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# Astrocyte Calcium Signaling Shifts the Polarity of Presynaptic Plasticity

Mathieu Letellier<sup>1\*</sup> and Yukiko Goda<sup>2\*</sup>

<sup>1</sup> University of Bordeaux, CNRS, Interdisciplinary Institute for Neuroscience, Bordeaux, France

<sup>2</sup> Okinawa Institute of Science and Technology Graduate University, Tancha, Onna-son, Okinawa, Japan

**Abstract**—Astrocytes have been increasingly acknowledged to play active roles in regulating synaptic transmission and plasticity. Through a variety of metabotropic and ionotropic receptors expressed on their surface, astrocytes detect extracellular neurotransmitters, and in turn, release gliotransmitters to modify synaptic strength, while they can also alter neuronal membrane excitability by modulating extracellular ionic milieu. Given the seemingly large repertoire of synaptic modulation, when, where and how astrocytes interact with synapses remain to be fully understood. Previously, we have identified a role for astrocyte NMDA receptor and L-VGCCs signaling in heterosynaptic presynaptic plasticity and promoting the heterogeneity of presynaptic strengths at hippocampal synapses. Here, we have sought to further clarify the mode by which astrocytes regulate presynaptic plasticity by exploiting a reduced culture system to globally evoke NMDA receptor-dependent presynaptic plasticity. Recording from a postsynaptic neuron intracellularly loaded with BAPTA, briefly bath applying NMDA and glycine induces a stable decrease in the rate of spontaneous glutamate release, which requires the presence of astrocytes and the activation of A1 adenosine receptors. Upon preventing astrocyte calcium signaling or blocking L-VGCCs, NMDA + glycine application triggers an increase, rather than a decrease, in the rate of spontaneous glutamate release, thereby shifting the presynaptic plasticity to promote an increase in strength. Our findings point to a crucial and surprising role of astrocytes in controlling the polarity of NMDA receptor and adenosine-dependent presynaptic plasticity. Such a pivotal mechanism unveils the power of astrocytes in regulating computations performed by neural circuits and is expected to profoundly impact cognitive processes. © 2023 The Author(s). Published by Elsevier Ltd on behalf of IBRO. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

**Key words:** astrocyte, NMDA receptor, mEPSC frequency, chemLTD, L-VGCC, A1 adenosine receptor.

## INTRODUCTION

Astrocytes, a major glial cell type in the brain, have many roles from providing structural and metabolic support to neurons, to actively shaping synaptic connectivity and function with behavioral consequences (Dityatev and Rusakov, 2011; Araque et al., 2014; Allen and Eroglu, 2017; Durkee et al., 2019; Nagai et al., 2021; Koizumi, 2022). Recent studies highlight diversity of astrocytes across various brain regions (Hoffer et al., 2015; Farmer et al., 2016; Miller, 2018; Batiuk et al., 2020; Bayraktar et al., 2020; Endo et al., 2022). Accordingly, astrocyte

functions are likely tuned to local circuits they serve (Khakh and Sofroniew, 2015; Nagai et al., 2021), while there may also be conserved mechanisms of astrocyte interaction with synapses that are shared across different brain regions (see for example, Saint-Martin and Goda, 2022).

In the mature brain, astrocytes form non-overlapping domains where they are found to tile the synaptic neuropil. Moreover, astrocytes have highly ramified processes, and through such finely branched processes, a single astrocyte can interact with over 100,000 synapses in a mouse brain or more than a million synapses in a human brain (Ventura and Harris, 1999; Bushong et al., 2002). Astrocytes have been shown to regulate synaptic transmission and plasticity (Singh and Abraham, 2017; Kofuji and Araque, 2021; Di Castro and Volterra, 2022). In response to specific neuronal activity patterns, over seconds to minutes time scales, astrocytes can alter presynaptic release positively or negatively via release of glutamate or through ATP/adenosine signaling (e.g. Covelos and Araque, 2018). In addition, astrocytes are capable of rapidly facilitating

\*Corresponding authors.

E-mail addresses: [mathieu.letellier@u-bordeaux.fr](mailto:mathieu.letellier@u-bordeaux.fr) (M. Letellier), [yukiko.goda@oist.jp](mailto:yukiko.goda@oist.jp) (Y. Goda).

**Abbreviation:** L-VGCC, L-type Voltage-gated calcium channel; NMDA, N-methyl-D-aspartate; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; HEPEs, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TTX, tetrodotoxin; D-AP5, D-2-amino-5-phosphonopentanoate; FAC, Fluoroacetate; CCPA, 2-chloro-N6-cyclopentyladenosine; mEPSC, miniature excitatory postsynaptic currents; CA, Cornu Ammonis.

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synaptic transmission over sub-second time scales by promoting multivesicular release (Myeong and Klyachko, 2022). Gliotransmitter release also targets postsynaptic strengths, such as D-serine that promotes synaptic plasticity through its action as an NMDA receptor co-agonist (Panatier et al., 2006). The full repertoire of synapse regulation by astrocytes remains to be established, especially in consideration of the diversity of astrocytes and their possible specialization for individual local circuits. Astrocytes do not generate action potentials, and intracellular calcium signaling is thought to play a major means by which astrocytes communicate with neuronal circuits into which they are embedded in (Araque et al., 2014). However, astrocyte calcium signals alone can be very diverse (Volterra et al., 2014). At one extreme, calcium increases can be highly localized to fine processes that are adjacent to synapses (Di Castro et al., 2011; Panatier et al., 2011; Arizono et al., 2020), and at another extreme, astrocyte calcium signals can be global, spreading through the astrocyte syncytium that is coupled via gap junctions (Cornell-Bell et al., 1990; Dani et al., 1992; Fujii et al., 2017). The relationship between the dynamics of astrocyte calcium signals to different synaptic states have yet to be fully understood (Zheng et al., 2015). Altogether, when and where a particular synaptic modulation by astrocytes is engaged and by what mechanism warrants further studies.

We previously identified a role for astrocytes in regulating presynaptic strength in hippocampal pyramidal cell synapses (Letellier et al., 2016; Chipman et al., 2021). This astrocyte-dependent regulation required GluN2C-containing NMDA receptors and calcium signaling in astrocytes and purinergic signaling to maintain the variability of presynaptic strengths received by a dendritic tree of a pyramidal neuron. To gain further insights into the mode by which astrocytes exert presynaptic strength regulation in hippocampal neurons, we sought to take advantage of a reduced cell culture system in which a form of NMDA receptor-dependent presynaptic plasticity could be elicited globally using pharmacological manipulation. Recording miniature excitatory postsynaptic currents (mEPSCs) from postsynaptic neurons loaded with BAPTA to prevent the induction of postsynaptic plasticity, a brief bath application of NMDA and glycine resulted in a stable decrease in mEPSC frequency, suggesting a decrease in release probability. This form of synaptic depression required the presence of astrocytes and activation of A1 adenosine receptors. Strikingly, when astrocyte calcium signaling or L-type voltage gated calcium channels (L-VGCC) were blocked, NMDA stimulation triggered an increase, rather than a decrease, in the rate of mEPSCs suggesting a shift from presynaptic depression to potentiation. Curiously, whereas A1 adenosine receptor agonist application also caused a stable decrease in mEPSC frequency, prior block of L-VGCCs no longer shifted the polarity of the mEPSC frequency change induced by the A1 receptor agonist. Altogether, our findings suggest that astrocyte calcium signaling and L-VGCCs gate NMDA receptor triggered plasticity to favor A1 adenosine receptor-dependent

presynaptic depression. More generally, our results underscore a remarkable property of astrocytes where they can generate divergent functional outputs from a same stimulus depending on their own physiological state.

## EXPERIMENTAL PROCEDURES

### Animals

Animal care and use protocols were approved by the UK Home Office and RIKEN Animal Experiment Committee, and all animal experiments were performed in accordance with the institutional regulations and guidelines.

### Cultures

For mixed cultures where neurons grow in contact with astrocytes, hippocampal neurons were obtained from P0–P1 Sprague Dawley rats and plated at low density onto an astrocyte monolayer maintained in Neurobasal-based culture media supplemented with B27 (ThermoFisher Scientific) (Morales et al., 2000; Letellier et al., 2016, 2019). For preparing cultures in which neurons do not grow in contact with glial cells (Kaeck and Banker, 2006), the neurons were obtained from E18 Sprague Dawley rat embryos and plated onto 18 mm glass coverslips at low density in Minimal Essential Medium (ThermoFisher Scientific) supplemented with 10% Horse serum. Three hours after plating, coverslips were flipped and suspended above 60 mm dishes containing DIV15 rat hippocampal glial cells cultured in Neurobasal-based culture medium supplemented with B27. Mixed or neuronal cultures were used for electrophysiological recordings at DIV9–14.

### Electrophysiology in hippocampal cultures

Whole cell patch-clamp recordings of miniature EPSCs (mEPSCs) were carried out from cultures placed on the stage of an Olympus IX71 inverted microscope at room temperature and using Axopatch 200B and multiclamp amplifiers (Axon Instruments). The recording chamber was continuously perfused with an artificial cerebrospinal fluid (aCSF) containing (in mM) 130 NaCl, 2.5 KCl, 2.2 CaCl<sub>2</sub>, 1.5 MgCl<sub>2</sub>, 10 D-glucose, 10 HEPES, 0.1 picrotoxin (pH 7.35, osmolarity adjusted to 290 mOsm). 0.5 μM TTX was added to block neurotransmitter release coupled to action potentials.

The micropipettes were made from borosilicate glass capillaries, with a resistance in the range of 3–5 mΩ. The intracellular solution contained (in mM) 100 Kgluconate, 17 KCl, 5 NaCl, 5 MgCl<sub>2</sub>, 10 HEPES, 0.5 EGTA, 4 ATPK<sub>2</sub>, 0.5 GTPNa (pH 7.3, osmolarity adjusted to 280 mOsm); 30 mM BAPTA was also included to prevent the induction of postsynaptic plasticity. Recorded neurons were held under V-clamp at –80 mV and series resistance was left uncompensated. Only cells with stable resting potential < –50 mV and series resistance < 20 mΩ were analyzed.

Recorded events were filtered at 2 kHz and sampled at 10 kHz using the pClamp software (Axon Instruments). Amplitude and frequency of the events were analyzed using mini analysis software (Synaptosoft). The detection threshold was set at  $-6$  pA. Cells with a noisy or unstable baseline were discarded. Analyzed traces were visually inspected to eliminate false events.

### Electrophysiology in hippocampal slices

Transverse hippocampal slices were obtained from young (P14–P21) male Sprague Dawley rats. Rats were anesthetized with isoflurane and decapitated. The brain was rapidly removed and placed in ice-cold dissection buffer containing (in mM): 252 sucrose, 3 KCl, 7 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 1.25NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 25 glucose (Lipton et al., 1995). The cerebellum was removed with a 20–30° angle between dorso-ventral and caudo-rostral directions and the resulting flat cut surface was glued onto a pre-cooled vibratome plate. 400  $\mu$ m sections were collected in the ice-cold dissection buffer using a Pelco Vibratome Series 1000 and maintained at 32 °C for 1–2 h in artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 2.5 KCl, 26 NaHCO<sub>3</sub>, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, and 25 glucose. Dissection buffer and aCSF were bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub>.

Whole-cell patch-clamp recordings were carried out from CA1 neurons in acute hippocampal slices placed on the stage of an Olympus BX51 upright microscope at room temperature and using Axopatch 200B and multiclamp amplifiers (Axon Instruments). The recording chamber was continuously perfused with aCSF (above for slices) containing 0.5  $\mu$ M TTX and bubbled with 95% O<sub>2</sub>/5% CO<sub>2</sub>. The internal solution was the same as for cultured neurons, and mEPSCs were recorded as described for cultures.

### Statistics

For normally distributed data (as determined by the d'Agostino-Pearson normality test), differences were tested using the paired or unpaired two-tailed Student's *t*-test or one way ANOVA. The Mann-Whitney test, the Wilcoxon rank test or the Kruskal-Wallis test were used when criteria for normality were not met. In figures, statistical significance is indicated by \*, for  $P < 0.05$ ; \*\*, for  $P < 0.01$ ; \*\*\*, for  $P < 0.001$  and \*\*\*\* for  $P < 0.0001$ . Graphpad Prism software was used for statistical analysis. Data were expressed as mean  $\pm$  SEM.

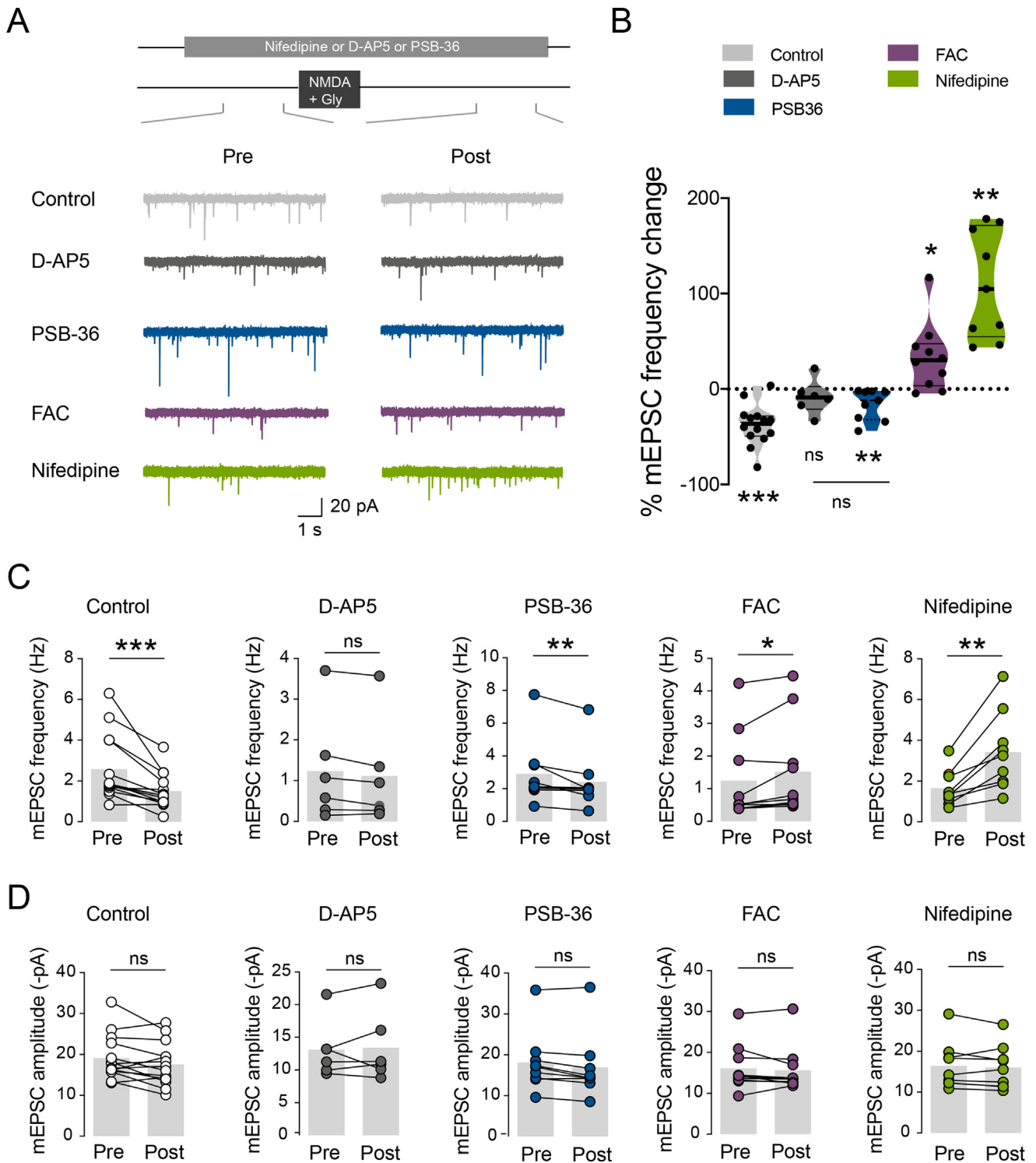
## RESULTS

To study the mechanism of presynaptic strength regulation by astrocytes that was NMDA receptor-dependent, we were motivated by two convergent sets of previous observations. First, cumulating studies pointed to a role for astrocytes in various forms of long-term synaptic depression (reviewed by Durkee et al., 2021). Second, strong pharmacological activation of NMDA receptors could trigger both astrocyte depolariza-

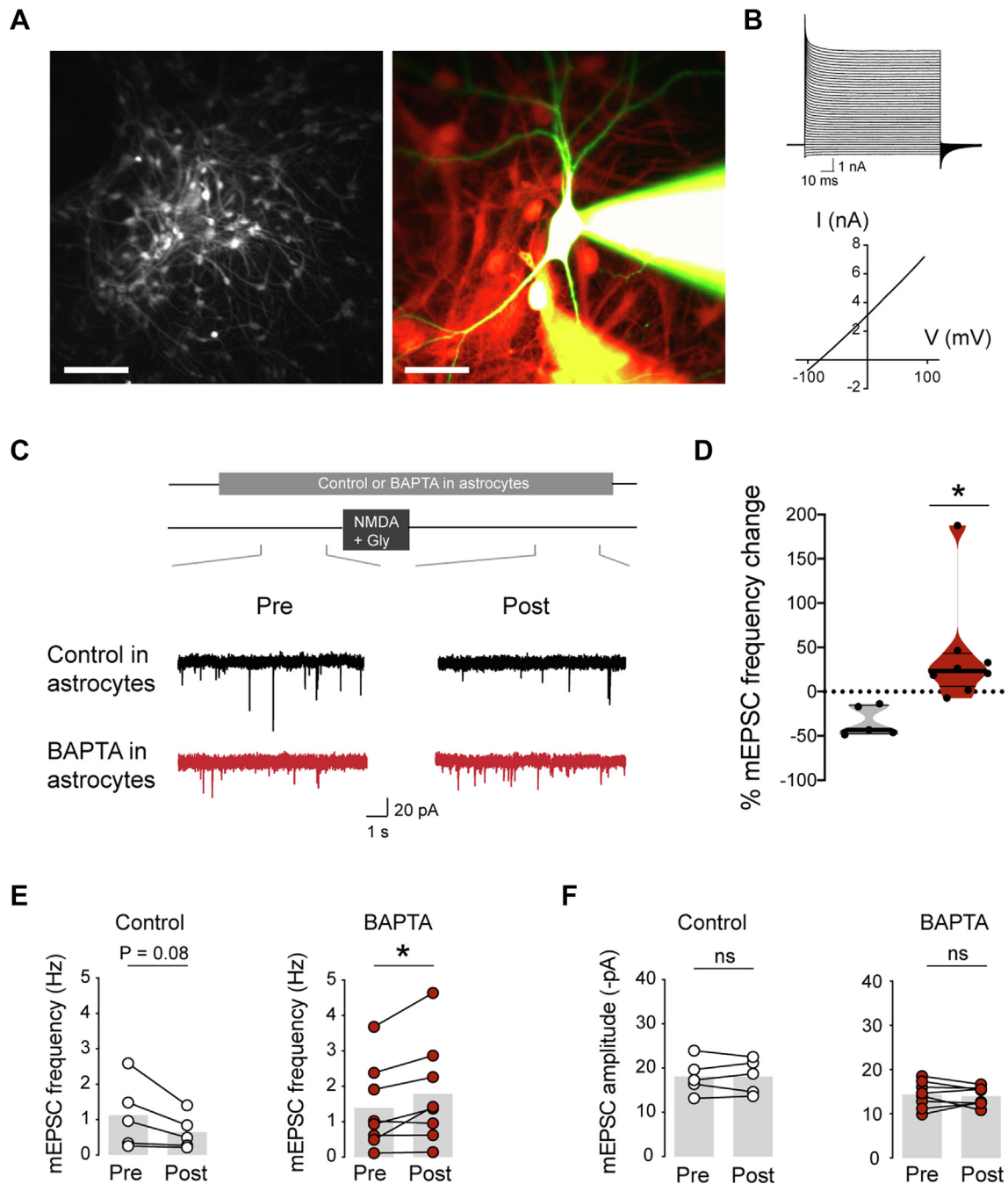
tion and intracellular Ca<sup>2+</sup> rise (Serrano et al., 2008; Letellier et al., 2016) along with presynaptic depression in hippocampal cultures and slices (Manzoni et al., 1994; Carroll et al., 1999; Serrano et al., 2006). In particular, dissociated hippocampal cultures offer a reduced system for examining molecular processes underlying synaptic strength regulation, in which changes in mEPSC frequency and amplitude indicate alterations in presynaptic release and postsynaptic receptor abundance, respectively. We therefore used dissociated hippocampal cultures to test if the pharmacologically induced NMDA receptor-dependent presynaptic depression could be also modulated by astrocyte activity. In order to isolate presynaptic plasticity, we recorded from a neuron infused with BAPTA to exclude postsynaptic NMDA receptor-mediated Ca<sup>2+</sup>-signaling. Bath applying NMDA and glycine (20  $\mu$ M each, “NMDA + Gly”) for 3 min rapidly decreased the frequency but not the amplitude of mEPSCs (Fig. 1, “untreated” group), suggestive of presynaptic depression. This decrease in mEPSC frequency was prevented in the presence of 50  $\mu$ M D-AP5 (Fig. 1, “D-AP5”), which supported the dependence of mEPSC frequency change on NMDA receptor activation.

After confirming the induction of NMDA receptor-dependent depression of presynaptic release in our hands, we then pharmacologically tested the contribution of astrocytes, purinergic signaling and L-VGCCs to this form of plasticity. Prior treatment of cultures with fluoroacetate (FAC, 5 mM for 30 min), an inhibitor of astrocyte metabolism (Zhang et al., 2003; Pougnet et al., 2014), not only attenuated the decrease in mEPSC frequency induced by NMDA + Gly application but instead increased mEPSC frequency without a change in mEPSC amplitude (Fig. 1, “FAC” group). In contrast, inhibiting A1 adenosine receptor with PSB-36 (10  $\mu$ M) partially inhibited the mEPSC frequency decrease, whose extent depression was not significantly different when compared to D-AP5 treatment group; mEPSC amplitude remained unaltered in the presence of PSB-36 (Fig. 1, “PSB-36” group). In the presence of nifedipine, a dihydropyridine L-VGCC blocker (10  $\mu$ M), NMDA + Gly stimulation caused an increase in mEPSC frequency without a change in mEPSC amplitude, similarly to FAC (Fig. 1, “nifedipine” group). These findings suggest that whereas A1 adenosine receptors are required for NMDA-induced presynaptic depression, compromising astrocyte metabolism and L-VGCCs shifts the polarity of NMDA-dependent plasticity.

We then wondered if following NMDA receptor activation, calcium-signaling in astrocytes played a role in the presynaptic depression. To directly test this possibility, we infused BAPTA into an astrocyte via a patch electrode; the internal solution also contained an Alexa dye to visualize the extent of gap junctional coupling between astrocytes (Fig. 2A, B). Simultaneously recording from a neuron whose dendritic arbor was in contact with the dye-labeled astrocyte syncytium infused with BAPTA, NMDA + Gly application produced an increase rather than a decrease in mEPSC frequency without a change in mEPSC



**Fig. 1. Brief activation of NMDA receptors stably reduces mEPSC frequency that is regulated by astrocytes, L-VGCCs, and A1 adenosine receptors** (A) Representative miniature excitatory postsynaptic current (mEPSC) traces before and after (> 15 min) NMDA and glycine treatment for 3 min (20  $\mu$ M each, "NMDA + Gly") in control or in the presence of indicated compounds. (B) Summary of the extent change in mEPSC frequency following NMDA + Gly application in control or with D-AP5 (50  $\mu$ M), PSB-36 (10  $\mu$ M), fluoroacetate (FAC, 5 mM for 30 min) and nifedipine (10  $\mu$ M) ( $n = 14, 6, 9, 10$  and 9 cells, respectively). The decrease in mEPSC frequency triggered by NMDA + Gly is reversed in the presence of nifedipine or after pre-treating the cultures with FAC for 30 min. (C, D) The effects of pharmacological inhibitors on mEPSC frequency (C) and amplitude (D) before and after NMDA + Gly stimulation for individual recordings summarized in B. Data were evaluated using a paired, two-tailed Student's  $t$  test or the Wilcoxon signed rank test. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , ns, not significant.



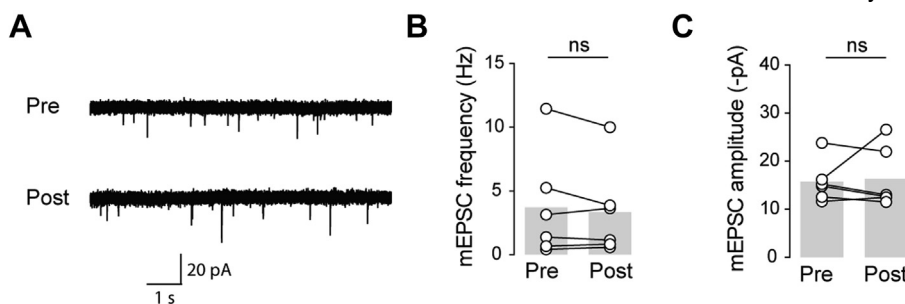
**Fig. 2. Blocking astrocyte calcium signaling shifts the polarity of NMDA receptor triggered plasticity of mEPSC frequency from depression to potentiation (A)** Fluorescence images of an example experiment showing (left) the intracellular spread of the dye across the astrocyte syncytium and (right) at higher magnification, a recorded neuron (green) on BAPTA-filled astrocytes (red). Scale bars = 120  $\mu\text{m}$  and 40  $\mu\text{m}$ . **(B)** The voltage clamp recording from an astrocyte shows characteristic linear current–voltage relationship. **(C)** Representative mEPSC traces before and after NMDA + Gly treatment from neurons on astrocytes filled with control (black) or BAPTA-containing (red) internal solution. **(D)** Summary of the extent change in mEPSC frequency following NMDA + Gly treatment in neurons contacting control ( $n = 5$  cells) or BAPTA-filled astrocytes ( $n = 8$  cells). **(E, F)** The effects of intracellular BAPTA infusion on mEPSC frequency **(E)** and amplitude **(F)** before and after NMDA + Gly stimulation for recordings shown in D. Data were evaluated using a paired, two-tailed Student's  $t$  test or the Wilcoxon signed rank test. \* $P < 0.05$ , ns, not significant.

amplitude (Fig. 2C–F), similarly to the effect observed with FAC and L-VGCC inhibitors. Thus, calcium-signaling in astrocytes plays a key role in controlling the polarity of NMDA receptor-dependent presynaptic plasticity.

Given that postsynaptic NMDA receptor signaling is blocked in our recording conditions, we speculated that NMDA + Gly likely acted on astrocyte NMDA receptors. Indeed, our previous studies revealed an unexpected role for NMDA receptors expressed in astrocytes for

both mediating astrocyte depolarization induced by NMDA + Gly treatment and regulating presynaptic strengths (Letellier et al., 2016; Chipman et al., 2021). Alternatively, NMDA receptors in presynaptic neurons (Sjöström et al., 2003; Dore et al., 2017; Di Castro and Volterra, 2022) could mediate presynaptic depression induced by NMDA + Gly application. To clarify this point, we prepared dissociated hippocampal cultures in which neurons develop on a coverslip suspended above an astrocyte feeder layer; this allowed us to isolate neurons from astrocytes for specific manipulations (Kaeck and Banker, 2006). Recording from neurons in such a preparation, application of NMDA + Gly in the absence of astrocytes failed to induce a reduction in mEPSC frequency; mEPSC amplitude also remained unchanged (Fig. 3). Therefore, astrocytes not only modulate the polarity of NMDA-induced mEPSC frequency change, astrocytes are requisite part of the triggering of NMDA-induced presynaptic plasticity.

Next, we sought to further clarify the relationship between A1 adenosine receptors, which appeared to be required for the expression of presynaptic depression, and L-VGCCs, whose inhibition reverted the polarity of NMDA-induced presynaptic plasticity from depression to potentiation. To this end, we tested if bypassing NMDA receptor activation and directly stimulating A1 adenosine receptors by applying its agonist, CCPA (100 nM, 5 min) was sufficient to produce presynaptic depression. After CCPA application, mEPSC frequency was reduced by ~30% ( $-30.5\% \pm 7.5\%$ ) compared to the baseline (Fig. 4A–C “control” group) without a change in mEPSC amplitude (Fig. 4D). When CCPA was applied in the presence of nifedipine, a reduction in mEPSC frequency occurred to an extent similar to the reduction observed in the absence of nifedipine ( $-27.2\% \pm 5.7\%$ ) compared to the baseline (Fig. 4A–C “nifedipine” group), again without a change in mEPSC amplitude. Therefore, inhibition of L-VGCCs was no longer effective in reversing the polarity of presynaptic depression induced by direct activation of A1 adenosine receptors, which suggested that L-VGCCs could modulate the direction of plasticity by acting at a step prior to presynaptic A1 receptor activation.



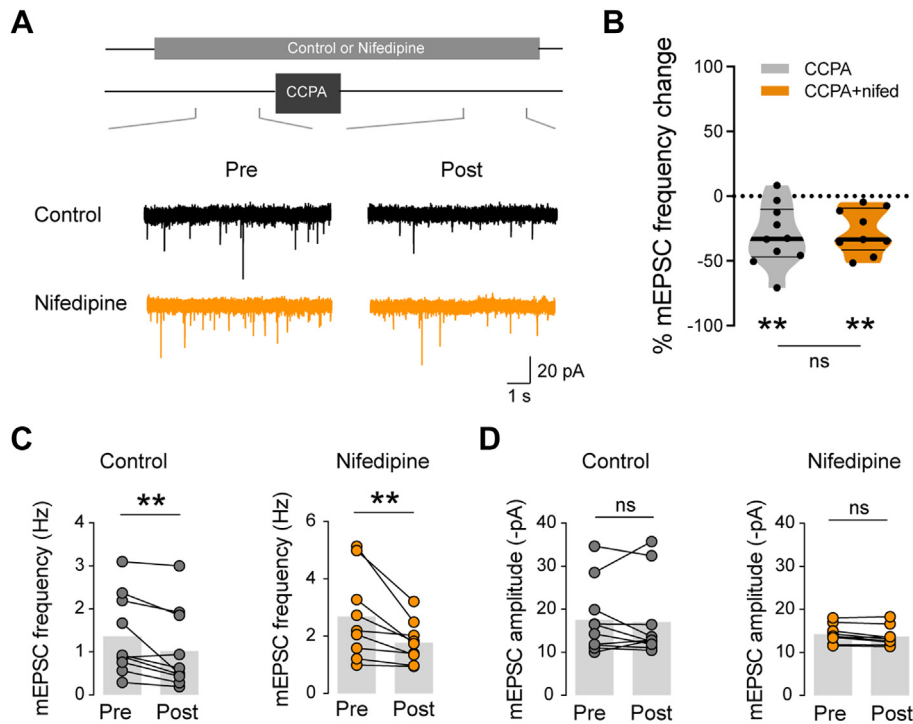
**Fig. 3. Brief activation of NMDA receptor fails to induce depression of mEPSC frequency in cultures largely devoid of astrocytes (A)** Representative mEPSC traces before and after NMDA + Gly treatment recorded in a neuron cultured under conditions that do not favor astrocyte growth. **(B)** Plots showing mEPSC amplitude (left) and frequency (right) before and after NMDA + Gly stimulation in cultures deficient in astrocytes ( $n = 6$  cells). Data were evaluated using the Wilcoxon signed rank test. ns, not significant.

Finally, we also confirmed that reversal of NMDA-triggered presynaptic depression to potentiation observed upon blockade of L-VGCCs is not a unique feature of dissociated hippocampal cultures by performing mEPSC recordings in acute hippocampal slices using the same protocol as cultured neurons. Recording from CA1 pyramidal neurons intracellularly perfused with BAPTA, bath applying NMDA + Gly triggered a reduction in mEPSC frequency without a change in mEPSC amplitude (Suppl. Fig. 1). In the presence of nifedipine, NMDA + Gly application induced an increase in mEPSC frequency which was blocked by NMDA receptor antagonist, D-AP5. Another dihydropyridine inhibitor, nimodipine, also promoted an increase in mEPSC frequency upon NMDA + Gly stimulation albeit less potently. None of the conditions affected mEPSC amplitude, which remained unchanged by the application of NMDA + Gly (Suppl. Fig. 1C).

## DISCUSSION

Dynamic changes in synaptic strength are crucial for neuronal circuit operations from computation to learning and memory. While astrocytes have been shown to modulate basal synaptic strength and synaptic plasticity, including the capacity of individual synapses to undergo synaptic plasticity and the extent of synaptic strength changes (Santello et al., 2019), the underlying mechanisms are far from fully understood. Here we have explored the basis of presynaptic modulation by astrocyte signaling using a global, chemical induction paradigm for eliciting long-term depression in dissociated hippocampal cultures. Our findings indicate a central role for astrocyte calcium and purinergic signaling for presynaptic depression of neurotransmitter release at hippocampal synapses that is consistent with prior studies (Manzoni et al., 1994; Serrano et al., 2006). Unexpectedly, however, we find that the polarity of presynaptic plasticity is gated by the activity of L-VGCCs and astrocyte calcium. Upon impairing L-VGCC or strongly buffering intracellular calcium in astrocytes, presynaptic potentiation is observed instead of depression. This raises a possibility that NMDA-triggered signaling is directed by L-VGCC and promotes rises in astrocyte calcium to release gliotransmitters,

including ATP that is subsequently metabolized into adenosine to mediate presynaptic depression (e.g. Covelo and Araque, 2018). That presynaptic depression triggered by direct activation of A1 adenosine receptors is not affected by L-VGCC inhibition suggests that L-VGCC acts upstream of the activation of A1 receptors. Considering the similar effect of buffering astrocyte calcium and blocking L-VGCCs, one possibility is that L-VGCCs directly modulate calcium signaling in astrocytes (Letellier et al., 2016).



**Fig. 4. Activation of A1 receptors promotes a stable decrease in mEPSC frequency which is unaffected by L-VGCC inhibition.** (A) Representative mEPSC traces before and after treatment with A1 receptor agonist CCPA (100 nM, 5 min) in control (black) or nifedipine (orange). (B) Summary of the extent change in mEPSC frequency after CCPA treatment in control ( $n = 10$  cells) or nifedipine ( $n = 9$  cells). (C, D) mEPSC frequency (C) and amplitude (D) before and after CCPA stimulation for individual recordings in the absence (left, untreated) or presence of nifedipine (right) for recordings shown in (B). Data were evaluated using a paired, two-tailed Student's  $t$  test or the Wilcoxon signed rank test. \*\* $P < 0.01$ , ns, not significant. Data are expressed as mean  $\pm$  SEM.

Where are NMDA receptors, whose pharmacological activation triggers presynaptic plasticity, located? While the infusion of BAPTA in the recorded neuron precludes a role for postsynaptic NMDA receptors (as supported by the lack of effect of NMDA + Gly application on mEPSC amplitudes), we have not directly tested the contribution of astrocyte versus presynaptic NMDA receptor signaling (Serrano et al., 2008; Letellier et al., 2016; Prius-Mengual et al., 2019). Yet, our results reveal that applying NMDA + Gly in the absence of astrocytes does not induce a reduction in mEPSC frequency, suggesting that the direct activation of neuronal NMDA receptors is not sufficient to mediate presynaptic depression. In contrast, compromising astrocyte calcium or metabolism strongly alters NMDA + Gly induced presynaptic plasticity. Therefore, if presynaptic NMDA receptors play any role in NMDA-triggered presynaptic depression, they would be expected to operate in parallel or act upstream of astrocyte signaling.

Recordings of mEPSCs provides a useful assay for monitoring the activity of synapses received by a given neuron without biasing activation to specific set of synapses. Given that spontaneous release of neurotransmitters shows a weak correlation to release probability (Prange and Murphy, 1999), mEPSCs detected are likely contributed by synapses that are above a certain threshold of release probability. Whether the switch in polarity observed in our culture system involves the same set of synapses contributing to mEPSC

depression remains to be determined, for instance by monitoring presynaptic strengths of individual synapses with fluorescent probes (e.g. Cook and Ryan, 2023) before and after NMDA + Gly application.

The switch in polarity of presynaptic plasticity that we observed following global NMDA receptor activation in both hippocampal cultures and acute slices is reminiscent of the shift in polarity of heterosynaptic plasticity that we observed previously in response to electrical stimulations in the same hippocampal preparations (Letellier et al., 2016). In both cases, presynaptic changes were dependent on NMDA receptor activation, astrocytes and L-VGCC activation, suggesting that this mechanism operates in response to a wide range of physiological stimuli and in various circuit configurations.

Interestingly, the critical role of astrocytes in controlling the polarity of presynaptic plasticity is evidenced during development at hippocampal CA3 to CA1 synapses, where a switch in spike-timing dependent plasticity from depression to potentiation has been recently observed (Falcón-Moya et al., 2020). Nevertheless, the similarity is limited to key players involved, and the mechanism of implementation appears to differ, including the lack of involvement of NMDA receptors in spike-timing dependent presynaptic potentiation.

Overall, our findings point to a crucial and surprising role of astrocytes in controlling the polarity of NMDA receptor and adenosine-dependent presynaptic plasticity. Such a pivotal mechanism reveals the power of astrocytes in regulating neuronal computations, and is therefore expected to profoundly impact cognitive processes, for instance by controlling the memory function of sleep which highly depends on purinergic signaling (Pascual et al., 2005; Halassa et al., 2009; Florian et al., 2011; Schmitt et al., 2012; Lalo et al., 2014). Furthermore, this mechanism may be compromised in pathological situations such as Alzheimer's disease (AD) or epilepsy where astrocyte calcium signaling is strongly altered. In particular, a pathological switch in the polarity of presynaptic plasticity, by dramatically altering glutamate release, may contribute to the aberrant neuronal activity synchronization that is observed in both AD (Kuchibhotla et al., 2009) and epilepsy (Gómez-Gonzalo et al., 2010; Carmignoto and Haydon, 2012). In this respect, our results provide new avenues to better understand the pathologies and development of new therapeutics through the specific targeting of astrocyte signaling.



## DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## APPENDIX A. SUPPLEMENTARY MATERIAL

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