFAP-CB1-KO mice. (E) Summary plots of normalized iEPSPs in GFAP-CB1-WT (n = 9) and GFAP-CB1-KO (n = 6) littermates. (F) Bar histograms of normalized iEPSPs from experiment in (E), 40 min after HFS. Traces on the right side of the summary plots in (C) and (E) represent 150 superimposed evoked iEPSPs before (1, black) and after (2, gray) HFS.
Data, mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.
See also Figure S1 and Table S1.
Figure 2. Activation of Astroglial CB₁ Receptors Enhances Intracellular Ca²⁺ Levels in Astrocytes and Extracellular D-Serine

(A) Representative image of a hippocampal astrocyte stained with SR101 and Fluo4 and pseudo-color images representing fluorescence intensities before and after WIN 515,212-2 (WIN) application, with the correspondent Ca²⁺ traces (numbers refer to different subcellular locations on the astrocyte).

(B) Somatic calcium event probability before and after WIN (at time = 0) in GFAP-CB₁-WT in control conditions (white), in the presence of AM251 (2 μM; gray), and in GFAP-CB₁-KO mice (black).

(C) Somatic calcium event probability before and after WIN in GFAP-CB₁-WT in control conditions (white; n = 9 slices and 79 somas), in the presence of AM251 (gray; n = 12 slices and 159 somas), and in GFAP-CB₁-KO mice (black; n = 16 slices and 145 somas).

(D) Calcium event probability in the processes before and after WIN (at time = 0) in GFAP-CB₁-WT in control conditions (white), in the presence of AM251 (2 μM; gray), and in GFAP-CB₁-KO mice (black).

(E) Calcium event probability in the processes before and after WIN in GFAP-CB₁-WT in control conditions (white; n = 8 slices and 171 processes), in the presence of AM251 (gray; n = 8 slices and 140 processes), and in GFAP-CB₁-KO mice (black; n = 10 slices and 189 processes).

(F–I) Determination of D-serine (F), glycine (G), glutamate (H), and GABA (I) as measured by capillary electrophoresis in extracellular solutions of acute mouse hippocampal slices treated with vehicle or WIN.

Data, mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

See also Figure S2 and Table S1.
Thus, astroglial CB1 receptors might control the activity of NMDARs and hippocampal LTP by regulating the synaptic levels of D-serine. Measurements of bulk extracellular amino acids as performed in Figures 2F–2I do not specifically address whether D-serine levels impact synaptic function. A direct way to evaluate the levels and functions of synaptic D-serine is to perform electrophysiological measurements to assess the occupancy of the NMDAR co-agonist binding site at CA3-CA1 synapses (Papouin et al., 2012). Thus, we measured the impact of exogenous applications of the D-serine on NMDAR-mediated fEPSPs at CA3-CA1 synapses in acute hippocampal slices (Papouin et al., 2012). Bath application of D-serine (50 \text{ mM}) increased NMDAR-dependent synaptic responses in both GFAP-CB1-WT and GFAP-CB1-KO mice (Figures 3A and 3B). Strikingly, the effect of D-serine was twice more pronounced in the absence of astroglial CB1 receptors (Figures 3A and 3B), indicating that these receptors are necessary to maintain appropriate concentrations of D-serine within the synaptic cleft and consequently ensuring a proper level of occupancy of the NMDAR co-agonist binding site.

Next, we asked whether astroglial CB1 receptor-dependent release of D-serine controls synaptic plasticity by regulating NMDAR activity. First, in vitro electrophysiological recordings of fEPSPs at CA3-CA1 synapses in hippocampal slices revealed that GFAP-CB1-WT and GFAP-CB1-KO have comparable input-output relationships (Figure S3A), indicating that the deletion of astroglial CB1 receptors did not alter basal glutamatergic synaptic transmission.

HFS is known to induce endocannabinoid mobilization through the activation of mGluR1/5 receptors, eventually leading to long-term depression of inhibitory transmission (I-LTD) in the hippocampus (Castillo et al., 2012; Chevalleyre and Castillo, 2003). Therefore, we asked whether mGluR1/5 receptors could be involved in HFS-induced LTP. Application of the mGluR1/5 antagonists LY367385 and MTEP,
Figure 4. Hippocampal Astroglial CB1 Receptors Are Necessary for Object Recognition Memory through D-Serine

(A) Memory performance of GFAP-CB1-WT and GFAP-CB1-KO mice injected with vehicle (n = 5 both groups) or D-serine (50 mg/kg; i.p; GFAP-CB1-WT, n = 4; GFAP-CB1-KO, n = 5).
(B) Memory performance of GFAP-CB1-WT and GFAP-CB1-KO mice injected with vehicle (GFAP-CB1-WT, n = 8; GFAP-CB1-KO, n = 9) or AS057278 (50 mg/kg; i.p; GFAP-CB1-WT, n = 9; GFAP-CB1-KO, n = 8).
(C) Memory performance of GFAP-CB1-WT and GFAP-CB1-KO mice intra-hippocampally injected with vehicle (GFAP-CB1-WT, n = 5; GFAP-CB1-KO, n = 7) or D-serine (25 µg/side; GFAP-CB1-WT, n = 5; GFAP-CB1-KO, n = 7).
(D) Immunofluorescence for s100β (green) and NeuN (white) in the hippocampus of mice injected with AAV-GFAP-CRE-mCherry (red). Filled arrows, cells co-expressing s100β and CRE. Empty arrows, cells expressing only CRE. Scale bar, 50 µm. Bottom right: quantification of co-expression indicating the percentage of neurons (NeuN-positive) and astrocytes (s100β-positive) containing CRE recombinase over the total CRE-positive cells (left superposed bars) and the percentage of CRE-positive cells over the whole population of neurons and astrocytes (right superposed bars). Data are from 2–3 sections per animal from 8 mice injected with AAV-GFAP-CRE.
(E) Memory performance of CB1-flox mice intra-hippocampally injected with either an AAV-GFAP-GFP or an AAV-GFAP-CRE and treated with vehicle (AAV-GFAP-GFP, n = 6; AAV-GFAP-CRE, n = 8) or D-serine (50 mg/kg; i.p; AAV-GFAP-GFP, n = 7; AAV-GFAP-CRE, n = 8).

Data, mean ± SEM. ***p < 0.001.
See also Figure S4 and Table S1.

respectively, did not alter LTP (Figures S3B and S3C), suggesting that mGluR1/5 receptors are not involved in this process. Similarly to in vivo electrophysiological results, HFS-induced LTP was significantly reduced in GFAP-CB1-KO mice as compared to GFAP-CB1-WT (Figure 3C). Whereas the exogenous application of D-serine (50 µM) had no effect in slices from GFAP-CB1-WT mice, it fully rescued in vitro LTP in GFAP-CB1-KO littermates (Figures 3D and 3E). Importantly, the lack of in vivo LTP observed in GFAP-CB1-KO was fully restored by the systemic administration of D-serine (50 mg/kg, i.p.; Figures 3F–3H).

Considering that activation of astroglial CB1 receptors increases Ca²⁺ in astrocytes, we asked whether this subpopulation of cannabinoid receptors is involved in the HFS-induced regulation of astroglial Ca²⁺ dynamics (Perea and Araque, 2005; Porter and McCarthy, 1996; Sherwood et al., 2017). While the Ca²⁺ activity evoked in both soma and large processes of astrocytes during the HFS was the same, GFAP-CB1-KO astrocytes displayed a reduction in the Ca²⁺ event probability after the HFS as compared to WT littermates (Figures S3D–S3J). Altogether, these results show that astroglial CB1 receptors regulate Ca²⁺ dynamics in astrocytes and determine the synaptic levels of the NMDAR co-agonist D-serine necessary for NMDAR-dependent in vitro and in vivo LTP.

Astrogial CB1 Receptors Determine NOR Memory via D-Serine

If, as shown above, astroglial CB1 receptors determine the activity of NMDARs via the control of synaptic D-serine levels, this mechanism might underlie the processing of NOR memory. Strikingly, a sub-effective dose of D-serine (i.e., having no effect on memory performance per se, 50 mg/kg, i.p.; Figure S4A) reverted the memory impairment of GFAP-CB1-KO mice (Figure 4A; see also Figures S4B and S4C). This effect of D-serine in GFAP-CB1-KO mice was not present when the injection occurred 1 hr after acquisition or immediately before test
Astroglial CB1 receptors have so far been described to impact synaptic plasticity in different ways. For instance, their activation by exogenous cannabinoids can promote NMDAR-dependent hippocampal LTD (Han et al., 2012), whereas their endogenous stimulation can, depending on experimental conditions, lead to heterosynaptic potentiation in the hippocampus, amygdala, and striatum (Martin et al., 2015; Martin-Fernandez et al., 2017; Navarrete and Araque, 2008, 2010; spike-timing depression in the neocortex (Min and Nevian, 2012); or hippocampal LTP (present results). The conditions through which the activation of astroglial CB1 receptors might lead to different synaptic effects are currently not known (Araque et al., 2017; Metna-Laur and Marsicano, 2015; Oliveira da Cruz et al., 2016). Future studies will surely reveal novel functions of astroglial CB1 receptors and will hopefully determine the physiological conditions and the cellular mechanisms leading to different forms of synaptic plasticity. In this context, the present data extend the value of astroglial CB1 receptors to the processing of object recognition memory through the regulation of D-serine, a key astrocyte-dependent modulator of synaptic functions.

The direct release of D-serine by astrocytes has recently been questioned, suggesting that astrocytes release L-serine, which, in turn, shuttles to neurons to fuel the neuronal synthesis of D-serine (Wolosker et al., 2016). Our data do not directly address this issue, but they support the idea that astrocyte functions and synaptic D-serine actions are required for hippocampal LTP (Henneberger et al., 2010; Papouin et al., 2017b, 2017c; Shepherd et al., 2017; Wolosker et al., 2016). Activation of hippocampal CB1 receptors by the agonist WIN induces a slight but significant increase of extracellular D-serine levels, which is specific among different amino acids. No clear evidence is currently present to explain the relative low amplitude of this pharmacological effect. However, it is possible that bulk measurement of amino acids lacks the power to detect specific changes at synaptic level. Unfortunately, it is currently technically impossible to obtain synaptic extracellular samples to directly measure amino acids in these tiny volumes. For this reason, we implemented another direct and reliable measure of synaptic D-serine levels by assessing the occupancy of synaptic NMDAR co-agonist sites (Henneberger et al., 2010; Papouin et al., 2012). The results clearly show that the occupancy of synaptic NMDAR co-agonist sites by D-serine is strongly reduced (more than 50%) in GFAP-CB1-KO mice. HFS-induced LTP is fully abolished in GFAP-CB1-KO mice in vivo but only partially reduced in ex vivo hippocampal slices. These slight discrepancies are likely due to uncontrollable factors that are necessarily different between

DISCUSSION

These results show that astroglial CB1 receptors are key determinants of physiological consolidation of object recognition memory in the hippocampus. Via Ca2+-dependent mechanisms, they provide the synaptic D-serine levels required to functionally activate NMDARs and to induce LTP in the hippocampal CA1 region. In turn, this process is necessary upon learning to consolidate long-term object recognition memory (Figure S5). By causally linking the functions of a specific subpopulation of CB1 receptors, astroglial control of NMDAR activity via the glutamatergic D-serine, and synaptic plasticity, these data provide an unforeseen physiological mechanism underlying memory formation.

By showing that astroglial CB1 receptors play a key role in the maintenance of the basal levels of D-serine in the synaptic cleft and thus in the control of NMDAR activity, these data shed light onto the pathway underpinning D-serine availability at synapses. Interestingly, it has been recently demonstrated that the amount of D-serine available during wakefulness depends on the activity of cholinergic fibers from the medial septum (Papouin et al., 2017b). Thus, synaptic D-serine levels are under the control of at least two sets of astroglial receptors, namely CB1 (present data) and α7-nicotinic acetylcholine receptors (Papouin et al., 2017b).

Astrocytes occupy non-overlapping domains of the neuropil, where they survey the activity of thousands of synapses (Bushong et al., 2002; Pannasch and Rouach, 2013; Papouin et al., 2017a). On the other hand, endocannabinoids are locally mobilized at synapses in an activity-dependent manner, and their actions are rather limited in space and time (Castillo et al., 2012; Kano et al., 2009; Piomelli, 2003). Therefore, it is tempting to speculate that astroglial CB1 receptors may act as sensors integrating the overall intensity of local synaptic activity within the territory of specific astrocytes, and this information may then be used to adjust the availability of D-serine and the activity of NMDARs. In this context, we propose that the astroglial CB1-dependent regulation of D-serine supply is a major mechanism determining how much D-serine each astrocyte contributes to NMDARs as a function of neuronal activity within its territory.
in vivo and ex vivo experimental conditions (Andersen, 2007; Windels, 2006), such as, for instance, the inevitable disruption of astroglial networks during slicing procedures or others. Importantly, however, the exogenous application of D-serine at the same doses, respectively, restoring learning in vivo and revealing the decrease of NMDAR occupancy in slices rescues LTP in both experimental settings. Thus, independently of their direct source, synaptic D-serine levels are under the control of CB1 receptors specifically expressed in astrocytes, whose activation increases astroglial Ca\(^{2+}\) levels and promotes D-serine occupancy of synaptic NMDARs, eventually controlling specific forms of in vivo and in vitro LTP and object recognition memory.

Generalized activation or inhibition of CB1 receptors does not reliably reflect the highly temporally- and spatially-specific physiological functions of the endocannabinoid system (Busquets-Garcia et al., 2018). Indeed, previous data showed that deletion of astroglial CB1 receptors abolishes the impairment of hippocampal working memory by cannabinoid agonists, but it does not alter this form of short-term memory per se (Han et al., 2012), thereby leaving open the question of the physiological roles of astroglial CB1 receptors in the hippocampus (Metna-Laurent and Marsicano, 2015; Oliveira da Cruz et al., 2016). This question could not be addressed using global genetic or pharmacological inactivation of CB1 receptors, because it is known that CB1 receptors expressed in different cellular subpopulations have often very diverse and even opposite impact on brain functions (Busquets Garcia et al., 2016; Busquets-Garcia et al., 2015, 2018), and this is particularly true between neurons and astroglial cells (Busquets-Garcia et al., 2018; Metna-Laurent and Marsicano, 2015; Oliveira da Cruz et al., 2016). Indeed, global pharmacological activation, blockade, and genetic deletion of CB1 receptors are not able to catch subtle but important effects of endocannabinoid signaling. For instance, recent data show that deletion of the CB1 gene in hippocampal GABAergic or glutamatergic neurons induces decreased and increased in vitro LTP, respectively, as compared to WT mice (Monery et al., 2015), suggesting that results obtained by global receptor manipulation might be confounded by contrary physiological functions of cell-type-specific subpopulations of CB1 receptors. Thus, the present results determine an unforeseen link between endogenous activation of astroglial CB1 receptor signaling and long-term memory consolidation. Moreover, by showing the involvement of D-serine and NMDAR in these processes, our data provide an unexpected synaptic mechanism for this physiological function.

The deletion of the CB1 gene in our study is induced in adult mice by tamoxifen treatment of GFAP-CB1\(^{-}\)KO mice or local injection of AAV-Cre under the control of a GFAP promoter into the hippocampus of CB1\(^{-}\)flox mice. These procedures occur a few weeks before testing, excluding potential compensatory confounding events during pre- and post-natal development. Moreover, the phenotypes of GFAP-CB1\(^{-}\)KO mice in NOR and LTP are rescued by increasing D-serine-dependent NMDAR signaling at the moment of memory acquisition/early consolidation or electrophysiological analysis. In particular, the behavioral effects of D-serine were present when it was administered systemically or locally immediately after task acquisition, but not 1 hr later or at recall. Thus, considering pharmacokinetic studies showing that the extracellular levels of D-serine are increased after exogenous administration for about 100 min in the brain (Fukushima et al., 2004), it is reasonable to conclude that the control of synaptic NMDAR plasticity and of NOR memory by astroglial CB1 receptors is due to acute alterations of hippocampal circuitries during memory formation and LTP induction. An additional potential confounding factor is the role played by both D-serine (Sultan et al., 2015) and CB1 receptors (Galve-Roperh et al., 2007) on adult neurogenesis. Due to the expression of GFAP in precursor neurons, we cannot fully exclude that neurogenesis might play a role in the mechanisms described. However, CB1 receptors expressed in GFAP-positive cells are necessary for LTP at CA3-CA1 hippocampal synapses that are likely not influenced by neurogenesis events, which are known to specifically impact dentate gyrus circuits (Massa et al., 2011).

The role of CB1 receptors expressed in GFAP-positive cells in NOR appears to be limited to the early phases of memory processing, namely early consolidation. Indeed, whereas the injection of D-serine immediately after memory acquisition fully rescues the phenotype of GFAP-CB1\(^{-}\)KO mice in NOR, the same treatment as soon as 1 hr after or just before memory retrieval has no effect. This is notable because it indicates a very early engagement of astrocyte signaling in memory processing, underlying the importance of glial-neuronal interactions at crucial phases of cognitive processes.

In conclusion, our data provide a novel neurobiological frame, in which the tight interaction between astrocytes and neurons required for the formation of object recognition memory is under the control of astroglial CB1 receptors. Thus, by determining the physiological availability of D-serine at NMDARs, astroglial CB1 receptors are key causal elements of spatial and temporal regulation of glia-neuron interactions underlying synaptic plasticity and cognitive processes in the brain.

STAR METHODS

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  - Measurement of Amino Acids in Hippocampal Slices
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at https://doi.org/10.1016/j.neuron.2018.04.034.
ACKNOWLEDGMENTS

We thank Nathalie Aubailly, Magali Dubuc, and all the personnel of the Animal Facility of the NeuroCentre Magendie for mouse care. We thank Delphine Gonzales and all the personnel of the Genotyping Facility of the NeuroCentre Magendie. We thank the Histology and Biochemistry platforms of the NeuroCentre Magendie for help in the experiments. We thank also C. de Rijck (Vrije Universiteit Brussel) for help with experiments, and all the members of Marsicano’s lab for useful discussions. This work was supported by INSERM (G.M. and S.H.R.O.), CNRS (S.H.R.O. and A.P.), EU–FP7 (PAINCAGE, HEALTH–603191, G.M.), European Research Council (Endofood, ERC–2010-StG–260515 and CannaPreg, ERC–2014-PoC–640923, G.M.), Fondation pour la Recherche Médicale (DRM20101220445 and DPP20151033974, G.M.; FDT20160435664, J.F.O.d.C.; DEQ 20130326519, S.H.R.O.; FDT20150532252, V.C.L.), Human Frontiers Science Program (RG10036/2014, G.M. and A.A.), Region Aquitaine (G.M.), Agence Nationale de la Recherche (ANR Blanc: NeuroNutriSens ANR–13–BSV4–0006, G.M., and BRAIN ANR–10–LABX–0043, G.M., S.H.R.O., L.M.R., Animal Facility, Genotyping Facility, Histology Platform, and Biochemistry Platform), Fyssen Foundation and CONACyT (E.S.-G.), EMBO and FRM post-doctoral fellowships (L.B.), and French Ministry of Higher Education and Research (L.M.R. and V.C.L.).

AUTHOR CONTRIBUTIONS

L.M.R., J.F.O.d.C., and V.C.L. performed behavioral, in vivo electrophysiology, and in vitro electrophysiology experiments, respectively, and wrote the manuscript. M.M.-L., A.B.-G., L.B., M.V., and E.S.-G. contributed to behavioral experiments. A.A. and M.M.-F. provided calcium measurements. B.B., I.S., L.M.R., J.F.O.d.C., and V.C.L. performed behavioral, electrophysiology experiments, respectively, and wrote the manuscript. M.M. conceived and supervised the whole project and wrote the manuscript. All authors edited and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources should be directed to the Lead Contact Giovanni Marsicano (giovanni.marsicano@inserm.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All experiments were conducted in strict compliance with the European Union recommendations (2010/63/EU) and were approved by the French Ministry of Agriculture and Fisheries (authorization number 3306369) and the local ethical committee (authorization number A50120118). Two to three months-old naive male C57BL/6N (JANVIER, France), CB1-flox (mice carrying the “floxed” CB1 gene (CB1f/f) and male GFAP-CB1-KO mutant mice and GFAP-CB1-WT littermates were used. Animals were housed in groups under standard conditions in a day/night cycle of 12/12 hr (light on at 7 am). Behavioral experiments were conducted between 2 and 5 pm. In vivo electrophysiological experiments were conducted during the light phase. Mice undergoing surgery were housed individually after the procedure.

GFAP-CB1-KO mice were generated using the CRE/loxP system as previously described (Han et al., 2012). Mice carrying the “floxed” CB1 gene (CB1f/f) (Marsicano et al., 2003) were crossed with GFAP-CreERT2 mice (Hirrlinger et al., 2006), using a three-step backcrossing procedure to obtain CB1f/f;GFAP-CreERT2 and CB1f/f littermates, called GFAP-CB1-KO and GFAP-CB1-WT, respectively. As CreERT2 protein is inactive in the absence of tamoxifen treatment (Hirrlinger et al., 2006), deletion of the CB1 gene was obtained in adult mice (7-9 weeks-old) by daily i.p. injections of tamoxifen (1 mg dissolved at 10 mg/mL in 90% sesame oil, 10% ethanol, Sigma-Aldrich, France) for 8 days. Mice were used 3-5 weeks after the last tamoxifen injection (Han et al., 2012).

METHOD DETAILS

Drug Preparation and Administration

For behavioral experiments, D-serine (Ascent Scientific, United Kingdom) was dissolved in 0.9% saline for systemic injections in order to inject 10 mL/kg of body weight in each mouse. For intra-hippocampal infusions, D-serine was dissolved in artificial cerebrospinal fluid (aCSF). AS057278 (Sigma-Aldrich, France) was dissolved in 0.9% saline added with 2% DMSO, 10% ethanol. D-AP5 (Sigma-Aldrich, France) was dissolved in aCSF. All vehicles contained the same amounts of solvents. All drugs were prepared freshly before the experiments. All drugs were injected either intraperitoneally (i.p.) or intra-hippocampally immediately after the acquisition phase of the NOR task (see below for exceptions), except for AS057278, which was injected 2 hr before, based on published data indicating the peak of endogenous D-serine at this time point (Adage et al., 2008). D-serine was also intraperitoneally injected 1 hr after the acquisition and right before the test session. D-AP5 was also injected intra-hippocampally 6 hr after the acquisition.

Intra-hippocampal drug infusions (see below) were performed with the aid of 30-gauge injectors protruding 1.0 mm from the end of the cannulae. The volume infused was: 0.3 μL at a rate of 0.3 μL/min. After infusion, injectors were kept in place for 60 s to prevent outflow of injected solutions.

Intra-hippocampal Drug Delivery

Mice (8-12 weeks of age) were anesthetized by intraperitoneal injection of a mixture of ketamine (100mg/kg, Imalgene 500, Merial, France) and Xylazine (10mg/kg, Rompun, Bayer, France) and placed into a stereotaxic apparatus (David Kopf Instruments, CA, USA) with mouse adaptor and lateral ear bars. For intra-hippocampal infusions of drugs, mice were bilaterally implanted with 23-gauge stainless steel guide cannulae (Bilaney, Germany) following stereotaxic coordinates (Paxinos and Franklin, 2001) aiming at the dorsal hippocampus (AP: -1.8, ML: ± 1, DV: -1.3 mm), guide cannulae were secured with cement anchored to the skull by screws. Mice were allowed to recover for at least one week in individual cages before the beginning of the experiments. During the recovery period, mice were handled daily.
Viral Vectors, Intra-hippocampal Delivery, and Histological Verification
AAV-GFAP-GFP control virus was produced in the lab as previously described (Hebert-Chatelain et al., 2016; Soria-Gomez et al., 2014, 2015) by using pAAV-GFAP-GFP plasmid (ADDGENE #50473) as vector backbone. AAV-GFAP-CRE-mCherry was acquired from UNC vector core (NC, USA). Both viral vectors were rAAV serotype 8 with titers 4.17*10^11 for AAV-GFAP-GFP and 1.2*10^12 for AAV-GFAP-CRE-mCherry, respectively. For viral intra-HPC AAV delivery, mice were submitted to stereotaxic surgery (as above) and AAV vectors were injected with the help of a microsyringe (0.25 mL Hamilton syringe with a 30-gauge beveled needle) attached to a pump (UMP3-1, World Precision Instruments, FL, USA). Mice were injected directly into the hippocampus (HPC) (0.5 µL per injection site at a rate of 0.5 µL per min), with the following coordinates: dorsal HPC, AP +1.8; ML ± 1; DV – 2.0 and –1.5; ventral HPC: AP +3.5; ML ± 2.7; DV – 4 and –3. Following virus delivery, the syringe was left in place for 1 min before being slowly withdrawn from the brain. CB1-flox mice were injected with AAV-GFAP-GFP (control) or AAV-GFAP-CRE (fused to mCherry, serotype 8, UNC Vector Core, USA) to induce deletion of the CB1 gene in hippocampal astroglial cells. Animals were used for experiments 4-5 weeks after injections. Mice were weighed daily and individuals that failed to regain the pre-surgery body weight were excluded from the following experiments. To verify the correct pattern of CRE expression and localization, mice were transcardially perfused with parafomaldehyde (Sigma Aldrich, France) and their brains were sliced with a vibratome. 40µm hippocampal sections incubated with primary antibody directed against s100β (Rabbit polyclonal, Sigma Aldrich, France) and NeuN (Mouse monoclonal, Millipore, France). Secondary antibodies incubation was performed in order to detect s100β with Alexa 488 (Thermo Scientific, France) and NeuN with Alexa 647 (Thermo Scientific, France). Single plane confocal images were acquired with an SP8 confocal microscope (Leica, France) and minimally processed with ImageJ software. Automatic quantification of mCherry (CRE positive), s100β and NeuN expressing cells was performed with ImageJ software as previously described (Bolte and Cordelières, 2006). Briefly, after threshold subtraction and crosstalk correction, the number of cells co-expressing mCherry/s100β or mCherry/NeuN was automatically obtained by the “particle analysis” tool of the same software. mCherry/s100β co-expressing cells were expressed in percentage of CRE positive cells as well as percentage of total s100β cells. On the other hand, mCherry/NeuN co-expressing cells were reported as percentage of CRE positive cells as well as percentage of total NeuN cells.

Novel Object Recognition Memory Task
We used the novel object recognition memory task in a L-maze (NOR) (Busquets-Garcia et al., 2011, 2013; Puighermanal et al., 2009, 2013). As compared to other hippocampal-dependent memory tasks, this test presents several advantages for the aims of the present study: (i) the acquisition of NOR occurs in one step and previous studies revealed that the consolidation of this type of memory is deeply altered by acute immediate post-training administration of cannabinoids via hippocampal CB1 receptors (Puighermanal et al., 2009, 2013); (ii) the NOR test performed in a L-maze decrease variability and give strong and replicable results; (ii) this test allows repeated independent measurements of memory performance in individual animals (Puighermanal et al., 2013), thereby allowing within-subject comparisons, eventually excluding potential individual differences in viral infection.

The task took place in a L-shaped maze made of dark gray polyvinyl chloride shaped by two identical perpendicular arms (35 cm and 30 cm long respectively for external and internal L walls, 4.5 cm wide and 15 cm high walls) placed on a white background (Busquets-Garcia et al., 2011; Puighermanal et al., 2009). The task occurred in a room adjacent to the animal house with a light intensity fixed at 50 lux. The maze was overhung by a video camera allowing the detection and scoring offline of animal’s behavior. The task consisted of 3 sequential daily trials of 9 min each. During the habituation session (day 1), mice were placed in the center of the maze and allowed to freely explore the arms in the absence of any objects. The acquisition session (day 2) consisted in placing the mice again in the center of the maze in the presence of two identical objects positioned at the extremities of each arm and let to freely explore the maze and the objects. The memory test occurred 24 hr later (day 3): one of the familiar objects was replaced by a novel object different in its shape, color, and texture and mice were left to explore both objects. The position of the novel object and the associations of novel and familiar were randomized. All objects were previously tested to avoid biased preference. The apparatus as well as objects were cleaned with ethanol (70%) before experimental use and between each animal testing. Memory performance was assessed by the discrimination index (DI). The DI was calculated as the difference between the time spent exploring the novel object (TN) and the familiar object (TF) divided by the total exploration time (TN+TF): DI = [TN-TF]/[TN+TF]. Memory was also evaluated by directly comparing the exploration time of novel and familiar objects, respectively. Object exploration was defined as the orientation of the nose to the object at a distance of less than 2 cm. Experienced investigators evaluating the exploration were blind to the treatment and/or genotype of the animals.

In Vivo Electrophysiology
GFAP-CB1−KO and WT littermate mice were anesthetized in a box containing 5% Isoflurane (Virbac, France) before being placed in a stereotaxic frame (model SR-6M-HT, Narishige International, United Kingdom) in which 1.0% to 1.5% of Isoflurane was continuously supplied via an anesthetic mask during the complete duration of the experiment. The body temperature was maintained at 37 °C using a homeothermic system (model 50-7087-F, Harvard Apparatus, MA, USA) and the complete state of anesthesia was assured through a mild tail pinch. Before surgery, 100 µL of the local anesthetic Lurocaine (Vetoquinol, France) was injected in the scalp region. Surgical procedure started with a longitudinal incision of 1.5 cm in length aimed to expose Bregma and Lambda. After ensuring correct alignment of the head, two holes were drilled in the skull to place: a glass recording electrode, inserted in the CA1 stratum radiatum, and one concentric bipolar electrode (Model NE-100, KOPF Instruments, Tujunga, CA, USA) in the CA3 region using the following...
coordinates: 1) CA1 stratum radiatum: A/P – 1.5 mm, M/L – 1.0 mm, DV 1.20 mm; CA3: A/P – 2.5 mm, M/L – 2.8, DV – 2.0 mm. The recording electrode (tip diameter = 1–2 μm, 4–6 MΩ) was filled with a 2% pontamine sky blue solution in 0.5M sodium acetate. At first the recording electrode was placed by hand until it reached the surface of the brain and then to the final depth using an automatic micromanipulator (UNI-Z, M2E, France). Both electrodes were adjusted to find the area with maximum response. In vivo recordings of evoked field excitatory postsynaptic potentials (fEPSPs) were amplified 10 times by Axoclamp 900A amplifier (Molecular Devices, CA, USA) before being further amplified 100 times and filtered (low pass at 1 Hz and high-pass at 5000Hz) via a differential AC amplifier (model 1700; A-M Systems, Sequim, WA, USA). fEPSPs were digitized and collected on-line using a laboratory interface and software (CED 1401, SPIKE 2; Cambridge Electronic Design, UK). Test pulses were generated through an isolated Constant Current Stimulator (DS3, Digitimer, UK) triggered by the SPIKE 2 output sequencer via CED 1401 and collected every 2 s at a 10 kHz sampling frequency and then averaged every 300 s. Test pulse intensities were typically between 50-250 μA with a duration of 500 μs. Basal stimulation intensity was adjusted to 30%–40% of the current intensity that evoked a maximum field response. All responses were expressed as percent from the average responses recorded during the 10 min before high-frequency stimulation (HFS). HFS was induced by applying 3 trains of 100 Hz (1 s each), separated by 20 s intervals. fEPSPs were then recorded for a period of 40 min. In the specific group of mice the following treatments were applied: 1) MK-801 (Abcam, United Kingdom; 3 mg/kg, i.p., dissolved in saline, approx. 60 min before HFS) or vehicle (saline, i.p., approx. 60 min before HFS) 2) D-serine (Ascent Scientific, United Kingdom; 50 mg/kg, i.p., dissolved in saline) approx. 2 hr before HFS or vehicle (saline, i.p.). At the end of each experiment, the positions of the electrodes were grouped in 10 s time bins. The time of occurrence of an event was considered to be at the onset of the Ca2+ event. The calcium event probability was calculated as the number of somas or processes starting a calcium event per time bin in a field of view, divided by the number of somas or processes in that field of view (8–12 somas and 15–20 processes in each field of view). Events were grouped in 10 s time bins. The time of occurrence of an event was considered to be at the onset of the Ca2+ event. The calcium event probability was calculated as the number of somas or processes starting a calcium event per time bin in a field of view, divided by the number of somas or processes in that field of view (8–12 somas and 15–20 processes in each field of view). Events were grouped in 10 s time bins. The time of occurrence of an event was considered to be at the onset of the Ca2+ event. The calcium event probability was calculated as the number of somas or processes starting a calcium event per time bin in a field of view, divided by the number of somas or processes in that field of view (8–12 somas and 15–20 processes in each field of view). Events were grouped in 10 s time bins. The time of occurrence of an event was considered to be at the onset of the Ca2+ event. The calcium event probability was calculated as the number of somas or processes starting a calcium event per time bin in a field of view, divided by the number of somas or processes in that field of view (8–12 somas and 15–20 processes in each field of view). Events were grouped in 10 s time bins. The time of occurrence of an event was considered to be at the onset of the Ca2+ event.
probability during 20 s before the WIN 515,212-2 (WIN, Sigma-Aldrich, USA) application (200 μM, 3 s, 10 psi) was compared with the calcium event probability in the time bin after the WIN application. WIN was dissolved in DMSO and then 36 μL of the DMSO-WIN solution was diluted in 1 mL of aCSF, obtaining a final concentration of 200 μM used in the pressure-pulse pipette. We estimate, based on quantifications of Alexa 594 fluorescence, that the WIN concentration becomes further diluted after being pressure ejected in the bath aCSF to approximately 1-10 μM around the recorded cells (Navarrete and Araque, 2008). In some cases, experiments were performed in the presence of the CB1 antagonist AM251 (2 μM, Tocris, United Kingdom). Mean values were obtained from at least 5 slices and 2 mice in each condition.

### Measurement of Amino Acids in Hippocampal Slices

For the simultaneous measurement of D-serine, glutamate, glycine and GABA, a capillary electrophoresis-laser induced fluorescence detection method was used.

Five hippocampi from adult C57BL/6N mice (10-12 weeks old) were isolated from 350 μm slices and incubated in 350 μL oxygenated aCSF containing 0.5 μM TTX (Tocris, United Kingdom) with either vehicle (1/4000 DMSO) or WIN (5 μM in DMSO) during 30 min at 31°C. Extracellular medium was quickly removed, frozen using liquid nitrogen and stored at −80°C. Extracellular levels of D-serine, glutamate, glycine and GABA were then determined. Briefly, pooled slices were deproteinized by addition of cold trichloroacetic acid (TCA) to a 4% final concentration. The suspension was centrifuged at 16,800 g for 10 min, the TCA was extracted from the supernatant with water-saturated diethyl ether and stored at −80°C. Samples were analyzed with a commercial laser-induced fluorescence capillary electrophoresis (CE-LIF) (CE: Beckman Coulter (Brea, California, US), P/ACE MDQ; LIF: Picometrics (Labège, France), LIF-UV-02, 410 nm 20 mW) as following: samples were processed for micellar CE-LIF and were fluorescently derivatized at RT for 60 min with napthalene-2,3-dicarboxaldehyde (NDA) before being analyzed by CE using a hydroxypropyl-β-cyclodextrin (HP-b-CD) based chiral separation buffer. All electropherograms data were collected and analyzed using Karat 32 software v8.0 (Beckman Coulter, France). The tissue amounts of D-serine, glutamate, glycine and GABA were normalized to the protein content determined from pooled hippocampal slices by the Lowry method using the BCA protein Pierce (ThermoScientific, CA, USA) assay with bovine serum albumin (BSA) as standards. The quantity of D-serine, glutamate, glycine and GABA in the samples was determined from a standardized curve while peak identification was made by spiking the fraction with the amino acid.

### QUANTIFICATION AND STATISTICAL ANALYSIS

Data were expressed as mean ± SEM or single data points and were analyzed with Prism 6.0 (Graphpad Software), using t test (paired or unpaired), Mann Whitney test or ANOVA (One- or Two-Way), where appropriate. Dunnet’s, Holm-sidak (One-Way ANOVA) or Bonferroni’s (Two-Way ANOVA) post hoc tests were used. Statistical details for each quantitative experiment are illustrated in Table S1.