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NMDA Receptors Containing GluN2B/2C/2D Subunits Mediate an Increase in Glutamate Release at Hippocampal CA3–CA1 Synapses

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Abstract

NMDA receptors (NMDARs) are involved in synaptic transmission and synaptic plasticity in different brain regions, and they modulate glutamate release at different presynaptic sites. Here, we studied whether non-postsynaptic NMDARs, putatively presynaptic (preNMDARs), are tonically active at hippocampal CA3–CA1 synapses, and if they modulate glutamate release. We found that when postsynaptic NMDARs are blocked by MK801, D-AP5 depresses evoked and spontaneous excitatory synaptic transmission, indicating that preNMDARs are tonically active at CA3–CA1 synapses, facilitating glutamate release. The subunit composition of these NMDARs was determined by studying evoked and spontaneous excitatory synaptic transmission in the presence of Zn^{2+}, Ro 25-6981, and PPDA, antagonists of NMDARs containing GluN2A, GluN2B, and GluN2C/D, respectively. We found that evoked and spontaneous release decreased when the activity of NMDARs containing GluN2B and GluN2C/D subunits but not GluN2A was impeded. In addition, we found that the increase in glutamate release mediated by these NMDARs requires protein kinase A (PKA) activation. We conclude that preNMDARs that contain GluN2B and GluN2C/2D subunits facilitate glutamate release at hippocampal CA3–CA1 synapses through a mechanism that involves PKA.

Keywords NMDA receptor · Presynaptic · Subunit composition · Tonic activation · Glutamate release · Protein kinase A

Introduction

NMDA receptors (NMDARs) belong to the family of ionotropic glutamate receptors, together with AMPA and kainate receptors [1]. As well as acting as ion channels, these ionotropic glutamate receptors have also been shown to have metabotropic functions [2–6]. NMDARs are distributed widely throughout the brain, and they fulfill important roles as mediators of synaptic transmission, in plasticity, and as modulators of neurotransmitter release. In addition, inappropriate activation of NMDARs causes different brain disorders [1, 7].

NMDARs have classically been described as postsynaptic receptors, although the existence of presynaptic NMDARs (preNMDARs) has also been firmly established [4, 8, 9]. While the roles of postsynaptic NMDARs are well-known, there is less information regarding their presynaptic functions, mainly due to the technical limitations in studying presynaptic receptors and their signaling. A role for preNMDARs has been established in the cortex [8, 9], cerebellum [4], and hippocampus [10–12], where they are tonically active, and they modulate neurotransmitter release at different synapses [8, 13, 14]. PreNMDARs are involved in plasticity in the visual [15–18] and somatosensory cortices [19–21], with direct evidence obtained from layer 4–layer 2/3 neurons [22, 23] and layer 2/3–layer 2/3 neurons of the somatosensory cortex [24]. In the hippocampus, preNMDARs might participate in the induction of LTP [10], although whether they are tonically active and modulate glutamate release at CA3–CA1 synapses remains to be determined.

In the present work, we studied the tonic activation and modulatory role of hippocampal preNMDARs, as well as their subunit composition and the involvement of protein kinases in their activity at CA3–CA1 synapses. We found that when postsynaptic NMDARs were blocked by MK-801 (loading the postsynaptic cell via the patch pipette), D-AP5 provoked a decrease in EPSP amplitude and slope, an effect that was not prevented when astrocytes were loaded with MK-801, indicating that preNMDARs are tonically active and that they facilitate glutamate release. To determine the subunit composition of these NMDARs, we studied the EPSP slope in the presence of Zn^{2+}, Ro 25-6981, and PPDA, antagonists of

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NMDARs containing GluN2A, GluN2B, and GluN2C/D, respectively. We found that NMDARs containing the GluN2B and 2C/2D subunits but not GluN2A are tonically active in this tissue, and that they mediate an increase in evoked and spontaneous glutamate release. In addition, we found that the increase in glutamate release mediated by the activation of NMDARs requires protein kinase A (PKA) but not PKC activation. We conclude that NMDARs (probably presynaptic) containing GluN2B and GluN2C/2D subunits mediate an increase in glutamate release, a process that requires the activation of PKA at hippocampal CA3–CA1 synapses.

Materials and Methods

Ethical Approval

All animal procedures were carried out in accordance with the European Union Directive 2010/63/EU regarding the protection of animals used for scientific purposes, and they were approved by the local Ethical Committees. C57BL/6 mice were obtained from Harlan Laboratories (Spain) and 13–21-day-old male mice were used in the experiments.

Slice Preparation

Hippocampal slices were prepared as described previously [11, 25, 26]. Briefly, mice were anesthetized with isoflurane (2%) and decapitated for slice preparation, and the brain of the mouse was then removed and placed in an ice-cold solution containing (in mM): NaCl, 126; KCl, 3; NaH_2PO_4, 1.25; $MgSO_4$, 2; $CaCl_2$, 2; $NaHCO_3$, 26; and glucose, 10 (pH 7.2, 300 mOsm L^{-1}). Transverse hippocampal slices (350 μm thick) were obtained on a vibrating blade microtome (Leica VT1000S), and they were maintained oxygenated (95% O_2/5% CO_2) in this solution for at least 1 h before use. All experiments were carried out at room temperature (22–25 °C), and during the experiments, the slices were continuously superfused with the solution indicated above.

Electrophysiological Recordings

Whole-cell patch-clamp recording of pyramidal cells located in the CA1 field of the hippocampus was obtained under visual guidance with infrared differential interference contrast (DIC) microscopy. The neurons were verified as pyramidal cells through their characteristic voltage response to a current step protocol, and they were recorded in current-clamp configuration with a patch clamp amplifier (Multiclamp 700B), acquiring the data using pCLAMP 10.2 software (Molecular Devices). Patch electrodes were pulled from borosilicate glass tubes, and they had a resistance of 4–7 MΩ when filled with (in mM): potassium gluconate, 110; HEPES, 40; NaCl, 4; ATP-Mg, 4; and GTP, 0.3 (pH 7.2–7.3, 290 mOsm L^{-1}). Only cells with a stable resting membrane potential below − 55 mV were assessed, and the cell recordings were excluded from the analysis if the series resistance changed by more than 15% during the recording. All recordings were low-pass filtered at 3 kHz and acquired at 10 kHz. The EPSPs induced by a monopolar stimulation electrode situated in the *stratum radiatum* using brief current pulses (200 μs, 0.1–0.2 mA) were recorded. Stimulation was adjusted to obtain the EPSP peak amplitude of approximately 4–5 mV in control conditions. Miniature responses were recorded in the presence of 500 nM tetrodotoxin (TTX).

Pharmacology

TTX and all the salts used to prepare the internal and external solutions were obtained from Sigma Aldrich. The pharmacological agents were purchased from Toctris Bioscience: (+)-MK-801 maleate, D-AP5, Zn^{2+}, PPDA, Ro 25-6981, calphostin C, and H-89.

Data Analysis

The data were analyzed using the Clampfit 10.2 software (Molecular Devices).

Statistical Analysis

A normality and equal variance test was performed before making statistical comparisons with a paired or unpaired Student's *t* test as appropriate. The data are expressed as the mean ± S.E.M., and p values < 0.05 were considered significant.

Results

Tonically Active, PreNMDARs Facilitate Glutamate Release at Hippocampal CA3–CA1 Synapses

Non-Postsynaptic NMDARs Regulate Evoked Release at CA3–CA1 Synapses

In light of what is currently known about preNMDARs, we first set out here to assess whether such receptors were tonically activated at hippocampal CA3–CA1 synapses. As such, in whole-cell recordings obtained from CA1 neurons in transverse hippocampal slices from postnatal day (P) 13–21 C57BL/6 mice, we studied the EPSPs evoked by stimulating CA3 Shaffer Collateral (SC) afferents. To ensure that the NMDARs modulating these synapses were not postsynaptic, we used the patch pipette to load the postsynaptic neuron with MK-801 (1 mM), blocking these receptors. In this experimental

set-up, bath application of D-AP5 (50 μM) provoked a decrease in the EPSP slope (to 67 ± 7%, $n = 7$), a modification that was reversed by the washout of D-AP5 (Fig. 1a). The tonic activation of NMDARs is not due to the performance of the experiments at room temperature (22–25 °C) as the results were the same when the experiments were performed at a more physiological temperature (32–33 °C, 55 ± 9%, $n = 6$). Hence, it appears that tonically active non-postsynaptic NMDARs exist, probably preNMDARs that mediate and enhance glutamate release.

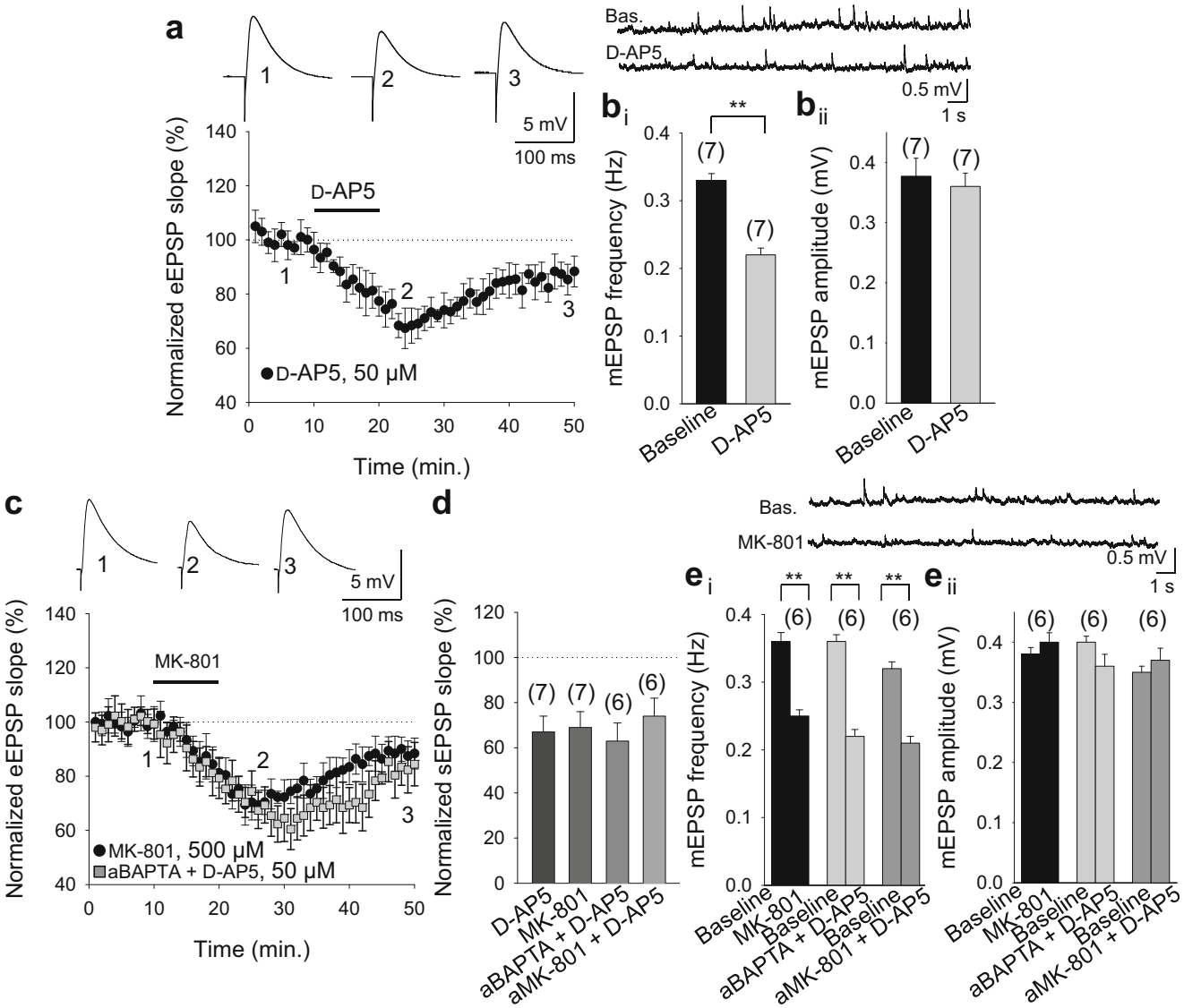

Fig. 1 Tonically active preNMDARs at CA3–CA1 synapses. **a** With the postsynaptic neuron loaded with MK-801, the addition of D-AP5 decreases the slope of evoked EPSPs, an effect that was reversed after D-AP5 washout. The inset shows the EPSP traces at baseline (1), in the presence of D-AP5 (2), and after D-AP5 wash-out (3). **b** Miniature EPSPs (mEPSPs) monitored during the baseline and after exposing neurons to D-AP5 in the presence of TTX (500 nM) and with the postsynaptic neuron loaded with MK-801 (1 mM). $\mathbf{b}_i$,$\mathbf{b}_{ii}$ Histograms showing that D-AP5 decreases the mEPSP frequency ($\mathbf{b}_i$), although it does not affect the mEPSP amplitude ($\mathbf{b}_{ii}$). **c** With the postsynaptic neuron loaded with MK-801, the addition of MK-801 to the bath decreases the slope of the evoked EPSPs, an effect that was reversed after MK-801 washout. The inset shows the EPSP traces at baseline (1), in the presence of MK-801 (2), and after MK-801 wash-out (3). With the astrocyte loaded with BAPTA, the addition of D-AP5 to the bath decreased the slope of the evoked EPSPs, an effect that was reversed after MK-801 washout. **d** Summary of the data. Note that the loading of astrocytes loaded MK-801 did not avoid the effect of D-AP5. **e** The mEPSPs were monitored during the baseline and after exposing neurons (or neurons and astrocytes) to MK-801 or D-AP5 in the presence of TTX (500 nM), and with the postsynaptic neuron loaded with MK-801 (1 mM). $\mathbf{e}_i$,$\mathbf{e}_{ii}$ Histograms showing that MK-801 and D-AP5 decrease the mEPSP frequency when MK-801 was loaded into the postsynaptic neuron and when astrocytes were loaded either with BAPTA or MK-801 ($\mathbf{e}_i$), but it does not affect the mEPSP amplitude ($\mathbf{e}_{ii}$). The error bars indicate the S.E.M., and the number of slices is shown in parentheses: ** $p < 0.01$, unpaired Student's t test

Non-Postsynaptic NMDARs Regulate mEPSP Frequency

To study the effect of these NMDARs on spontaneous glutamate release at SCs, we again blocked postsynaptic NMDARs by introducing MK-801 (1 mM) into the postsynaptic neuron before measuring the effects of D-AP5 (10 min) on miniature EPSP (mEPSP) frequency and amplitude in the presence of TTX (500 nM). We found that D-AP5 produced a decrease in mEPSP frequency (baseline 0.33 ± 0.01 Hz, $n = 7$; D-AP5 0.22 ± 0.01 Hz, $n = 7$, Fig. 1b), with no effect on their amplitude (baseline 0.38 ± 0.03 mV, $n = 6$; D-AP5 0.36 ± 0.02 mV, $n = 7$, Fig. 1b). This effect of D-AP5 on mEPSP is not due to the performance of the experiments at room temperature (22–25 °C) as the results were the same when the experiments were performed at a more physiological temperature (32–33 °C, baseline, 0.36 ± 0.01 Hz, D-AP5, 0.24 ± 0.01 Hz, $n = 6$). These results indicate that, as for evoked release, non-postsynaptic NMDARs are tonically active, modulating spontaneous release.

To check for a possible metabotropic role of NMDARs mediating these effects [4], we monitored the EPSP slope over time by adding extracellular MK-801 to the bath (500 μM) with the postsynaptic neuron loaded with MK-801 (1 mM). In these experimental conditions, the EPSP slope clearly decreased in the presence of MK-801 (69 ± 7%, $n = 7$, Fig. 1c,d), which reduced mEPSP frequency but not amplitude (frequency: baseline 0.36 ± 0.01 Hz, $n = 6$; MK-801 0.25 ± 0.01 Hz, $n = 6$; amplitude: baseline 0.38 ± 0.01 mV, $n = 6$; MK-801 0.40 ± 0.02 mV, $n = 6$, Fig. 1e) to a similar extent as D-AP5. These results indicate that the effects observed are mediated by non-postsynaptic ionotropic NMDARs.

Astrocytes Are Not Involved in the Increase in Glutamate Release Mediated by Activation of NMDARs

Glutamate can be released by astrocytes as a gliotransmitter, and it is possible that NMDARs exist in astrocyte membranes [27, 28]. To check for a possible role of astrocytes in the increase of glutamate observed with D-AP5 treatment, we repeated the experiments with astrocytes close to recorded neurons loaded with BAPTA (20 mM) that prevents vesicular release [27, 28]. In this experimental condition, D-AP5 reduced eEPSP slope to a similar extend than when BAPTA was not present (63 ± 8%, $n = 6$, Fig. 1c,d) indicating that glutamate from astrocytic origin is not involved in the tonic activation of NMDARs. D-AP5 also reduced mEPSP frequency but not amplitude (frequency: baseline 0.36 ± 0.01 Hz, $n = 6$; D-AP5 0.22 ± 0.01 Hz, $n = 6$; amplitude: baseline 0.40 ± 0.01 mV, $n = 6$; D-AP5 0.36 ± 0.02 mV, $n = 6$, Fig. 1e) To check for the possible existence of NMDARs in astrocytes mediating the observed increase in glutamate release, we repeated the experiments performing dual recordings, with MK-801 (1 mM) loaded into the postsynaptic neuron and into the astrocytes. In this experimental conditions, the EPSP slope clearly decreased in the presence of D-AP5, 74 ± 8%, $n = 6$ (Fig. 1c) and reduced mEPSP frequency but not amplitude (frequency: baseline 0.32 ± 0.01 Hz, $n = 6$, D-AP5 0.21 ± 0.01 Hz, $n = 6$; amplitude: baseline 0.35 ± 0.01 mV, $n = 6$; D-AP5 0.37 ± 0.02 mV, $n = 6$, Fig. 1e) to a similar extent as when MK-801 was loaded only into the postsynaptic neuron. These results indicate that NMDARs mediating a decrease in glutamate release are not situated in the postsynaptic neuron or in the astrocytes and strongly suggest that NMDARs are located in the presynaptic neurons.

Tonically Active NMDARs Containing GluN2B and GluN2C/D Subunits Mediate the Increase of Glutamate Release

To test whether the tonic activation and facilitation of glutamate release observed depends on receptors containing the GluN2A subunit, we used the preferred GluN2A subunit antagonist Zn^{2+} [11, 29]. The EPSP slope remained unaffected in the presence of 300 nM Zn^{2+} (104 ± 3%, $n = 6$ vs D-AP5, 67 ± 6%, $n = 6$, Fig. 2a,b), and thus, we studied the effect of Zn^{2+} on spontaneous glutamate release at CA1 pyramidal neurons, again blocking postsynaptic NMDARs by introducing MK-801 (1 mM) into the postsynaptic neuron. When the effects of Zn^{2+} (10 min) on mEPSP frequency and amplitude were measured in the presence of TTX (500 nM), we found that Zn^{2+} did not alter the mEPSP frequency (baseline 0.40 ± 0.05 Hz, $n = 7$; Zn^{2+} 0.41 ± 0.07 Hz, $n = 7$ vs D-AP5, baseline 0.38 ± 0.015 Hz, $n = 6$; D-AP5 0.28 ± 0.011 Hz, $n = 6$, Fig. 2c) or amplitude (baseline 0.38 ± 0.03 mV, $n = 7$; Zn^{2+} 0.42 ± 0.043 mV, $n = 7$, D-AP5: baseline 0.40 ± 0.04 mV, $n = 6$; D-AP5 0.39 ± 0.03 mV, $n = 6$, Fig. 2c). Thus, like the evoked release, spontaneous glutamate release is mediated by tonically active NMDARs that do not contain GluN2A subunits.

In light of this data, we assessed whether NMDARs containing GluN2B subunits were involved in the tonic activation and the facilitation of glutamate release using the selective, non-competitive GluN2B subunit antagonist Ro 25-6981 [9, 11, 30, 31]. With MK-801 loaded into the postsynaptic cell to block postsynaptic NMDARs, Ro 25-6981 (0.5 μM) induced a decrease in the slope of evoked EPSPs to a similar extent as D-AP5 (69 ± 8%, $n = 6$ vs D-AP5, 64 ± 6%, $n = 6$, Fig. 3a,b). The effect of Ro 25-6981 on spontaneous glutamate release at CA1 pyramidal neurons was assessed through its influence on mEPSP frequency and amplitude in the presence of TTX (500 nM) when postsynaptic NMDARs were blocked again by introducing MK-801 (1 mM) into the postsynaptic neuron. Ro 25-6981 (10 min) reduced mEPSP frequency (baseline 0.39 ± 0.02 Hz, $n = 6$; Ro 25-6981 0.24 ± 0.01 Hz, $n = 6$; Fig. 3c), but it had no effect on mEPSP amplitude (baseline 0.38 ± 0.05 mV, $n = 7$; Ro 25-6981 0.39 ± 0.04 mV, $n = 6$,

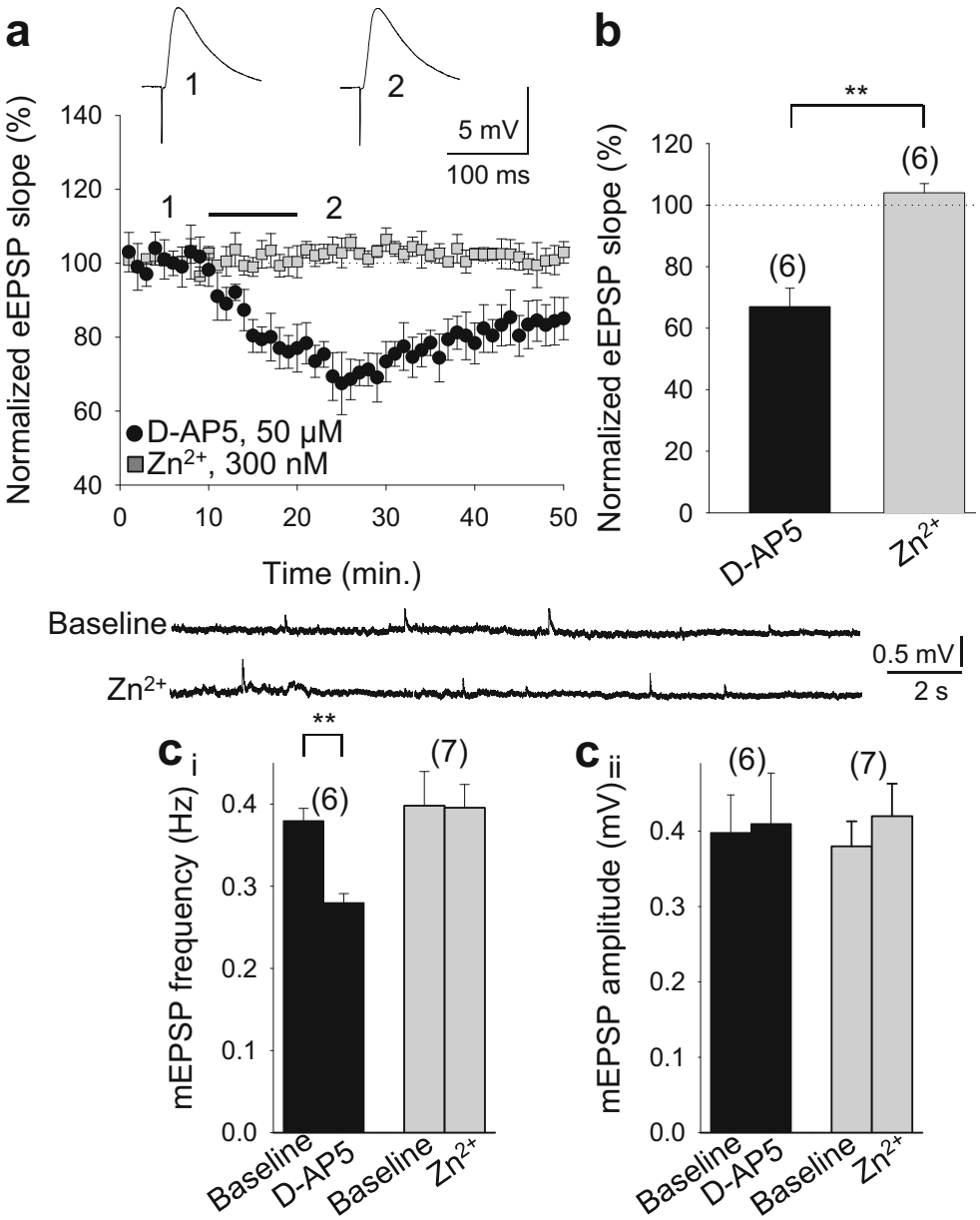

Fig. 2 The tonic activation and facilitation of glutamate release do not involve NMDARs containing GluN2A subunits at CA3–CA1 synapses. **a** With the postsynaptic neuron loaded with MK-801, the addition of Zn^{2+} does not affect the slope of evoked EPSPs. The inset shows the EPSP traces at baseline (1) and in the presence of Zn^{2+} (2). **b** Summary of the data. **c** The miniature EPSPs (mEPSPs) monitored at baseline and after exposing neurons to Zn^{2+} in the presence of TTX (500 nM) and with the postsynaptic neuron loaded with MK-801 (1 mM). $\mathbf{c}_i$,$\mathbf{c}_{ii}$ Histograms showing that Zn^{2+} does not affect the mEPSP frequency ($\mathbf{c}_i$) or amplitude ($\mathbf{c}_{ii}$). The error bars indicate the S.E.M., and the number of slices is shown in parentheses: ** $p <$ 0.01, unpaired Student's *t* test

Fig. 3c) as observed for D-AP5 (frequency: baseline 0.42 ± 0.02 Hz, $n = 6$; D-AP5 0.26 ± 0.02 Hz, $n = 6$, amplitude: baseline 0.40 ± 0.06 mV; D-AP5 0.42 ± 0.05 mV, $n = 6$, Fig. 3c). Hence, tonically active NMDARs that modulate evoked and spontaneous release contain the GluN2B subunit.

The possible involvement of GluN2C/D subunits in these phenomena was also investigated using PPDA, a competitive antagonist of GluN2C/D subunit containing NMDARs [32]. Bath application of PPDA (10 μM) diminished the EPSP slope to a similar extent as D-AP5 (63 ± 6%, $n = 6$ vs D-AP5, 72 ± 7%, $n = 7$, Fig. 4a,b), and thus, we studied the effect of PPDA on spontaneous glutamate release at CA1 synapses, again blocking postsynaptic NMDARs by introducing MK-801 (1 mM) into the postsynaptic neuron. In the presence of TTX (500 nM), PPDA (10 min) reduced the frequency of mEPSPs (baseline 0.41 ± 0.02 Hz, $n = 6$; PPDA 0.26 ± 0.02 Hz, $n = 7$, Fig. 4c) but not their amplitude (baseline 0.39 ± 0.03 mV; PPDA 0.41 ± 0.04 mV, $n = 7$, Fig. 4c) as occurred with D-AP5 (frequency: baseline 0.44 ± 0.03 Hz, $n = 6$; D-AP5 0.28 ± 0.02 Hz, $n = 6$, amplitude: baseline 0.43 ± 0.03 mV; D-AP5 0.40 ± 0.04 mV, $n = 6$, Fig. 4c). These results indicate that the tonically active NMDARs that enhance evoked and spontaneous release contain the GluN2C and/or GluN2D subunits. Thus, the tonically active preNMDARs that facilitate glutamate release at CA3-CA1 synapses contain GluN2B and GluN2C/D but not GluN2A subunits.

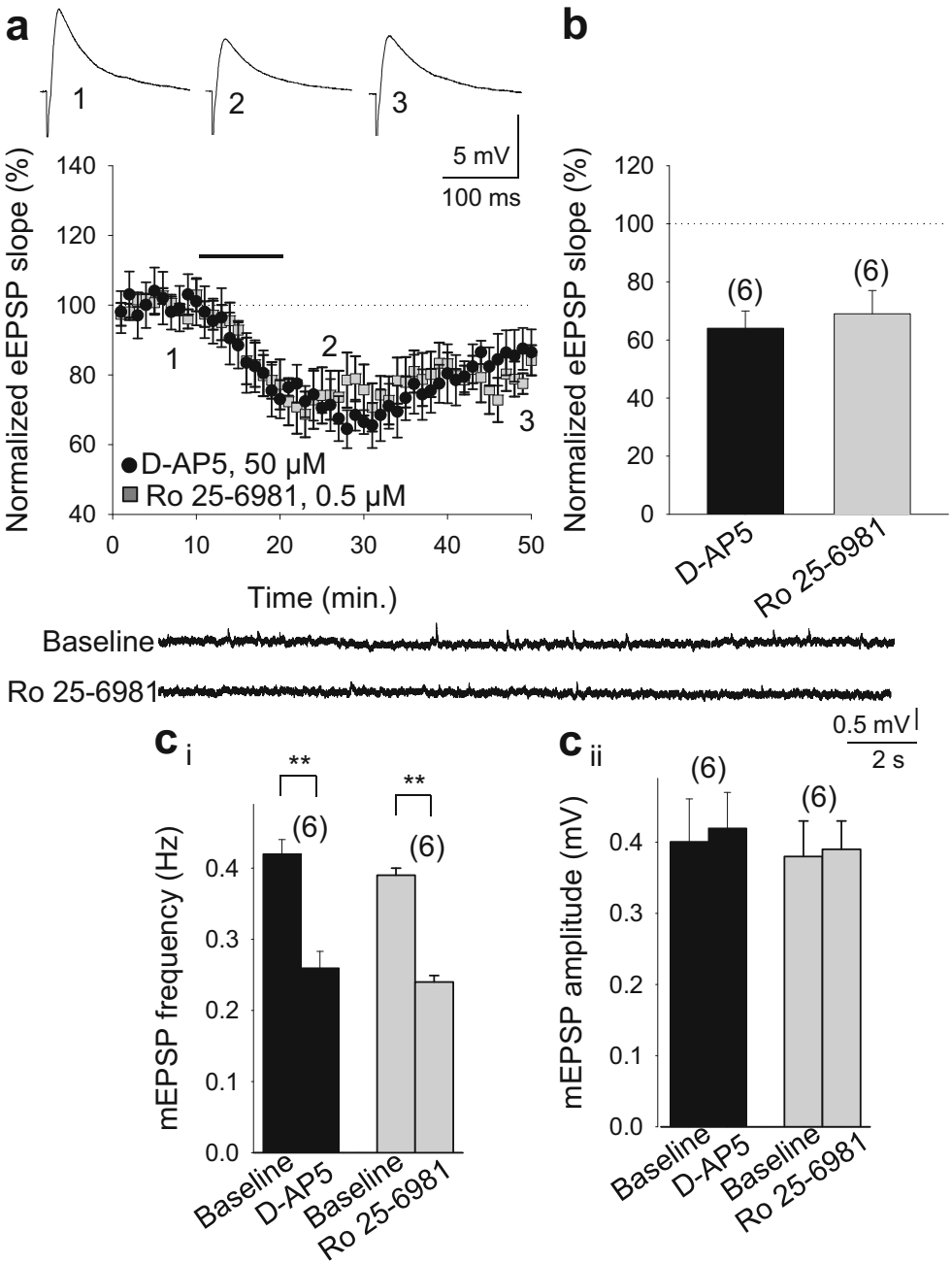

Fig. 3 The tonic activation and facilitation of glutamate release at CA3–CA1 synapses involve NMDARs containing the GluN2B subunit. **a** With the postsynaptic neuron loaded with MK-801, the addition of Ro 25-6981 (0.5 μM) decreased the slope of evoked EPSPs. The inset shows the EPSP traces relative to the decrease observed with D-AP5, at baseline (1), in the presence of Ro 25-6981 (2), and after Ro 25-6981 wash-out (3). **b** Summary of the data. **c** The miniature EPSPs (mEPSPs) monitored at baseline and after exposing neurons to Ro 25-6981 in the presence of TTX (500 nM) and with the postsynaptic neuron loaded with MK-801 (1 mM). $\mathbf{c}_i$,$\mathbf{c}_{ii}$ Histograms showing that Ro 25-6981 decreases the mEPSP frequency ($\mathbf{c}_i$), but it does not affect the mEPSP amplitude ($\mathbf{c}_{ii}$). The error bars indicate the S.E.M., and the number of slices is shown in parentheses: ** $p <$ 0.01, unpaired Student's *t* test

The Mechanism Underlying Glutamate Release Facilitated by Non-Postsynaptic NMDARs at CA3–CA1 Synapses

Tonic activation of preNMDARs is mediated by protein kinase C in the visual cortex [33], yet it is unclear if protein kinases are involved in the modulation of glutamate release in the hippocampus. To gain some insight into how activation of preNMDARs enhances glutamate release, we examined the influence of protein kinase inhibitors on these events. In slices incubated with 0.5 μM calphostin C (a PKC inhibitor), the effect of D-AP5 on the evoked EPSPs remained unaffected, D-AP5 producing a decrease in the evoked EPSP slope similar to that observed in the absence of calphostin C (70 ± 7% in calphostin C, $n = 6$ vs 67 ± 6%, $n = 6$ in non-treated interleaved slices, Fig. 5a,b). Likewise, the effect of D-AP5 on spontaneous release was not altered by the presence of calphostin C (baseline 0.42 ± 0.022 Hz; D-AP5 0.26 ± 0.02 Hz, $n = 7$; Fig. 5c) vs non-treated slices (baseline 0.40 ± 0.01 Hz, $n = 6$; D-AP5 0.25 ± 0.02 Hz, $n =$ 6; Fig. 5c). Hence, the glutamate release facilitated by the tonic activation of NMDARs does not require PKC

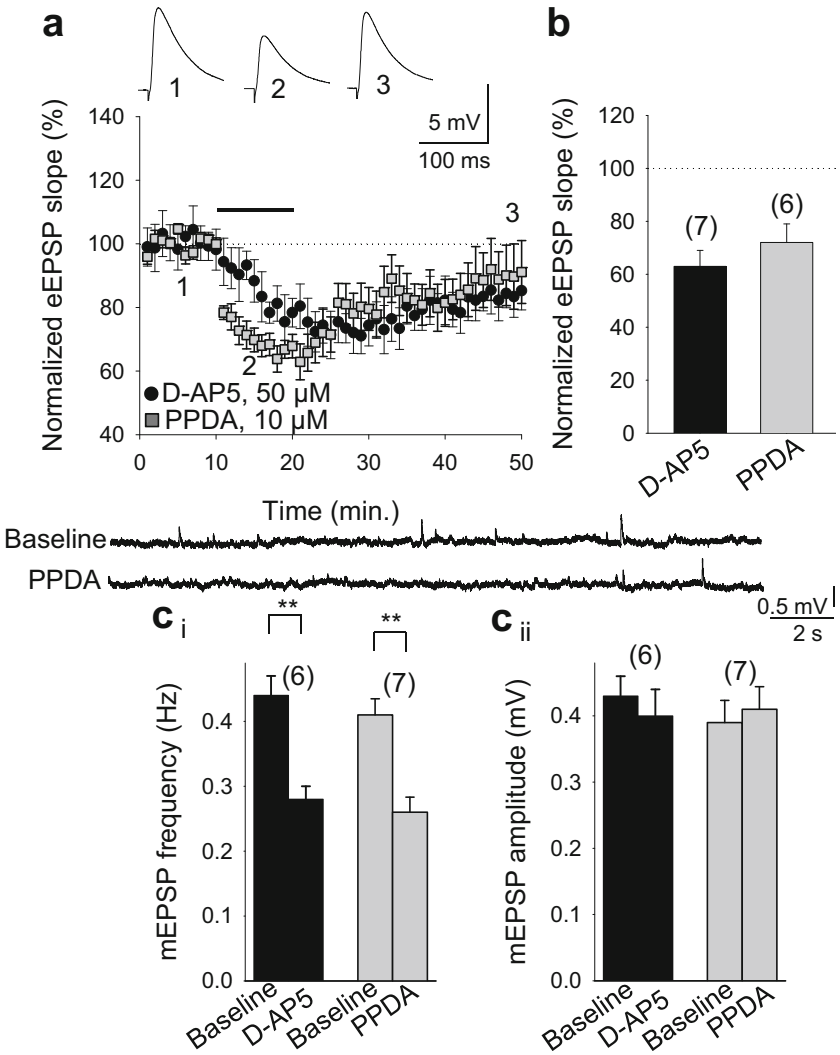

Fig. 4 The tonic activation and facilitation of glutamate release at CA3–CA1 synapses involve NMDARs containing GluN2C/D subunits. **a** With the postsynaptic neuron loaded with MK-801, the addition of PPDA (10 μM) decreased the slope of evoked EPSPs. The inset shows the EPSP traces relative to the decrease observed with D-AP5 at baseline (1), in the presence of PPDA (2), and after PPDA wash-out (3). **b** Summary of the data. **c** The miniature EPSPs (mEPSPs) monitored at baseline and after exposing neurons to PPDA in the presence of TTX (500 nM), and with the postsynaptic neuron loaded with MK-801 (1 mM). $\mathbf{c}_i$,$\mathbf{c}_{ii}$ Histograms showing that PPDA decreases the mEPSP frequency ($\mathbf{c}_i$), but it does not affect the mEPSP amplitude ($\mathbf{c}_{ii}$). The error bars indicate the S.E.M., and the number of slices is shown in parentheses: ** $p <$ 0.01, unpaired Student's *t* test

activation at CA3–CA1 hippocampal synapses, in contrast to the visual cortex.

As PKC was not involved in facilitating glutamate release, we assessed whether PKA may be required for the tonic activation observed, especially since it is involved in glutamate release and LTP at different synapses [34, 35]. When slices were maintained in the presence of the PKA inhibitor H-89 (2 μM), D-AP5 had no effect on the EPSP slope (102 ± 7%, $n = 7$ in H-89 vs 67 ± 6%, $n = 6$ in non-treated interleaved slices, Fig. 5a,b) or on spontaneous (mEPSP) release (baseline 0.38 ± 0.01 Hz; D-AP5 0.41 ± 0.03 Hz, $n = 7$; Fig. 5c vs non-treated slices (baseline 0.40 ± 0.01 Hz, $n = 6$; D-AP5 0.25 ± 0.02 Hz, $n = 6$; Fig. 5c). Hence, PKA activation is required for NMDARs to facilitate glutamate release at hippocampal CA3–CA1 synapses. To further characterize the intracellular pathway involved in the increase of glutamate release mediated by the activation of preNMDARs, we explored whether the direct activation of adenylate cyclase (AC) by forskolin (30 μM) affected this modulation of glutamate release. Slices were preincubated for 1 h with the diterpene in these experiments. In this condition, the effect of D-AP5 on eEPSP slope was prevented (97 ± 6%, $n = 6$, Fig. 5b), indicating that the facilitation of glutamate release mediated by the activation of preNMDARs is manifested through an AC/cAMP/PKA signaling pathway. Similarly, when slices were incubated with forskolin, D-AP5 failed to affect mEPSP frequency or amplitude (frequency: baseline 0.36 ± 0.01 Hz, $n = 6$; D-AP5 0.38 ± 0.01 Hz, $n = 6$; amplitude: baseline 0.39 ± 0.01 mV, $n = 6$; D-AP5 0.38 ± 0.01 mV, $n = 6$, Fig. 5c). These results indicate that an AC/cAMP/PKA signaling pathway is mediating the observed increase in glutamate release mediated by the activation of preNMDARs.

To determine whether PKA is necessary for NMDARs containing GluN2B and GluN2C/2D subunits, we carried out experiments on slices incubated with calphostin C or H-89, assessing the effect of Ro 25-6981 and PPDA on

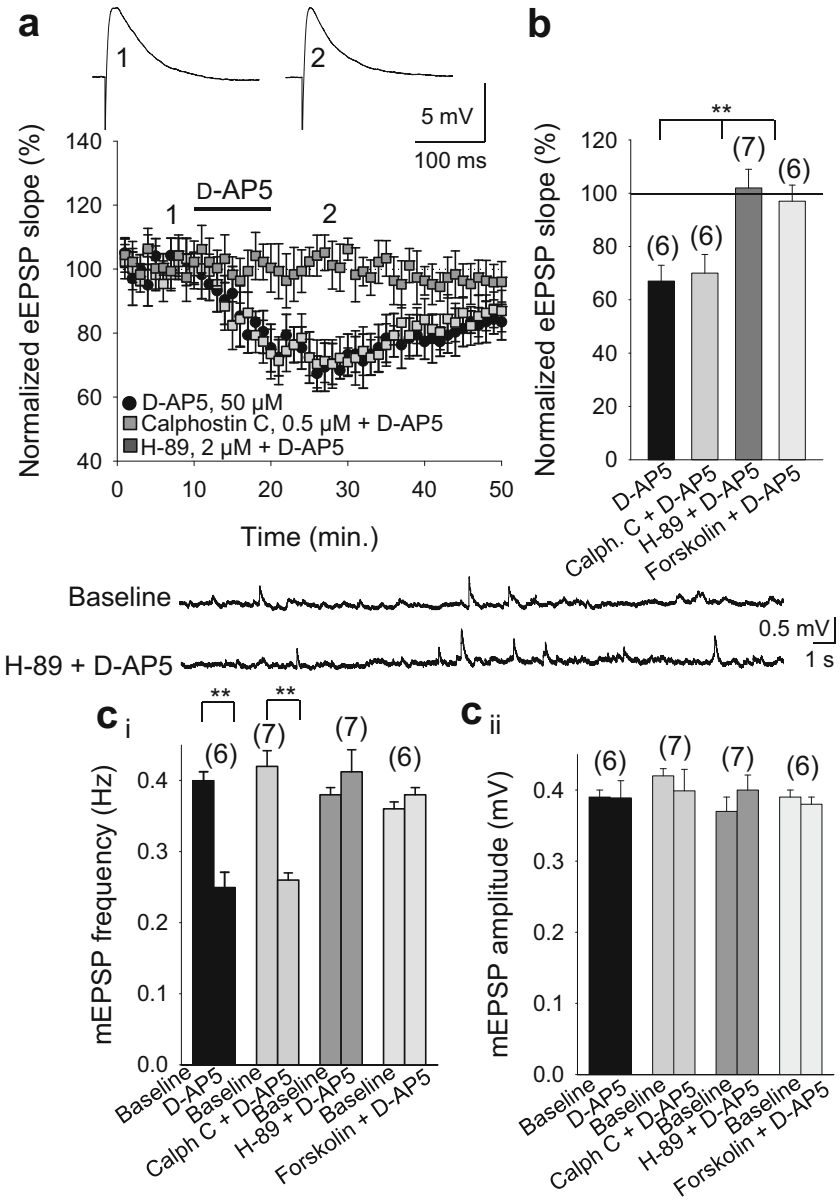

Fig. 5 The facilitation of glutamate release at CA3–CA1 synapses involve protein kinase A. **a** In slices incubated with calphostin C (0.5 μM) and with the postsynaptic neuron loaded with MK-801, the addition of D-AP5 (50 μM) decreases the slope of the evoked EPSPs. The inset shows the EPSP traces at baseline (1) and in the presence of D-AP5 (2). In slices incubated with H-89 (2 μM), D-AP5 did not affect the EPSP slope. **b** Summary of the data. Note that D-AP5 effect was prevented in the presence of forskolin. **c** The miniature EPSPs (mEPSPs) monitored at baseline and after exposing neurons in slices treated with H-89 and forskolin to D-AP5 in the presence of TTX (500 nM) and with the postsynaptic neuron loaded with MK-801 (1 mM). $\mathbf{c}_i$,$\mathbf{c}_{ii}$ Histograms showing that in H-89 and forskolin-treated slices, D-AP5 did not affect the mEPSP frequency ($\mathbf{c}_i$) or amplitude ($\mathbf{c}_{ii}$). The error bars indicate the S.E.M., and the number of slices is shown in parentheses: ** $p <$ 0.01, unpaired Student's *t* test

evoked and spontaneous release. The effect of Ro 25-6981 and PPDA on evoked and spontaneous release in slices was prevented by H-89 to a similar extent as H-89 prevented D-AP5 effect (Ro 25-6981: evoked: 70 ± 5% in calphostin C, $n = 7$; 104 ± 8%, $n = 6$ in H-89 vs 66 ± 6%, $n = 8$ in non-treated interleaved slices, Fig. 6a,b; spontaneous release, frequency: baseline 0.38 ± 0.02 Hz, 0.22 ± 0.01 Hz in calphostin C, $n = 6$; baseline 0.37 ± 0.01, 0.42 ± 0.03 Hz, $n = 6$ in H-89 vs baseline 0.38 ± 0.01 Hz, Ro-256891 0.21 ± 0.02 Hz, $n = 6$, in non-treated slices, Fig. 6c). Spontaneous release, amplitude: baseline 0.40 ± 0.01 mV, 0.41 ± 0.03 mV in calphostin C, $n = 6$; baseline 0.40 ± 0.01 mV, 0.41 ± 0.03 mV, $n = 6$ in H-89 vs baseline 0.41 ± 0.01 mV, Ro-256891 0.39 ± 0.02 mV, $n = 6$, in non-treated slices, Fig. 6c. PPDA: evoked: 70 ± 8%, $n = 6$ in calphostin C; 98 ± 6%, $n = 6$ in H-89 vs 65 ± 7%, $n = 7$ in non-treated interleaved slices, Fig. 6d,e; spontaneous release, frequency: baseline 0.40 ± 0.01 Hz, 0.24 ± 0.02 Hz in calphostin C, $n = 7$; baseline 0.39 ± 0.01 Hz, 0.41 ± 0.02 Hz, $n = 6$ in H-89 vs baseline 0.42 ± 0.01, PPDA 0.25 ± 0.02 Hz, $n = 6$, in non-treated slices, Fig. 6c. Spontaneous release, amplitude: baseline 0.39 ± 0.01 mV, 0.40 ± 0.02 mV in calphostin C, $n = 7$; baseline 0.41 ± 0.01 mV, 0.41 ± 0.02 mV, $n = 6$ in H-89 vs baseline 0.38 ± 0.01 mV, PPDA 0.41 ± 0.01 mV, $n = 6$, in non-treated slices, Fig. 6f).

These results indicated that preNMDARs, containing GluN2B and/or GluN2C/2D subunits, mediate a facilitation of glutamate release, which involves PKA but not PKC activation at hippocampal CA3–CA1 synapses.

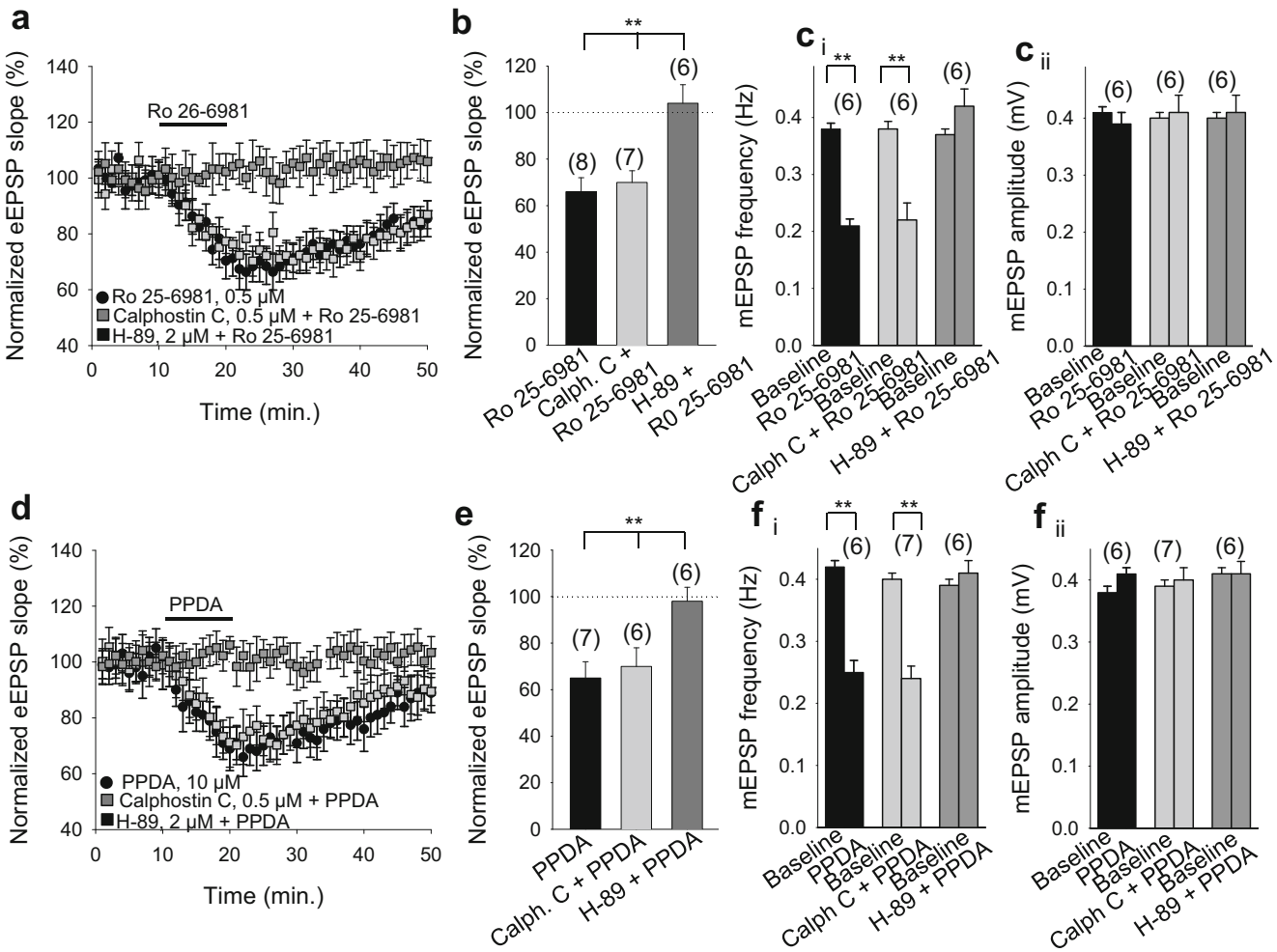

Fig. 6 The facilitation of glutamate release at CA3–CA1 synapses involves GluN2B, and GluN2CD NMDARs mediated activation of protein kinase A. **a** In slices incubated with calphostin C (0.5 μM) and with the postsynaptic neuron loaded with MK-801, the addition of Ro 25-6981 (0.5 μM) decreased the slope of evoked EPSPs. In slices incubated with H-89 (2 μM), Ro 25-6981 did not affect the EPSP slope. **b** Summary of data. $\mathbf{c}_i$,$\mathbf{c}_{ii}$ Histograms showing that in H-89-treated slices, Ro 25-6981 did not affect the mEPSP frequency ($\mathbf{c}_i$) or amplitude ($\mathbf{c}_{ii}$). **d** In slices incubated with calphostin C (0.5 μM) and with the postsynaptic neuron loaded with MK-801, the addition of PPDA (10 μM) decreased the slope of evoked EPSPs. In slices incubated with H-89 (2 μM), PPDA did not affect the EPSP slope. **e** Summary of the data. ($\mathbf{f}_i$,$\mathbf{f}_{ii}$) Histograms showing that in H-89 treated slices, PPDA did not affect the mEPSP frequency ($\mathbf{f}_i$) or amplitude ($\mathbf{f}_{ii}$). The error bars indicate the S.E.M., and the number of slices is shown in parentheses: ** $p < 0.01$, unpaired Student's t test

Discussion

Tonically Active PreNMDARs Increase the Evoked and Spontaneous Glutamate Release at CA3–CA1 Synapses

There is evidence that preNMDARs can physiologically modulate transmitter release by acting as autoreceptors at different synapses [10, 13, 14]. Indeed, here, we have found that D-AP5 can decrease glutamate release even when postsynaptic or possible astrocytic NMDARs are blocked, confirming the tonic activity of probably preNMDARs in the hippocampus and their role as autoreceptors at CA3–CA1 synapses. Moreover, this tonic activity of preNMDARs was observed at P13–P21, coincident with a critical period of plasticity.

It is known that synapses frequently show a high probability of neurotransmitter release early in development, as witnessed in the somatosensory [36], auditory [37], visual [38], and prefrontal [39] cortices. PreNMDARs are also thought to be tonically active during development at some synapses in the entorhinal [40], visual [17], and somatosensory cortices [20]. We now extend these results to the CA3–CA1 synapses of the hippocampus. Importantly, it was recently suggested that evoked and spontaneous release are mediated by different NMDARs in the cortex [41]. However, the results we present here indicate that this is not the case in the hippocampus,

where the same NMDARs appear to modulate evoked and spontaneous release.

The Subunit Composition of Tonically Activated NMDARs Involved in the Facilitation of Glutamate Release

The presence of different subpopulations of NMDARs in distinct brain regions has led to the suggestion that different subtypes fulfill different roles in the brain [42]. Using subunit-specific pharmacological agents, it was possible to dissociate different forms of plasticity in the hippocampus, with LTP being dependent on receptors containing GluN2A but not GluN2B subunits and LTD requiring receptors containing GluN2B but not GluN2A subunits [43]. This type of receptor specificity was also evident in the perirhinal cortex [44], although this situation has since been shown to be more complex in both these regions with postsynaptic and presynaptic NMDARs possibly fulfilling different roles [45–48]. Here, we used different antagonists to determine the subunit composition of the NMDARs [49]. While the GluN2A antagonist Zn^{2+} did not affect evoked or spontaneous release at hippocampal CA3–CA1 synapses, the antagonists Ro 25-6981 and PPDA that preferentially act on GluN2B, and on GluN2C and GluN2D subunits, completely prevented tonic activation and the facilitation of evoked or spontaneous glutamate release. GluN2C/D subunits are expressed postnatally in the hippocampus [50] and while PPDA does not distinguish between the activation of NMDARs containing GluN2C or GluN2D subunits, there are no other antagonists suitable to distinguish between these subunits at present. As such, either the GluN2C or GluN2D subunits could be involved in these processes. Interestingly, the fact that Ro 25-6981 and PPDA do not have an additive effect but rather, that they produce a similar effect as D-AP5 alone, suggests that preNMDARs contain GluN2B and GluN2C/D subunits, and that they are possibly heterotrimers.

Adenylate Cyclase/cAMP/Protein Kinase A Activity Is Necessary for the Increase in Glutamate Release Mediated by NMDARs Modulating Glutamate Release

Protein kinases and phosphatases appear to be required for several effects of preNMDARs in the neocortex [33, 51] and hippocampus [11]. In the visual cortex, PKC appears to be necessary to facilitate glutamate release [33], yet this does not appear to be the case in the hippocampus. PKA has been implicated in the facilitation of glutamate release and LTP [34], which led us to test its possible involvement in the tonic activation of NMDARs and in the facilitation of glutamate release observed here. In the presence of an inhibitor of the PKA catalytic subunit, the effect of D-AP5 on evoked and spontaneous release was impaired, indicating that the tonic activation and facilitation of glutamate release require PKA activation. This result was also confirmed using Ro 25-6981 and PPDA, indicating that PKA is part of the intracellular cascade responsible for the increase in glutamate release at CA3–CA1 synapses. Similarly, and congruently, direct activation of AC by preincubation with forskolin, produced refractoriness of the facilitatory effect of tonically active NMDARs. Collectively, these results consistently suggest that AC/cAMP/PKA signaling underpins the observed modulation of synaptic transmission by preNMDARs at CA3–CA1 synapses.

Presynaptic, Ionotropic NMDARs in the Hippocampus

The data obtained here suggest that the tonic activation of NMDARs is mediated by preNMDARs in the hippocampus [4], as ionotropic NMDARs are involved but not postsynaptic or astrocytic NMDARs (postsynaptic or astroglial MK-801 do not block the effects of D-AP5). In addition, the effect is ionotropic as M-801 in the bath has a similar effect as D-AP5. Indeed, synaptic plasticity requires preNMDAR activity in the somatosensory cortex [8, 16–19, 22, 52]. While we aimed to define the subunit composition of the NMDARs involved in the tonic activation and enhanced glutamate release in the hippocampus, and to provide insights into the mechanisms facilitating glutamate release, the exact location of the NMDARs involved in tonic hippocampal activation has not yet been demonstrated. Future studies of paired recordings at CA3–CA1 synapses that include the administration of MK-801 to presynaptic as well as postsynaptic neurons will unequivocally demonstrate the nature and location of these NMDARs. The intracellular release of caged MK-801 could more precisely identify the location of these receptors in cells, as shown in layer 4–layer 2/3 synapses in the somatosensory cortex [23, 53].

The presence of preNMDARs in the hippocampus was suggested by experiments monitoring noradrenaline release by synaptosomes [54–56], and immuno-electron microscopy demonstrated NMDAR labelling of presynaptic elements in the hippocampus [14, 57, 58]. Physiological roles for presynaptic NMDARs have also been proposed, serving as autoreceptors to modulate transmitter release [8, 13, 14]. PreNMDARs may also be involved in spike timing-dependent plasticity in the visual [16–18] and somatosensory cortices [19–21], with direct evidence obtained for layer 4–layer 2/3 neurons in the somatosensory cortex [22, 23]. In the hippocampus, preNMDARs have been proposed to participate in the induction of LTP [10] and LTD [11], yet further studies will be necessary to elucidate their precise

location and physiological roles at hippocampal CA3–CA1 synapses.

What Is the Physiological Role of the Tonic Facilitation of Glutamate Release?

PreNMDARs may serve to maintain high probabilities of glutamate release, as observed generally during early development. This high probability of transmitter release may be necessary for terminals to establish connections with postsynaptic cells and to maintain neurotransmission when such postsynaptic neurons are still not fully developed [59]. We show here that likely preNMDARs can be activated by evoked and spontaneous glutamate release, indicating that ambient glutamate activates preNMDARs at these synapses during early developmental stages. This study focused on synapses at P13–P21, a critical period of development and plasticity. Hence, the high probability of release might be necessary for the establishment of correct synapses and to mediate different forms of plasticity during development.

In summary, we show here that probably preNMDARs are tonically active at hippocampal CA3–CA1 synapses and that they mediate an increase in the evoked and spontaneous glutamate release. These receptors are composed of GluN2B and GluN2C/D subunits, and they require PKA activity to drive the increase in glutamate release observed.

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Author Contributions J.P.-M., M.P.-R., and Y.A.-T. performed the electrophysiological experiments and analyzed the data. A.R.-M. conceived the study and wrote the manuscript. All the authors have read and approved the final version of the manuscript submitted.

Compliance with Ethical Standards

Competing Financial Interests The authors declare that they have no competing financial interests.

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