CELLULAR AND MOLECULAR MECHANISMS OF SYNAPTIC PLASTICITY AT HIPPOCAMPAL AND CORTICAL SYNAPSES

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CELLULAR AND MOLECULAR MECHANISMS OF SYNAPTIC PLASTICITY AT HIPPOCAMPAL AND CORTICAL SYNAPSES

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Editorial: Cellular and molecular mechanisms of synaptic plasticity at hippocampal and cortical synapses

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Editorial on the Research Topic
Cellular and Molecular Mechanisms of Synaptic Plasticity at Hippocampal and Cortical Synapses

One of the most fascinating properties of the nervous system is the ability to modify its structure and function in order to adapt to the continuously changing environment. The efficiency of the communication between two neurons is modulated by the plasticity of synapses, the strengthening or weakening over time of their function in response to changes in synaptic activity. The idea that neuronal circuits could change in an activity-dependent manner was first proposed by Donald Hebb in 1949. Nowadays, plasticity processes have been identified in almost all species, in primary modalities but also in multimodal functions. It is also well-established that plastic changes can span along different timescales and are required for the correct development of the nervous system as well as for experience-dependent memory and learning. Moreover, many neurological diseases and neuropsychiatric disorders are today interpreted as alterations in plasticity mechanisms.

The main objective of this Research Topic is to collect research articles and reviews to provide new insights and explanatory models capable of accounting for the complexity of the plasticity mechanisms used by the hippocampus and cerebral cortex to modulate the strength of neuronal circuits under physiological and pathological conditions. Based on the articles and reviews detailed below, the multi-level analysis becomes relevant to support our understanding of synaptic plasticity in the brain.

Vannini et al., use different methodological approaches (a combination of photoconversion and electron microscopy to assess changes of synaptic vesicle pools

in vivo, electrophysiology and proteomics) to identify at nanoscale level alterations of homeostatic synaptic plasticity (HSP) mechanisms. They took advantage of a well-characterized model of chronic, focal epilepsy in the visual cortex of the mouse. Their data contribute to the characterization of the complex release machinery and the molecular modifications promoted by epileptic networks.

Chen et al., focus on a very relevant aspect of synaptic plasticity, which is the dynamic trafficking of AMPARs into and out of the synaptic membrane. In their work, they focus on Spastin, a microtubule-severing protein, whose mutations are considered the most common cause of hereditary spastic paraparesis. By using functional, molecular and biochemical strategies in hippocampal cultures they were able to better characterize the role of Spastin in AMPAR trafficking and advance our understanding of the synaptic plasticity and cognitive dysfunction underlying this pathology.

Cui et al., deepen the analysis at molecular level of the role of Rac1, a small GTPase of the Rho family, on the induction and maintenance of long-term potentiation (LTP) in the rodent hippocampus. By means of electrophysiological methods combined with biochemical and pharmacological approaches, they were able to show that during different stages of LTP, the activation of Rac1 can modulate different signaling pathways (activation of PKCι/λ by PI3K, and inhibition of PKMζ by LIMK), which leads to an opposing effect on the induction and maintenance of LTP in the hippocampus.

Chaloner and Cooke, based on previous research on long-lasting stimulus-selective response potentiation (SRP) in the primary mouse visual cortex, the authors explore the neocortical processes of plasticity occurring during habituation at distinct timescales. Using *in vivo* recordings and genetic manipulations, they show that cortical plasticity accompanying behavioral habituation occurs across seconds, minutes, and days of repeated stimulus experience. Moreover, they characterized the role of NMDA receptors and parvalbumin-positive interneurons in such processes, identifying a range of mechanistically separable forms of plasticity occurring at different timescales in the same learning mouse.

dos Santos Cardoso et al., focus on the analysis of the potentially beneficial effect of photobiomodulation (transcranial near-infrared laser treatment) on the aging brain. By investigating the expression and activation of distinct intracellular signaling proteins in the cerebral cortex and hippocampus of aged rats treated with the transcranial near-infrared laser, they identify that this experimental approach improves intracellular signaling pathways linked to cell survival, memory, and glucose metabolism.

Reyes-García and Escobar provide an overview of the experimental evidence supporting the relationship between long-term depression (LTD) and synaptic depotentiation with extinction in different models and summarized the established cellular and molecular mechanisms underlying this process. They discuss the role of calcineurin in the association between hebbian and HSP during new learning or re-learning processes.

Taylor and Jeans discuss the experimental evidence linking deficits in HSP with the onset and/or progression of major neurodegenerative diseases and describe the contribution of different HSP-associated proteins in neurodegeneration. By summarizing evidence obtained mainly in studies on Alzheimer, Parkinson, and Huntington disease and on amyotrophic lateral sclerosis, they suggest a distinct role of HSP in each of these major diseases associated with neurodegeneration.

Inglebert and Debanne review the relevance of physiological concentrations of extracellular Ca^{2+} in spike timing-dependent plasticity (STDP). They discuss experimental data and mathematical models that address the requirement of postsynaptic Ca^{2+} entry for the induction and/or maintenance of this form of long-term plasticity. They open the debate regarding whether synaptic plasticity rules inferred from *in vitro* studies could be applied to *in vivo* conditions and, in this sense, they question if different forms of STDP persist under physiological concentrations of Ca^{2+}. They finally sum up a variety of experimental data, obtained from different animal models exploring the rules of STDP *in vivo*.

Meza et al., present a quite exhaustive revision of the role of a particular complex channel, the Transient Receptor Potential Vanilloid 1 (TRPV-1), in the modulation of synaptic function in different brain regions. The authors emphasized the main mechanisms described for the plasticity-related role of TRPV-1 at the pre and postsynaptic level, in glial cells, in interaction with the endocannabinoid system, development, mental disorders and neurological diseases such as epilepsy, anxiety, and depression, as well in drug-addiction disorders.

Ruggiero et al., focus on the relevance of the main neurotransmitter systems (acetylcholine, dopamine, noradrenaline, serotonin, and endocannabinoids) in long and short-term synaptic plasticity in the hippocampus-prefrontal cortex (HPC-PFC) pathway. In this comprehensive review they also discuss the implications of HPC-PFC disruption in synaptic plasticity and functional connectivity and thus in neuropsychiatric disorders, such as schizophrenia, major depression and anxiety, and in Alzheimer disease.

Manuscript contribution

Neuronal plasticity is a fundamental aspect in the whole nervous system functioning. We believe that the manuscripts integrating this Research Topic contributed to a deeper characterization of the extremely complex mechanisms regulating plasticity in its different forms, in both physiological and pathological conditions.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Synaptic Vesicles Dynamics in Neocortical Epilepsy

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Neuronal hyperexcitability often results from an unbalance between excitatory and inhibitory neurotransmission, but the synaptic alterations leading to enhanced seizure propensity are only partly understood. Taking advantage of a mouse model of neocortical epilepsy, we used a combination of photoconversion and electron microscopy to assess changes in synaptic vesicles pools *in vivo*. Our analyses reveal that epileptic networks show an early onset lengthening of active zones at inhibitory synapses, together with a delayed spatial reorganization of recycled vesicles at excitatory synapses. Proteomics of synaptic content indicate that specific proteins were increased in epileptic mice. Altogether, our data reveal a complex landscape of nanoscale changes affecting the epileptic synaptic release machinery. In particular, our findings show that an altered positioning of release-competent vesicles represent a novel signature of epileptic networks.

Keywords: epilepsy, synaptic vesicles, hyperexcitability, visual cortex, visual processing, tetanus neurotoxin

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INTRODUCTION

Epilepsy is a disorder of the central nervous system that affects around 50 million people worldwide and it is characterized by recurrent spontaneous seizures, that are the clinical manifestation of an excessive hypersynchronous discharge of a population of neurons (Bromfield and Cavazos, 2006). Despite all the available treatments, one third of patient develop a drug-resistant form of epilepsy (Kwan et al., 2011; Sharma et al., 2015). The propensity to develop seizures is due to brain damage, in lesional epilepsy (Pitkänen and Immonen, 2014; Wasilewski et al., 2020; Wie Børsheim et al., 2020), or to an altered synaptic function at excitatory and/or inhibitory terminals, in non-lesional epilepsy (Wykes et al., 2012; Farisello et al., 2013; Corradini et al., 2014; Ferecskó et al., 2015; Snowball et al., 2019). Specifically, chronic epilepsy is thought to result from a synaptic reorganization that leaves permanent marks on cortical networks and may lead to network dysfunction, cognitive deficits and impaired information processing (Pitkänen et al., 2013, 2015; Holmes, 2015; Vannini et al., 2016). Epileptic networks display several modifications in synaptic function and structure at the level of presynaptic boutons, post-synaptic structures, and the glial processes enwrapping them (Bernard, 2010). In the presynaptic compartment, recent technological advances have allowed a detailed characterization of the size and spatial organization of functional vesicle pools. These parameters correlate with measures of synaptic strength and are altered following plasticity-inducing stimuli (Rey et al., 2020). However, such ultrastructural readouts of

synaptic function have not been applied thus far to the study of epileptogenic modifications or their consequences.

The correct functioning of neuronal networks requires precise modulation of excitatory and inhibitory activity (Xue et al., 2014; Rossi et al., 2017; Rubin et al., 2017; Sohal and Rubenstein, 2019). When network activity is tipped out of balance, a number of cellular processes take place to re-establish its normal function (Turrigiano, 2012). The processes underlying homeostatic plasticity can affect cellular activity and synaptic output (Wefelmeyer et al., 2016). Following a perturbation, neurons attempt to restore their baseline firing rates and dynamic range by regulating their intrinsic excitability, probability of neurotransmitter release and neurotransmitter receptor expression (Davis and Müller, 2015). Central synapses have the ability to plastically adapt to new conditions by dynamically scaling up and down their output. Such regulation has been demonstrated to occur during development, sleep and learning. However, much less is known about the mechanisms of homeostatic scaling as a consequence of a pathological, system-level perturbation *in vivo* (Turrigiano, 2008, 2012; González et al., 2019).

Here we took advantage of a well-characterized model of chronic, focal epilepsy in the visual cortex (Mainardi et al., 2012; Chang et al., 2018) to investigate synaptic changes in hyperexcitable networks. Tetanus neurotoxin (TeNT) is a metalloprotease that cleaves the synaptic protein VAMP/synaptobrevin leading to the establishment of a focal cortical hyperexcitability, with electrographic seizures that persist for several weeks after TeNT wash out (Nilsen et al., 2005; Jiruska et al., 2010; Mainardi et al., 2012; Vannini et al., 2016; Snowball et al., 2019). Despite many studies characterizing TeNT-induced epilepsy (i.e., seizures manifestation), very little is known about the persistent synaptic changes that underpin chronic, spontaneous seizures. The absence of neuronal loss and gliosis, together with the persistence of spontaneous seizures, chronically altered neural processing and structural modifications of both dendritic spines and branches suggest that synaptic modifications occur and last after TeNT clearance (Mainardi et al., 2012; Vannini et al., 2016). Here, using FM1-43FX and activity-dependent labeling of synaptic vesicles, we simultaneously investigated function and ultrastructure of both excitatory and inhibitory terminals, in acute and chronic phases of TeNT-induced epilepsy. We combined this approach with an unbiased measure of proteins content in the two phases of hyperexcitability, isolating synaptosomes at different time points after TeNT injection, and assessing by electrophysiological recordings the impact of inhibiting Carboxypeptidase E (CPE), upregulated in epileptic mice, on seizures occurrence.

MATERIALS AND METHODS

Animals and TeNT Injections

Adult (age > postnatal day 60) C57BL/6J mice used in this study were reared in a 12 h light-dark cycle, with food and water available *ad libitum*. All the experiments were performed in compliance with the EU Council Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the Italian Ministry of Health. TeNT (Lubio; Lucerne, Switzerland; 0.1–0.2 ng) or RSA (Rat Serum Albumin) solutions in PBS were intracranially injected into the primary visual cortex (i.e., 0.0 mm anteroposterior, 2.7 mm lateral to the lambda suture and at a cortical depth of 0.65 mm); of anesthetized (ketamin/Xylazine 100–10 mg/Kg) mice. After surgery, a glucose solution (5% in saline) was subcutaneously administered and recovery of animals was carefully monitored. Paracetamol was added in drinking water for 3 days. Additional details can be found in Mainardi et al. (2012); Vallone et al. (2016), and Vannini et al. (2016, 2017). No behavioral seizures are detectable in those animals, as already reported in Mainardi et al. (2012) and Vannini et al. (2016).

FM 1-43FX Injection and Visual Stimulation

Control and epileptic mice, deeply anesthetized with urethane (7 ml/kg; 20% solution in saline, i.p.; Sigma) and placed in a stereotaxic apparatus, received an injection of FM 1-43FX dye into the primary visual cortex, layers II–III (i.e., 0.0 mm anteroposterior and 2.7 mm lateral to the lambda suture, 0.7 mm depth). FM1-43FX is a non-permeable styryl dye that labels the cell membrane. Once synaptic vesicles fuse with the cell membrane, molecules of FM1-43FX diffuses laterally along the membrane previously comprising the vesicle. As vesicles undergo endocytosis, as part of their recycling, the dye is trapped inside the recently released vesicles (Marra et al., 2014). Three minutes later, animals were stimulated for 10 min with square-wave gratings (1 Hz, 0.06 c/deg, contrast 90%) and flashes of light. All visual stimuli were computer-generated on a display (Sony; 40 x 30 cm; mean luminance 15 cd/m2) by a VSG card (Cambridge Research Systems). Mice, still under anesthesia, were kept in the dark and perfused through the heart with a fresh solution of 6% glutaraldehyde, 2% formaldehyde in PBS, as described in Jensen and Harris (1989) right after the end of the visual stimulation.

Photoconversion and Electron Microscopy Analysis

All the following procedures were made in the dark. The protocol followed is described in detail in Marra et al. (2014). Briefly, embedded in EPON, slices were collected with an ultramicrotome serial sections (70 nm thickness) and placed in grids at RT. Thereafter, sections could be viewed with a transmission electron microscope fitted with a cooled CCD camera. Images were acquired using local landmarks and analyzed using Image J/Fiji (NIH) and a custom script in Python (Python.org). At ultrastructural level, target synapses (visual cortex, layers II–III) were randomly chosen and synaptic vesicles were scored based on their vesicle luminal intensity using methods outlined previously (Marra et al., 2014), image names were changed to ensure that the experimenters were blind to the experimental condition of each electron micrograph. A terminal was considered inhibitory if no spine or postsynaptic density could be observed in the middle section and in at least one of the adjacent sections. As expected (Meyer et al., 2011; Tremblay et al., 2016; van Versendaal and Levelt, 2016; Lim et al., 2018), inhibitory terminals were estimated to be 15–25% of the total.

Synaptosomes Extraction and Proteomic Analysis

Synaptosomes were extracted using a slightly modified protocol taken from Giordano et al. (2018). Visual cortices were gently homogenized in 500 µl of ice cold homogenizing buffer (0.32 M sucrose, 1 mM EDTA, 1 mg/ml BSA, 5 mM HEPES pH 7.4, proteases inhibitors) and centrifuged 10 min at 3,000 g at 4°C; supernatant was recovered and centrifuged again for 15 min at 14,000 g at 4°C. After discarding supernatant, the pelleted synaptosomes were suspended in 110 µl of Krebs-Ringer Buffer and 90 µl of Percoll (Sigma-Aldrich) were added. A 2 min spin (14,000 rpm, 4°C) was performed and enriched synaptosomes were recovered from the surface of the solution with a P1000 tip and resuspended in 1 ml of Krebs-Ringer buffer. After an additional spin of 2 min (14,000 rpm, 4°C), the supernatant was discarded and the pellet resuspended in 20 µl of RIPA buffer.

Proteomics Sample Preparation and Data Analysis

Trypsin/LysC mix Mass Spec grade was purchased from Promega (Madison, WI). Tandem Mass Tags (TMT 10-plex) kits and microBCA protein assay kit were purchased from Thermo Fisher Scientific (Rockford, IL). All other reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO). Synaptosomes proteome extracts were quantified with a micro BCA protein assay and aliquots of 3.5 µg of proteins were diluted to 40 µL of RIPA/Trifluoroethanol (TFE) 50/50. Paramagnetic beads were added to each sample and further processed following a modified SP3 protocol for ultrasensitive proteomics as previously described (Pellegrini et al., 2019). Synaptosomes proteins were reduced alkylated and digested with a mixture of trypsin/Lys-C (1:20 enzyme to protein ratio). Digested peptides were then quantified, and labeled with TMT 10-plex: samples were block randomized[1] over 5 TMT sets. Each TMT set included two normalization channels for batch corrections built pooling an aliquot from each digested synaptosome sample (Plubell et al., 2017). TMT sets underwent high pH fractionation on an AssayMap Bravo (Agilent technologies) and fractions run on a nano-LC (Easy1000 Thermo Fisher Scientific) equipped with a 50 cm EasySpray column and coupled with an Orbitrap Fusion for MS3 analysis (Thermo Fisher Scientific). Experimental details regarding sample fractionation and LC-MS/MS runs have been already reported elsewhere (Pellegrini et al., 2019). Data were analyzed using Proteome Discoverer 2.1. TMT data were normalized by internal reference scaling (Plubell et al., 2017).

Electrophysiological Recordings and Drugs Administration

Surgery was performed as described in Spalletti et al. (2017), but the small craniotomy was centered at 3 mm lateral to Lambda and performed in TeNT/RSA-injected hemisphere. Neuronal activity was recorded with a NeuroNexus Technologies 16-channel silicon probe with a single-shank (A1x16-3mm-50-177) mounted on a three-axis motorized micromanipulator and slowly lowered into the portion of visual cortex previously injected with TeNT or RSA solution. The tip of the probe was positioned at the depth of 1 mm so that the electrode contacts (spaced by 50 microns) sampled activity from all cortical layers. Before the beginning of the recording, the electrode was allowed to settle for about 10 min. Local Field Potentials (LFP) signals were acquired at 1 kHz and bandpass filtered (0.3–200 Hz) with a 16-channel Omniplex recording system (Plexon, Dallas, TX). Local Field Potentials (LFP) were computed online and referred to the ground electrode in the cerebellum. In order to verify whether interacting with Carboxypeptidase E (CPE) would change epileptic activity, we topically applied over the craniotomy 2 µL of PBS containing 25 µM of 2-guanidinoethylmercaptosuccinic acid (GEMSA; Sigma-Aldrich) without removing the electrode. Neural signals were acquired at regular time intervals up to 30 min after GEMSA delivery to verify the effect and the penetration of the drug in the cortical layers. At the end of the experiment animals were sacrificed. Data were analyzedanalysed offline with NeuroExplorer software (Plexon Inc., United States) and with custom made Python interfaces[2]. Movement artifacts were removed offline. The coastline analysis was calculated as the sum of the absolute difference between successive points (Wykes et al., 2012).

Statistical Analysis

Statistical analysis was performed with Graph Pad (version 8) except for proteomics analysis, in which we used Perseus. Normality of distributions was assessed with D'Agostino test and appropriate test was chosen accordingly.

RESULTS

Ultrastructural Investigation of Synaptic Vesicle Function in Hyperexcitable Networks

Our studies included three groups of C57BL/6 mice with injections into the primary visual cortex (V1): a control group injected with vehicle (Control), an Acute epileptic group tested 10 days after TeNT injection in V1 and a Chronic epileptic group tested 45 days after TeNT injection in V1. Synaptic vesicles from three animals in each of these groups were labeled by infusing FM1-43FX in the visual cortex while presenting a series of visual stimuli (**Figure 1A**). After fixation, photoconversion and processing for electron microscopy, we were able to label individual vesicles at excitatory (asymmetrical) and inhibitory (symmetrical) synapses (**Figure 1A**). This approach allowed us to identify individual synaptic vesicles, released and recycled in the presence of FM1-43FX, as having an electron dense lumen, while non-released vesicles present a clear lumen and a darker membrane (Marra et al., 2012). First, we investigated the length of the active zone as a readout of synaptic activity

[1] www.sealedenvelope.com

[2] http://python.org/

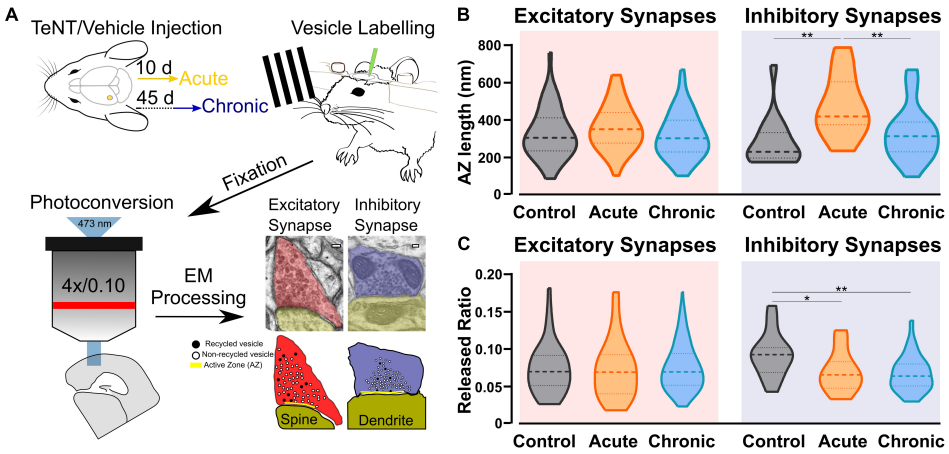

FIGURE 1 | Ultrastructural and functional changes at presynaptic terminals of TeNT-injected mice. **(A)** Diagrammatic representation of labeling protocol. Visual cortices from mice in Control, Acute, and Chronic groups were infused with FM1-43FX during visual stimulation. Brains were rapidly fixed and sliced to allow photoconversion of FM1-43FX signal before processing for electron microscopy. Individual presynaptic terminals were classified as excitatory (asymmetrical synapses, red) or inhibitory (symmetrical synapses, blue); size, position and numbers of active zone (AZ, yellow), non-released vesicles (open circles) and released vesicles (black circles) were analyzed (scale bars 100 nm). **(B)** Left: Active zone (AZ) length in Control (gray), Acute (orange), and Chronic (blue) epileptic mice at excitatory synapses; no differences between the groups (Kruskal-Wallis test, $p = 0.10$). Distribution, median and quartiles shown for each group; Control $n = 41$; Acute $n = 46$; Chronic $n = 118$. Right: Active zone (AZ) length in Control (gray), Acute (orange), and Chronic (blue) epileptic mice at inhibitory synapses (Kruskal-Wallis test, $p < 0.01$, Control vs. Acute $p < 0.01$, Control vs. Chronic $p > 0.05$, Chronic vs. Acute $p < 0.01$). Distribution, median and quartiles shown for each group; Control $n = 15$; Acute $n = 14$; Chronic $n = 29$. **(C)** Left: Released fraction of synaptic vesicles (labeled vesicles/total vesicles) in Control (gray), Acute (orange), and Chronic (blue) epileptic mice at excitatory synapses; no differences between the groups (Kruskal-Wallis test, $p = 0.67$). Distribution, median, and quartiles shown for each group; Control $n = 47$; Acute $n = 57$; Chronic $n = 118$. Right: Released fraction of synaptic vesicles of Control (gray), Acute (orange), and Chronic (blue) epileptic mice at inhibitory synapses (Kruskal-Wallis test, $p < 0.05$, Control vs. Acute $p < 0.05$, Control vs. Chronic $p < 0.01$, Chronic vs. Acute $p > 0.05$). Distribution, median, and quartiles shown for each group; Control $n = 16$; Acute $n = 16$; Chronic $n = 30$.

independent of our labeling protocol (Harris and Weinberg, 2012). Surprisingly, we found an increase in the length of inhibitory synapses' active zone in the acute phase (**Figure 1B**), normally associated with increased release. However, the released fraction of inhibitory vesicles (number of released vesicles over total number of vesicles) is reduced in animals injected with TeNT (**Figure 1C**), shown to preferentially impair inhibitory release (Schiavo et al., 2000). No differences in active zone length and released fraction of vesicles were found at excitatory synapses (**Figures 1B,C**). Interestingly, the total number of vesicles across control and epileptic groups does not change significantly neither at excitatory (Control: mean = 52.16, *SD* = 35.47, *n* = 49; Acute: mean = 56.23, *SD* = 29.81, *n* = 59; Chronic: mean = 51.28, *SD* = 19.39, *n* = 124; data not shown) nor inhibitory synapses (Control: mean = 40.76, *SD* = 14.38, *n* = 17; Acute: mean = 54.33, *SD* = 36.12, *n* = 18; Chronic: mean = 54.33, *SD* = 19.91, *n* = 30; data not shown).

Changes in Docking and Positioning of Activated Vesicles at Excitatory Synapses in Chronic Epilepsy

After quantifying direct and indirect measures of vesicular release, we examined the spatial distribution of released and non-released vesicles within presynaptic terminals. We started by analysing the released fraction in the docked and non-docked populations of vesicles. As described before for excitatory synapses (Marra et al., 2012), the Control group showed a higher released fraction in the docked population, similar results were found in the Acute group. Conversely, in the Chronic group the released fraction was higher in the non-docked population at excitatory synapses (**Figure 2A**). We also report that inhibitory synapses have a higher released fraction in the docked population, which does not seem to be affected by the induction of epilepsy (**Figure 2A**). To gain insight on the effect observed at excitatory synapses of the Chronic group, we compared the distance of released and non-released vesicles from the active zone (**Figure 2B**). We reasoned that if the effect is specific to the ability of released vesicles to dock, their position within the terminal should not be affected. We examined the cumulative fraction of the distance of released and non-released vesicles from their closest point on the active zone. At excitatory synapses, in Control and Acute groups the released vesicle population are closer to the active zone compared to the non-released population. However, in the Chronic group released excitatory vesicles do not show a spatial bias toward the active zone, that was instead observed in the other groups (**Figure 2B**). At inhibitory synapses, the distances of vesicular populations to the active zone has a different pattern, with no difference between released and non-released population in Control and Acute groups and with a spatial bias of released vesicles toward the active zone in the Chronic group (**Figure 2B**). As a visual representation of the distribution of released vesicles at excitatory and inhibitory across the three conditions, we generated 2D histograms of the distribution of released vesicles within spatially normalized terminals, with the center of the active zone at the

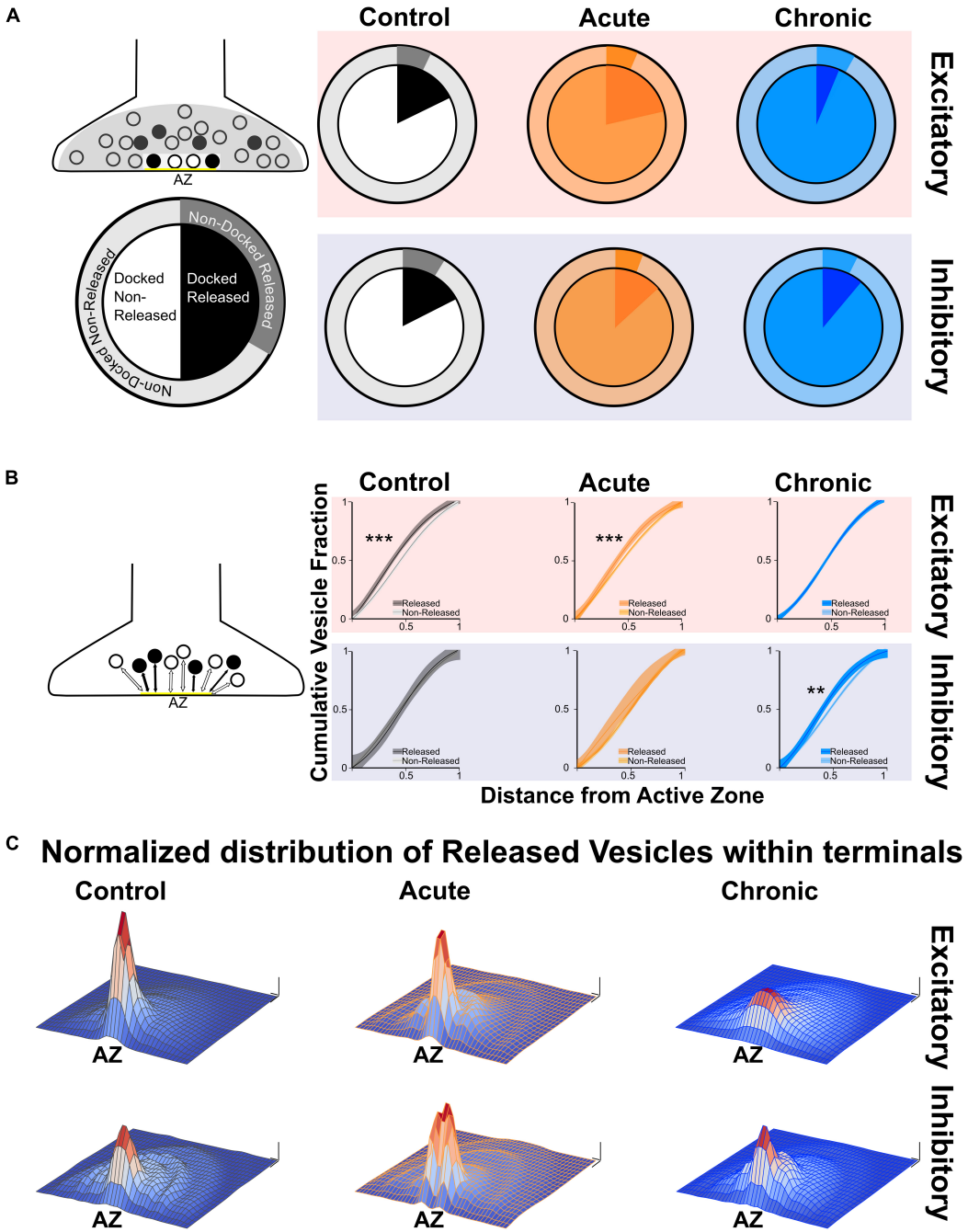

FIGURE 2 | Changes in released vesicles' docking and spatial organization in chronic phase of epilepsy. **(A)** Ratio of released vesicles in the docked and undocked population. Left: Diagram and legend for each pie chart. Top: Excitatory synapses' ratio of released vesicles (darker) in docked (inner pie chart) and undocked population (outer pie chart) in Control (gray), Acute (orange), and Chronic (blue) groups. Only the Chronic group shows a significant difference from expected frequencies based on control observation (Chi-squared test: $p < 0.001$). Bottom: Inhibitory synapses' ratio of released vesicles (darker) in docked (inner pie chart) and undocked population (outer pie chart) in control (gray), acute (orange), and chronic (blue) groups. **(B)** Distance of released or non-released vesicles to the closest point on the active zone. Left: Diagram representing of how distance measures were taken at each synapse. Top: Sigmoid fit and 95% confidence interval of cumulative fraction of distance between released and not-released synaptic vesicles to the active zone at excitatory synapses in Control (gray), Acute (orange), and Chronic (blue) epileptic mice. Bottom: Sigmoid fit and 95% confidence interval of cumulative fraction of distance between released and not-released synaptic vesicles to the active zone at inhibitory synapses in Control (gray), Acute (orange), and Chronic (blue) epileptic mice. Paired *t*-test, Excitatory synapses: Control mice $p = 0.0002$ ($n = 40$), Acute mice $p = 0.0006$ ($n = 41$), Chronic mice $p = 0.298$ ($n = 112$). Paired *t*-test, Inhibitory synapses: Control mice $p = 0.06$ ($n = 14$), Acute mice $p = 0.135$ ($n = 13$), Chronic mice $p = 0.001$ ($n = 28$). **(C)** 2D histograms of released vesicles distribution at excitatory (top) and inhibitory (bottom) synapses across the three conditions with active zone at the origin of the XY plane. Control (gray), Acute (orange), and Chronic (blue). Each synapse was spatially normalized (*X*- and *Y*-axis) and frequency is plotted on the *Z*-axis. Scale bars: 0.1 normalized size X and Y; 0.1 fraction *Z*-axis.

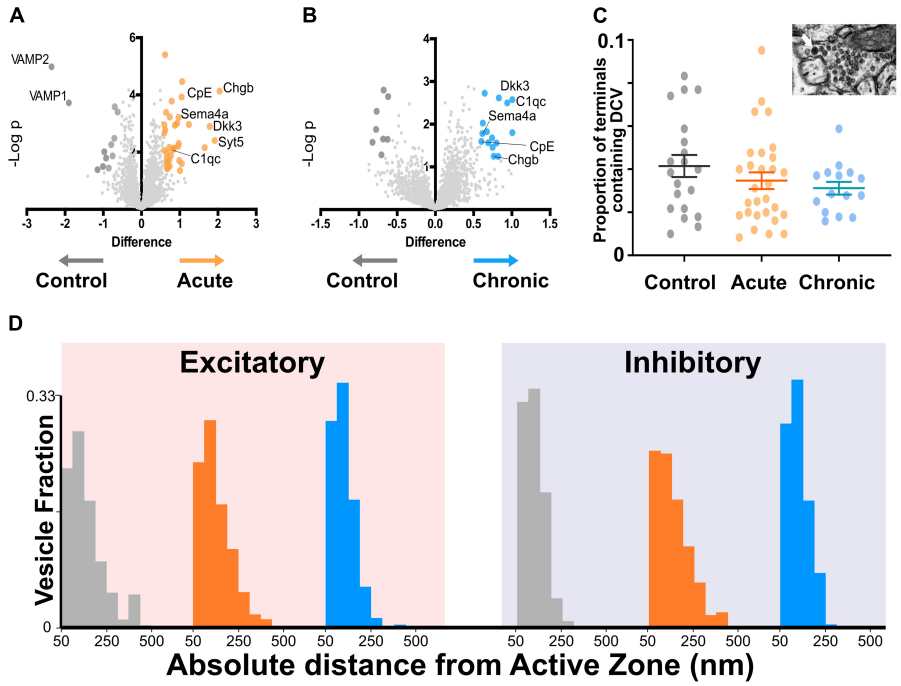

FIGURE 3 | Proteomics analysis of synaptosomes reveal an increase of proteins involved in vesicular positioning. **(A,B)** Differentially expressed proteins in Control vs. Acute **(A)** and Chronic epileptic phase **(B)**. Volcano plots are built plotting average ratio of TeNT vs. corresponding control against their *t*-test log *P*-values; significance thresholds: FDR > 0.05 and fold change > 0.6. Proteins significantly upregulated in Acute and Chronic tetanic animals are highlighted, respectively, in orange and light blue; proteins significantly downregulated are in dark gray. Proteins abbreviations are Dkk3, Dickkopf-related protein 3; Sema4a, Semaphorin 4A; Cpe, carboxypeptidase e; Chgb, chromogranin b; Syt5, synaptotagmin5; VAMP1, Vesicle-associated membrane protein 1; VAMP2, Vesicle-associated membrane protein 2; C1qc, Complement C1q C Chain. **(C)** Proportion of presynaptic terminals containing Dense Core Vesicles in different non-overlapping sampled areas of Control (gray; $n = 20$), Acute (orange; $n = 29$), and Chronic (blue; $n = 15$) groups. No differences between groups (One Way ANOVA, $p = 0.2869$). Data are represented as mean ± SEM. Inset, a representative image of Dense Core Vesicles. **(D)** Right: Distribution of distances of non-released vesicles from active zone at excitatory synapses in Chronic (gray; $n = 2140$), Acute (orange; $n = 2503$), and Chronic (blue; $n = 5705$) groups (One-way ANOVA; $F = 238.15$, $p < 0.0001$, Control vs. Actute: $p < 0.0001$; Control vs. Chronic: $p < 0.0001$). Left: Distribution of distances of non-released vesicles from active zone at inhibitory synapses in Chronic (gray; $n = 543$), Acute (orange; $n = 717$), and Chronic (blue; $n = 1520$) groups ($F = 75.57$, $p < 0.0001$, Control vs. Actute: $p < 0.0001$; Control vs. Chronic: $p > 0.05$).

origin of the *X*-axis (**Figure 2C**). This representation shows a clear broadening of the distribution of released excitatory vesicles in the chronic phase.

Upregulation of Synaptic Proteins Involved in Vesicle Positioning in Acute and Chronic Epilepsy Phases

To better understand molecular changes taking place in epileptic synapses, we performed an in-depth proteomic analysis of visual cortex synaptosomes. The expression profile of 1991 synaptic proteins extracted from animals in the acute and chronic phase of epilepsy was compared with controls. Using a fold change cut-off of 0.6, we found a total of 70 regulated proteins (51 proteins upregulated and 19 downregulated; **Figures 3A,B**). As expected following TeNT injection, the Acute group showed a significant downregulation of VAMP1 and VAMP2 (Mainardi et al., 2012; Vannini et al., 2016). Interestingly, a few synaptic proteins remained upregulated at both stages of epilepsy, suggesting that one single TeNT injection is sufficient to induce persistent plastic changes. Proteins involved in synthesis of regulatory peptides, WNT pathway, immune response and membrane-trafficking were upregulated in hyperexcitable mice (i.e., Dickhopf related protein 3, Complement component 1q, Synaptotagmin 5, Semaphorin 4a, Carboxypeptidase E - CPE, Chromogranin B). The upregulation of neuropeptides was in line with previous reports (Vezzani and Sperk, 2004; Kovac and Walker, 2013; Clynen et al., 2014; Dobolyi et al., 2014; Nikitidou Ledri et al., 2016). These data prompted us to quantify the incidence of Dense Core Vesicles (DCV) in synaptic terminals. To this aim, we performed electron microscopy on samples collected from control and experimental animals and found no difference in the number of DCV across the three conditions (**Figure 3C**). However, we found a tightening of synaptic vesicle clusters at excitatory synapses in both the Acute and Chronic groups and at inhibitory terminals in the Acute group (Fig**Figure 3D**). We limited our analysis to non-released vesicles, whose position is less likely to have been affected by recent recycling. Since the increase in CPE levels did not affect DCV incidence and given the changes in synaptic vesicles clustering at excitatory terminals in epileptic mice, we speculated that CPE might principally act through the pathways involved in vesicles organization (Ji et al., 2017).

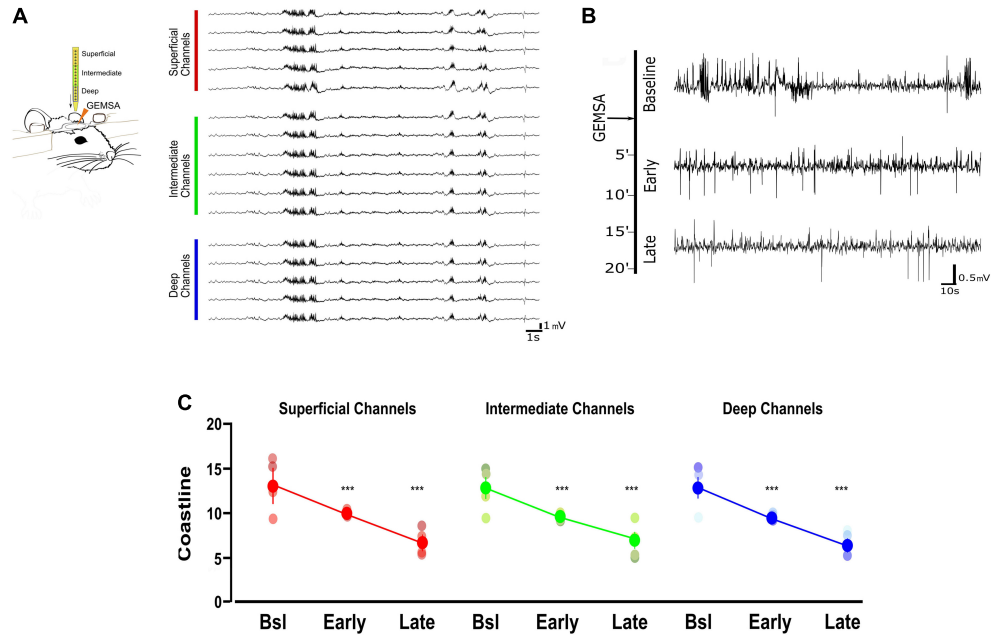

FIGURE 4 | Acute inhibition of Carboxypeptidase (CPE) decreases hyperexcitability in TeNT-injected mice. **(A)** Left: diagram of experimental design: a 16-channel silicone probe was used to record LFP in different layers of the primary visual cortex, channels were analyzed in three groups according to their recording sites in relation to the surface of the cortex: the five most superficial, the five deepest, and the six intermediate channels. GEMSA was applied locally to inhibit CPE activity. Right: Examples of LFP traces obtained with a 16-channels probe from the visual cortex of an Acute epileptic mouse. **(B)** LFP traces of an Acute epileptic mouse before (baseline, top) and after GEMSA administration at two different time points: early (5–10 min) and late (10–20 min). **(C)** Coastline analysis of LFP signals recorded before (baseline) and after GEMSA administration at early and late time points. The analysis was differentially performed for superficial (left, red), intermediate (middle, green), and deep (right, blue) channels (Two-way ANOVA, Channel factor $p > 0.05$, Time factor $p < 0.001$; Baseline vs. Early: $p < 0.01$, Baseline vs. Late: $p < 0.001$, Early vs. Late: $p < 0.001$, $n = 4$). The mean, SEM, and value of individual recordings are shown for each group. ***$p < 0.001$.

Acute Carboxypeptidase E Inhibition Reduces Seizure Activity in Epileptic Mice

Based on the indication that CPE is upregulated in TeNT-injected mice, and given its potential involvement in vesicle positioning (Ji et al., 2017), we decided to perform *in vivo* electrophysiological recordings in acute epileptic mice before and after pharmacological inhibition of CPE. We performed local field potential (LFP) recordings using a 16-channel silicon probe, spanning the whole cortical thickness in awake epileptic mice. Recording channels were divided in superficial (channels 1–5), intermediate (6–11), and deep (12–16) according to their position in the primary visual cortex. After baseline recording of seizures, we topically administered on the visual cortex GEMSA, a CPE inhibitor (**Figures 4A,B**). The recording sessions following GEMSA administration showed a significant decrease in LFP coastline (**Figure 4C**), indicating that CPE inhibition reduces indicators of seizure activity in epileptic mice.

DISCUSSION

This study provides new insights into functional and ultrastructural synaptic changes in epileptic neuronal networks. Using a well-established model of epilepsy, we observed differential regulation of vesicular positioning and active zone size at excitatory and inhibitory synapses (**Figures 1**, **2**). We identified a homeostatic increase in active zone length specifically at inhibitory synapses, consistent with previous reports and previous findings that GABA release is preferentially impaired by TeNT (Schiavo et al., 2000; Ferecskó et al., 2015). These early changes at inhibitory synapses are also consistent with previous observations made in the acute phase, when TeNT catalytic activity can still be detected (i.e., 10 days after TeNT-injection) (Mainardi et al., 2012; Vannini et al., 2016). We suggest that active zone length at inhibitory synapses is homeostatically upregulated in an attempt to restore baseline GABAergic release in spite of TeNT activity. We speculate that the lengthening of inhibitory active zones is later "discarded," as a homeostatic mechanism, given its inability to overcome TeNT-induced reduction in vesicle release, as demonstrated by the reduced ratio of released GABAergic vesicles in both Acute and Chronic groups (**Figure 1B**).

Ultrastructural changes of release competent vesicle positioning at excitatory synapses can only be detected at a later stage. In the chronic phase, excitatory terminals contained a smaller proportion of docked release-competent vesicles, consistent with the reported loss in spatial bias. A similar spatial reorganization of release competent vesicles can be achieved pharmacologically by stabilizing actin, leading to a slower

release rate during 10 Hz stimulation (Marra et al., 2012), its opposite, a tightening of synaptic vesicles, can be observed following Long-Term Potentiation in slices (Rey et al., 2020). Here, we report that spatial organization of release-competent synaptic vesicles can be modulated *in vivo*. During the chronic phase, released glutamatergic vesicles are positioned farther away from the active zone, potentially to limit their re-use during high-frequency activity. This loss of spatial bias may reduce the likelihood of generating spontaneous discharges in hyperexcitable networks. While a direct measure of the functional impact of this spatial reorganization is not possible with currently available methods, we can speculate that the reduction of released vesicles at the active zone of excitatory synapses may fit with the models of occupancy and two-step release proposed over the years by the Marty's lab (Trigo et al., 2012; Pulido et al., 2015; Pulido and Marty, 2017; Miki et al., 2018). Interpreted in the light of Marty's work, excitatory synapses in the chronic phase, although not changing in release fraction, may have a broader range of release latencies due to a reduction in occupancy at rest (Pulido et al., 2015; Pulido and Marty, 2017; Miki et al., 2018). Thus, in chronic epileptic mice the spatial organization of release-competent vesicles farther from active zone may represent an attempt to homeostatically reduce networks' synchronicity without affecting the total number of vesicles released. While not sufficient to block seizures in TeNT epileptic model, this spatial rearrangement may account for the reported reduction of seizures observed in the chronic phase (Vallone et al., 2016; Vannini et al., 2016; Chang et al., 2018). To dissect the molecular mechanisms underlying this change in spatial bias, we performed an unbiased analysis of synaptosomes content in the two different phases of epilepsy. Unsurprisingly, we found upregulation of several proteins involved in DCV trafficking as expected during intense synaptic remodeling. However, we did not find any statistical differences in the number of DCV present in the three experimental groups. We focussed our study on CPE, a protein that is involved in many different pathways, including neuropeptides' synthesis and WNT/BDNF signaling, that was also hypothesized to regulate synaptic vesicles trafficking and positioning (Bamji et al., 2006; Staras et al., 2010; Skalka et al., 2016). Although the exact role of CPE is not completely clear, we reported a reduced hyperexcitability of acute epileptic mice after the administration of its inhibitor (**Figure 4**). We also showed a tightening of synaptic vesicle clusters, measured from the active zone (**Figure 3D**). Therefore, our loss of spatial bias in recently released vesicles might happen on a background of overall contraction of vesicular clusters. This observation offers a possible interpretation for the effect of CPE inhibition on epileptiform activity, that was electrophysiologically measured *in vivo* (**Figure 4**). The mechanisms by which CPE inhibition impacts on seizures remain to be fully clarified. CPE is involved in several different biosynthetic and signaling pathways (i.e., WNT, BDNF) which may account for the anti-epileptic effects. Moreover, CPE impact is likely to be indirect and very hard to dissect *in vivo*. However, further studies on other epileptic models are necessary to better elucidate CPE role in epilepsy. Taken together, our results suggest a complex landscape of molecular and ultrastructural changes evolving over time, opening intriguing questions regarding the temporal evolution of homeostatic changes in response to the induction of hyperexcitability. It would be particularly interesting to observe how homeostatic regulation of excitability adapts over development, for example in a model of genetic epilepsy, where epileptogenic factors are present since the very early formation of the nervous system (Lignani et al., 2020). Since TeNT-induced epilepsy is pharmacoresistant (Nilsen et al., 2005) and refractory seizures represent a major unmet medical need, drugs acting on CPE levels and other regulators of synaptic pools warrant further investigation as possible therapeutic treatments in currently intractable epilepsy.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD022270.

ETHICS STATEMENT

All the experiments were performed in compliance with the EU Council Directive 2010/63/EU on the protection of animals used for scientific purposes and were approved by the Italian Ministry of Health.

AUTHOR CONTRIBUTIONS

EV, MC, VM, and LM conceived and designed the experiments. EV, LR, and MC supplied the animal models. MC and VM supervised the work. EV, LR, MD, and VM performed the experiments. EV, MD, and VM analyzed the data. EV and VM wrote the manuscript. All authors contributed to the critical revision of the manuscript.

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REVIEW
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Calcineurin Participation in Hebbian and Homeostatic Plasticity Associated With Extinction

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In nature, animals need to adapt to constant changes in their environment. Learning and memory are cognitive capabilities that allow this to happen. Extinction, the reduction of a certain behavior or learning previously established, refers to a very particular and interesting type of learning that has been the basis of a series of therapies to diminish non-adaptive behaviors. In recent years, the exploration of the cellular and molecular mechanisms underlying this type of learning has received increasing attention. Hebbian plasticity (the activity-dependent modification of the strength or efficacy of synaptic transmission), and homeostatic plasticity (the homeostatic regulation of plasticity) constitute processes intimately associated with memory formation and maintenance. Particularly, long-term depression (LTD) has been proposed as the underlying mechanism of extinction, while the protein phosphatase calcineurin (CaN) has been widely related to both the extinction process and LTD. In this review, we focus on the available evidence that sustains CaN modulation of LTD and its association with extinction. Beyond the classic view, we also examine the interconnection among extinction, Hebbian and homeostatic plasticity, as well as emergent evidence of the participation of kinases and long-term potentiation (LTP) on extinction learning, highlighting the importance of the balance between kinases and phosphatases in the expression of extinction. Finally, we also integrate data that shows the association between extinction and less-studied phenomena, such as synaptic silencing and engram formation that open new perspectives in the field.

Keywords: extinction, calcineurin, Hebbian and homeostatic plasticity, long-term depression, kinases and phosphatases, depotentiation

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INTRODUCTION

Extinction from the perspective of classical conditioning can be defined as the reduction or temporal inhibition of the conditioned response (CR) that takes place once established a CS-US (conditioned stimulus-unconditioned stimulus) association and the CS is repeatedly presented in the absence of the US, while for instrumental learning, extinction represents the reduction in responses, when a response (R) that was previously followed by an appetitive outcome (O) is not followed by that outcome anymore (Myers and Davis, 2002; Myers et al., 2006; Herry et al., 2010).

This reduction of a previously learned behavior is a natural and adaptive process that allows animals to re-learn associations about their environment. Originally, extinction was thought to implicate the elimination of a behavior previously acquired (Rescorla and Wagner, 1972). However, the sudden reappearance of the extinguished behavior (spontaneous recovery), its reappearance by a reminder (reinstatement) or by changing the context (renewal), provided evidence that extinction is not the erasure of certain learning. Instead, extinction refers to a new learning or a re-learning process (Bouton, 2004; Bouton et al., 2011), that also presents acquisition, consolidation, and retrieval phases (Quirk and Mueller, 2008). The mechanisms involving this type of learning are not entirely explored and are yet to be defined.

Behaviorally it has been proposed that during extinction the CS acquires inhibitory properties that suppress the CR (Bouton et al., 2006), or alternatively, that the original CS-US association is modified (Bouton and Todd, 2014). As extinction refers to a new learning, the neural basis of acquisition of conditioning (i.e., synaptic plasticity) have also been associated as underlying mechanisms of extinction, as we will discuss later. Particularly, long-term depression (LTD) has been proposed as the underlying mechanism of extinction, while the protein phosphatase calcineurin (CaN) has been widely related to both the extinction process and LTD.

Calcineurin is a Ca^{2+}/calmodulin (CaM)-dependent serine/threonine phosphatase consisting of two subunits: one catalytic (CaN-A, ∼61 kDa) and one regulatory (CaN-B, ∼19 kDa). The mammalian CaN-A has three isoforms: α-isoform is neuron specific, β-isoform has wide distribution and γ-isoform is predominantly located in cortical neurons. Meanwhile, CaN-B has two isoforms: CaN-B1 and CaN-B2, only CaN-B1 binds to CaN-Aα and CaN-Aβ, while CaN-B2 was found only in testes. Each CaN subunit isoform is encoded by an individual gene, which are located in different chromosomes: CaN-Aα in chromosome 4, CaN-Aβ in chromosome 10, and CaN-Aγ in chromosome 8 (Tarasova et al., 2018). In addition to calcineurin, the serine/threonine protein phosphatase family members include protein phosphatases 1 (PP1), 2A (PP2A), and 2C (PP2C) and have different roles on signal transduction in eukaryotic cells (Rusnak and Mertz, 2000). Calcineurin is widely distributed in the body and it is selectively enriched within the post-synaptic densities and cell soma of neurons of the central nervous system (Groth et al., 2003) where primarily modulates synaptic transmission associated with memory (Tarasova et al., 2018). Calcineurin was originally described for its role on the activation of the nuclear factor of activated T-cells (NFAT), this regulation is linked to apoptosis, cardiac pathology and immune response in the kidney (Azzi et al., 2013).

MECHANISMS UNDERLYING EXTINCTION: HEBBIAN AND HOMEOSTATIC PLASTICITY

Hebbian synaptic plasticity refers to the activity-dependent modification of the strength or efficacy of synaptic transmission at preexisting synapses and has a central role in the capacity of the brain to incorporate transient experiences into persistent memory traces. In this regard, the synaptic plasticity phenomena known as long-term potentiation (LTP) and long-term depression (LTD), are believed to underlie memory formation and maintenance. LTP refers to the prolonged activity-dependent increment of the synaptic efficacy, usually generated by the application of high frequency stimulation (HFS) and it is associated with the induction and maintenance of conditioning. LTD, in turn, refers to the activity-dependent decrement of the synaptic efficacy, commonly induced by low frequency stimulation (LFS) (Bear et al., 2001; Abraham, 2008; Citri and Malenka, 2008). Similar decrements can be generated after the induction of LTP, in which case the decrease is called depotentiation referring to the reversal of synaptic strength from a potentiated LTP state. In particular, LTD as well as synaptic depotentiation (**Figure 1A**) have been closely associated with extinction learning (Lin et al., 2003a; Saito et al., 2012; Li et al., 2016).

In this context, the hebbian forms of plasticity would need some type of homeostatic regulation to preserve its adequate function (Turrigiano and Nelson, 2004). Homeostatic forms of plasticity might provide the global regulation necessary to maintain synaptic strength and plasticity within a functional dynamic range. These forms of plasticity also operate through diverse mechanisms: by detecting changes in neuronal firing and scaling excitatory synaptic strengths up or down while preserving their relative weights (synaptic scaling); by strengthening pre- or post-synaptic properties as a compensatory mechanism (synaptic redistribution) or by altering the ability of synapses to undergo subsequent Hebbian modifications (metaplasticity). In this framework, metaplasticity is thought to be essential not only to maintain synapses within a dynamic functional range but also for the maintenance of memory traces (**Figure 1B**; Abbott and Nelson, 2000; Pérez-Otaño and Ehlers, 2005; Abraham, 2008; Abraham and Richter-Levin, 2018; Lee and Kirkwood, 2019).

Among the evidence supporting the association between LTD and extinction, Dalton and collaborators showed that the inhibition of *N*-Methyl-D-Aspartate receptors (NMDAR)-dependent LTD in the amygdala elicits an impairment of fear extinction training (Dalton et al., 2008), whilst Amano et al. (2010) showed that fear conditioning was associated with an enhancement of field excitatory post-synaptic potentials (fEPSPs) in the basolateral amygdala (BLA), which was partially reverted by the extinction of this task. Similarly, it was reported that the fear conditioning-induced potentiation of EPSCs was reverted by extinction in the thalamus-amygdala and cortico-lateral amygdala pathways (Kim et al., 2007; Hong et al., 2009). Interestingly, Li and collaborators showed that the inhibition of the acid-sensing ion channel 1a (ASIC1a) attenuated the LTD induction in the insular cortex, as well as the extinction of the conditioned taste aversion (CTA) (Li et al., 2016). It was also shown that the application of low-frequency stimulation (LFS) in the hippocampal CA1 area reduced the expression of freezing just like extinction does (Saito et al., 2012). More recently it was observed that optogenetically induced LTD in the thalamus-lateral amygdala

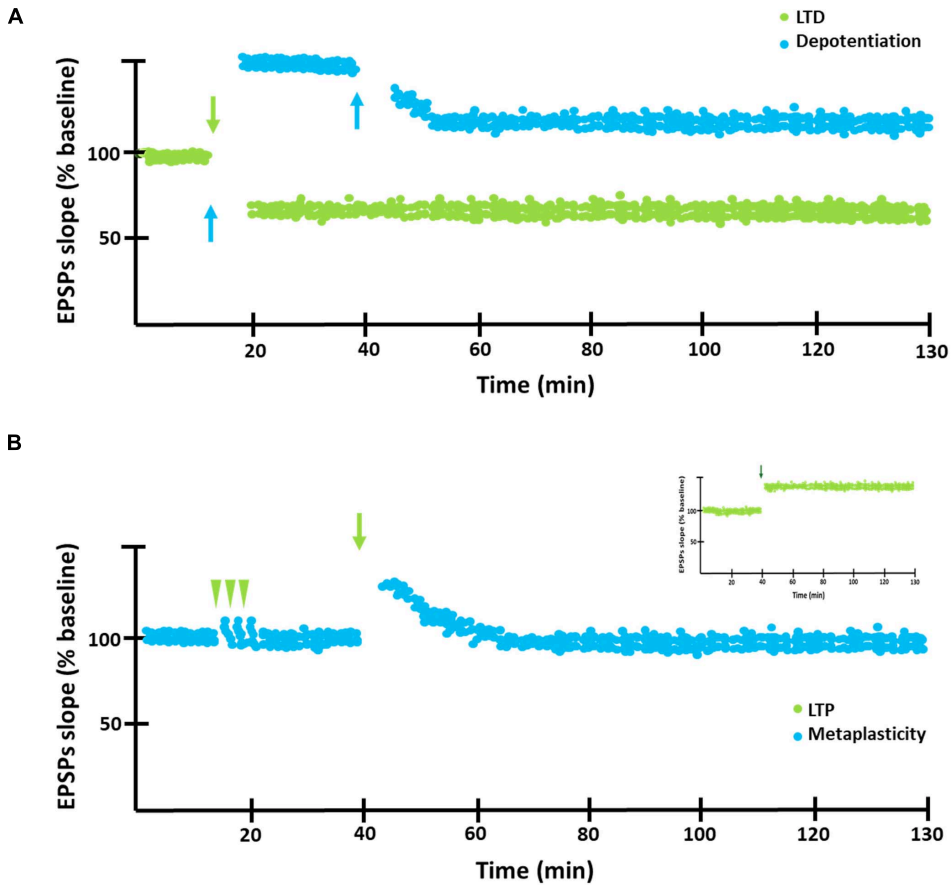

FIGURE 1 | LTD, depotentiation and metaplasticity graphic representation. **(A)** Graphic representation of LTD (green circles) and depotentiation (blue circles). Blue arrows indicate application of low frequency stimulation in order to induce LTD or depotentiation. **(B)** Metaplasticity. After receiving a weak stimulation (green arrow heads), LTP cannot be induced by applying a high frequency stimulation (green arrows) capable of inducing this phenomenon in a control preparation as observed in the inset. That is, the previous stimulation modifies the threshold to induce LTP.

(LA) pathway reduced fear conditioning (Lee et al., 2016; Klavir et al., 2017), as well as drug and alcohol-seeking behaviors (Ma et al., 2018; Rich et al., 2019). These findings exemplify the remarkable relationship between LTD and extinction, and strongly suggest that the former represents a mechanism underlying the latter.

There is also a series of studies emphasizing the relationship between the processes of extinction and depotentiation. In this sense, it was proven that several conditioned fear extinction paradigms induce synaptic depotentiation in the lateral amygdala (Lin et al., 2003a; Kim et al., 2007; Hong et al., 2009). Zhang and collaborators, for example, showed that failure in spatial memory extinction is accompanied by impairment in both LTD and synaptic depotentiation (Zhang et al., 2011). Likewise, it was observed that optical depotentiation of auditory pathways to the amygdala generates amnesia of fear conditioning induced by optogenetic stimulation (Nabavi et al., 2014), and optogenetically induced depotentiation of the auditory pathways to LA suppressed conditioned fear responses to the CS (Kim and Cho, 2017). Similarly, Song et al. (2017) described that the administration of the antipsychotic olanzapine in the CA3 area of the hippocampus impaired depotentiation as well as reversal learning in the Morris water maze test.

Taken together, the findings described above clearly show that decrements in synaptic efficacy (either LTD or depotentiation) constitute mechanisms that underlie extinction.

Nowadays, learning and memory research widely accepts that the trace of memory refers to the formation of the engram. In this sense, Lacagnina and collaborators showed that fear extinction suppresses the reactivation of contextual fear engram cells while activating a second ensemble in the hippocampus (Lacagnina et al., 2019). More recently, Zhang and collaborators showed that fear extinction memory requires forming a new engram in the basolateral amygdala (Zhang et al., 2020). Furthermore, chronic stimulation of engram cells associated with fear memories in the hippocampus produced a reduction of fear responses, suggesting that optogenetic manipulation of a fear engram is sufficient to induce an extinction-like behavior (Chen et al., 2019; Cincotta et al., 2021). Likewise, the inhibition of the assembly activated during cue-paired alcohol self-administration in the medial prefrontal cortex (mPFC), 1 month later prevented relapse of alcohol-seeking (Visser et al., 2020), and optogenetic activation

of infralimbic cortex-basolateral amygdala pathway during fear extinction trials, resulted in a stronger extinction compared to non-optogenetic activation (Bukalo et al., 2021). From these data, it can be inferred that in fact extinction represents new learning that requires the formation of a new engram. Nevertheless, it has alternatively been proposed that extinction implies the silencing of the original engram. The term silent engram refers to those engrams that cannot be retrieved by natural retrieval cues but can be retrieved with direct optogenetic stimulation (Josselyn and Tonegawa, 2020). Engram silencing also diminishes the previously learned behavior and seems to share synaptic mechanisms with extinction (Josselyn and Tonegawa, 2020). In this sense, it was shown that the chemogenetic reactivation of a fear engram evoked the extinguished behavior (Yoshii et al., 2017). On the other hand, the fact that reconsolidation and extinction seem to share similar signal transduction cascades, including the CaN participation (Fukushima et al., 2014), could imply that memory reconsolidation constitutes a component of extinction. However, these are quite novel ideas that require more experimental support.

So far, we have mentioned evidence showing that LTD and depotentiation are mechanisms underlying extinction. There is also interesting evidence demonstrating the participation of homeostatic plasticity in the regulation of the extinction process. In this sense, it was shown that fear extinction induces LTP instead of LTD in the hippocampal CA1 area, through the activation of mGlu receptors (Stansley et al., 2018), and that CTA extinction impairs LTD induction in the insular cortex (Li et al., 2016). Our group has shown that LTP in the insular cortex promotes CTA retention (Escobar and Bermúdez-Rattoni, 2000). Additionally, we recently showed that the extinction of CTA is bidirectionally modulated by LTP and LTD. While the induction of LTP reinforces the retention of learning, the induction of LTD facilitates its extinction (Rodríguez-Durán et al., 2017). More recently, we also showed that CTA extinction allows the induction but not the maintenance of LTP in the insular cortex *in vivo* (Rivera-Olvera et al., 2018). In like manner, homeostatic plasticity triggered by optogenetic stimulation in the hippocampus altered the balance between excitation and inhibition, thus favoring the extinction expression (Mendez et al., 2018).

Furthermore, it was reported that the odor preference paradigm in rat pups leads to up-regulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptor levels. However, when pups are re-trained 3 h later with another odor, the levels of AMPA and NMDA receptors are reset to the baseline condition through a metaplastic mechanism. This adjustment in receptor levels is associated with down-regulation of the NMDA receptor subunit GluN1 and leads to unlearning of the first odor. In addition, when the phosphatase calcineurin is inhibited in the anterior piriform cortex during training or retraining, the downregulation of GluN1, as well as the unlearning originated by metaplasticity, are prevented (Mukherjee et al., 2017; Bhattacharya et al., 2018). In a similar manner, the negative effect of isoflurane administration in the hippocampus and amygdala on fear memory, was prevented when CaN was inhibited after exposure to isoflurane in these areas (Yang et al., 2017). These results suggest that both calcium levels and calcineurin activity participate in the homeostatic regulation exerted by extinction.

ON THE CELLULAR BASIS OF EXTINCTION

Derived from the aforementioned evidence, the understanding of the cellular and molecular mechanisms underlying synaptic plasticity expressions becomes essential to comprehend the plastic mechanisms underlying extinction learning.

Concerning the NMDAR-dependent LTP, the binding of glutamate (Glu) and the post-synaptic depolarization allow the calcium ion (Ca^{2+}) influx to the postsynaptic membrane. In high concentrations, Ca^{2+} forms a complex with calmodulin, which modifies and activates the protein Ca^{2+}/calmodulin-dependent kinase II (CaMKII). CaMKII then phosphorylates the AMPA receptors at residue S831 of the GluA1 subunit, in concert with this, phosphorylation of GluA1 S818 by PKC and S845 by PKA, lead to AMPARs insertion in the post-synaptic membrane (Dunning and During, 2003; Diering and Huganir, 2018). In addition, phosphorylation of GluA1 S831 by CaMKII or PKC regulates the conductance of AMPA receptors (Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997; Jenkins and Traynelis, 2012; Coultrap et al., 2014; Diering et al., 2016). Moreover, phosphorylation of auxiliary transmembrane AMPA receptor regulatory proteins (TARPs) subunits by CaMKII and PKC play prominent roles in regulating AMPAR synaptic localization during LTP and LTD (Rouach et al., 2005; Tomita et al., 2005; Menuz et al., 2008; Kristensen et al., 2011; Jenkins and Traynelis, 2012; Park et al., 2016). However, it has been described that CaMKII can also be activated by LFS, but in this case its activity elicits the phosphorylation of the GluA1-S567, thus promoting the removal of AMPA receptors toward extrasynaptic sites, which in turn promotes the induction of LTD (Coultrap et al., 2014; Woolfrey et al., 2018). On the other hand, low levels of Ca^{2+} activate calcineurin (CaN), a serine/threonine protein phosphatase, which dephosphorylates the inhibitor 1 (I-1) that normally acts as an inhibitor of the protein phosphatase (PP1). When I-1 is dephosphorylated by CaN, the now active PP1 dephosphorylates CaMKII thereby causing its inhibition. Additionally, CaN can dephosphorylate GluA1 S845 and TARPs to control LTD (Tavalin et al., 2002; Tomita et al., 2005; Diering et al., 2014; Itakura et al., 2014). This generates the removal of AMPA receptors from the membrane and its internalization, which leads to a decrement of the synaptic efficiency that can generate LTD.

The mechanisms that generate NMDAR-dependent LTD are also involved in the induction of depotentiation, and are generally triggered by LFS, but can also be activated by spike timing-dependent plasticity (STDP) protocols, a phenomenon in which the precise timing of spikes affects the direction and magnitude of changes in synaptic strength. Typically, a pre-synaptic spike preceding a post-synaptic spike within a narrow time window leads to LTP, if the order is reversed, LTD results (Wittenberg and Wang, 2006; Shouval et al., 2010).

Long-term depression also exists in a metabotropic receptor (mGluR) dependent variant, which can be induced by paired-pulse LFS, or by the application of mGluR agonists such as 1-amino-1,3-dicarboxycyclopentane (ACPD) or (S)-3,5-dihydroxyphenylglycine (DHPG) (Collingridge et al., 2010). DHPG is commonly used for characterizing the induction and expression mechanisms underlying hippocampal mGluR-LTD. The mGluR-LTD has different mechanisms from NMDAR-LTD, but also leads to AMPAR internalization (Gladding et al., 2009). The stimulation of group 1 mGluRs leads to activation of the phosphoinositide-specific phospholipase C (PLC), which allows the activation of the protein kinase C (PKC); PKC can then phosphorylate the AMPA receptors thus allowing their removal from the synaptic space. The canonical signaling pathway of group I mGluRs involves the hydrolysis of phosphatidyl inositol to generate inositol trisphosphate (IP3) and diacylglycerol (DAG), which in turn can activate PKC. This pathway is involved in mGluR-LTD triggered by both mGlu1 and mGlu5 receptors in the hippocampus. The protein interacting with C kinase 1 (PICK1) is also required for mGluR-LTD at different synapses. In the perirhinal cortex PICK1 forms a complex with the prototypic member of the neuronal calcium sensor (NCS) family NCS-1, which could act as a high-affinity Ca^{2+} sensor for mGluR-LTD. As PICK1 can also bind PKCα, it is possible that NCS-1 attracts the PICK1–PKC complex to the GluA2 subunit of AMPARs in response to Ca^{2+} signals, resulting in phosphorylation of the subunit and dissociation from the AMPAR-binding protein–glutamate receptor interacting protein (ABP–GRIP) (Lin and Huganir, 2007; Collingridge et al., 2010). Depending on the developmental stage of the animal, mGluR-LTD can be induced in presence of protein synthesis inhibitors (Huber et al., 2000; Nosyreva and Huber, 2005). Other molecular actors that have been implicated in the expression of mGluR-LTD are the p38 mitogen-activated protein kinase (p38 MAPK) and, to a lesser extent, extracellular signal-regulated kinases (ERKs), protein tyrosine phosphatases (PTPs) and Arc, though, the downstream effectors have not been fully described (Collingridge et al., 2010).

Other forms of LTD have also been described, such as endocannabinoid-LTD (eCB-LTD) which is the form of long-term reduction of the neurotransmitter release at the same or nearby synapses by activation of presynaptic cannabinoid receptor type 1 (CB1R) (Xu and Chen, 2015). Induction of eCB-LTD requires an increase in intracellular Ca^{2+} and activation of postsynaptic mGluRs in most brain regions (Xu and Chen, 2015). In the hippocampus, the endocannabinoids act as mediators of a form of heterosynaptic mGluR induced LTD. Endocannabinoid release during LTP can also lead to LTD of GABA (γ-aminobutyric acid)-mediated synaptic transmission and this affects the subsequent plasticity of the network (Collingridge et al., 2010; Fontaine et al., 2020). Endocannabinoids can also function as retrograde messengers in the striatum, neocortex and cerebellum (Collingridge et al., 2010). It is known that activation of CB1R on GABAergic nerve terminals inhibits adenylcyclase, decreases cAMP levels, reduces PKA activity, and increases intraterminal Ca^{2+} thus activating calcineurin. Together, these actions promote dephosphorylation of proteins necessary for transmitter release, leading to inhibitory LTD of transmission (Bennett et al., 2017). However, the exact mechanism of sustaining the long-term depression of a neurotransmitter release after activation of CB1R within the short-time scale (minutes) is still unknown, but the distribution of CB1R largely determines the strength of eCB-mediated short and long-term synaptic plasticity (Xu and Chen, 2015). The activation of CB1R can also be modulated by glucocorticoids and BDNF, which could associate eCB-LTD with different types of learning, including reward-seeking behavior and fear learning (Bennett et al., 2017; Bilbao et al., 2020; Gunduz-Cinar, 2021). Though CB1R activity seem to be also associated to extinction, maybe through the endogenous release of BDNF (Bennett et al., 2017). Nevertheless, NMDAR-LTD has been further explored and predominantly associated with extinction learning (Huang et al., 2016).

THE MOLECULAR BALANCE: ON CALCINEURIN, KINASES AND OTHER PHOSPHATASES

At present, it is of common knowledge in the field that the activity of kinases, such as CaMKII, has been identified as a molecular mechanism underlying LTP, while the activity of phosphatases, such as CaN, has been associated with LTD expression. Although, as we will discuss later, this perspective begins to be nuanced by experimental evidence.

Calcineurin Modulation of Learning: Hebbian and Homeostatic Plasticity

The evaluation of the protein phosphatases' participation in extinction has provided valuable information about the cellular mechanisms that underlie this process. Notably, the protein phosphatase 2B (PP2B) or calcineurin plays an essential role in a variety of biological processes, including extinction and LTD, as mentioned earlier. Calcineurin is a serine/threonine phosphatase consisting of two subunits: a catalytic subunit (CaN-A) and a regulatory subunit (CaN-B). The catalytic subunit, in turn, consists of the catalytic region, one binding domain for CaN-B, a binding domain for calmodulin and an autoinhibitory domain (AID). On the other hand, the regulatory CaN-B subunit possesses four domains for calcium, two with low and two with high affinity (**Figure 2A**; Rusnak and Mertz, 2000; Tarasova et al., 2018).

Regarding the participation of CaN during the extinction process, it was shown that the administration of two selective inhibitors of CaN, cyclosporine A (CsA) or tacrolimus (FK506) before extinction training, blocked fear extinction (Lin et al., 2003b; Fuente et al., 2014; Almeida-Correâ et al., 2015). Shaw and collaborators showed that CaN also plays an important role in the extinction of spatial memories (Shaw et al., 2012). We have recently reported that after CTA extinction, the calcineurin expression is increased in the insular cortex (Rivera-Olvera et al., 2018). Similarly, increases of CaN-A were observed after fear extinction (Alvarez-Ricartes et al., 2018). Furthermore, a recent study shows that the overactivation of CaN, by the administration

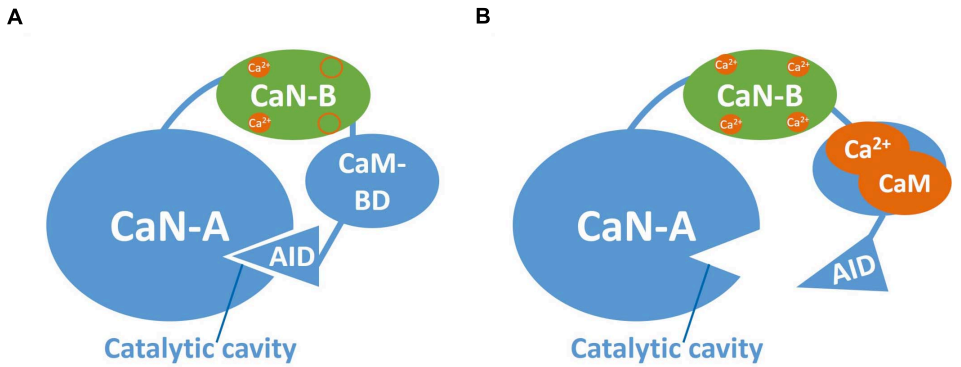

FIGURE 2 | Calcineurin structure. **(A)** Inactivated CaN. Representation of CaN-A catalytic subunit (blue) and the regulatory subunit CaN-B (green). CaN-A consists of a catalytic cavity, a CaN-B binding domain, a calmodulin binding domain (CaM-BD) and an autoinhibitory domain (AID). CaN-B possesses four binding sites for Ca^{2+}, two of high affinity typically occupied by Ca^{2+} at basal levels. The AID occupies the catalytic cavity when CaN is inactive. **(B)** Activated CaN. At low Ca^{2+} influx, Ca^{2+} binds to the low affinity sites at CaN-B and the Ca^{2+}/CaM complex binds to CaM-BD allowing AID removal from the catalytic cavity.

of chlorogenic acid (CGA) after extinction training, facilitates the extinction and protects from the reinstatement of a cocaine cue memory; this effect was blocked by FK506 (Rich et al., 2020).

The effects of CaN inhibition seem to be phylogenetically conserved, as the administration of protein phosphatases inhibitors blocked the spike frequency decline observed after an *in vitro* extinction procedure of classical conditioning in *Hermissenda crassicornis* (Cavallo et al., 2014).

Calcineurin was also reported to have a role in synaptic plasticity. The administration of cyclosporine or FK506 in a slice preparation of the CA1 area of the hippocampus showed a diminished LTD compared with controls (Mulkey et al., 1994). In a similar preparation, mice lacking the regulatory subunit of CaN also showed a diminished LTD (Zeng et al., 2001). Moreover, the infusion of calcineurin inhibitors blocked the induction of depotentiation in the lateral nucleus of the amygdala (Lin et al., 2003a).

The manipulation of the endogenous regulators that modulate CaN activity has also stressed the role of this phosphatase in synaptic plasticity. In this sense, Zhu and collaborators showed that the knock-out of calpain-1 (a protease that degrades the regulatory subunit of CaN) impaired hippocampal mGluR-LTD, as well as fear extinction (Zhu et al., 2017). Furthermore, the inhibition of calpain before or after extinction training impaired the extinction of fear memory in mice (Song et al., 2018). In addition, the phosphorylation of the endogenous regulator of CaN (RCAN1) by glycogen synthase kinase 3 beta (GSK3β) was proven to be necessary for LTD expression in CA1 synapses, while the phosphorylation of RCAN1 at PKA sites blocked CaN activity, thus allowing the induction of LTP (Dudilot et al., 2020).

There is experimental evidence showing that CaN is also capable of modulating LTP. Funauchi and collaborators showed that CaN inhibition facilitated the induction of LTP in the rat visual cortex (Funauchi et al., 1994). On the other hand, Moradpour and collaborators showed that the inhibition of CaN prevents impairment of hippocampal CA1 LTP by the steroid nandrolone (Moradpour et al., 2019). It is important to mention that it was demonstrated that LTP at the parallel fiber (PF)-Purkinje cell synapses, requires the activation of calcineurin as well as other phosphatases (Belmeguenai and Hansel, 2005; Jörntell and Hansel, 2006; Grasselli and Hansel, 2014). In addition, Fuji and Hirano showed that a late phase of LTD at Purkinje neurons requires a downregulation of calcineurin (Fuji and Hirano, 2002). There is also some behavioral evidence that CaN is involved in the formation of learning since the chronic administration of the CaN inhibitor CsA impaired visuospatial learning (Speigel et al., 2019). It was also recently shown that the maintenance of the long-term memory in an object recognition paradigm is regulated in part by CaN, since the daily systemic administration of FK506 showed extended memory on the task (Sachser et al., 2016).

Dephosphorylation of the AMPAR subunit GluA1 by CaN seems to be a molecular event that may modulate both hebbian and homeostatic plasticity. It was reported that cultured cortical neurons in the presence of tetrodotoxin (TTX) presented reduced CaN activity and high levels of phosphorylated GluA1, associated with increases in the average mEPSC amplitude, thus suggesting that the decreased activity of CaN lead to increased phosphorylation of GluA1, allowing the expression of homeostatic plasticity and favoring in turn the expression of LTP (Kim and Ziff, 2014). Furthermore, it was shown that the balance of AKAP5 (AKAP79/150)-anchored PKA and CaN signaling regulate GluA1 S845 phosphorylation to control homeostatic synaptic plasticity in both hippocampal and cortical neurons (Diering et al., 2014; Sanderson et al., 2018). Indeed, the inhibition of CaN by FK506 leads to an increase in the levels of GluA1 in the olfactory bulb only after an odor preference learning (Bhattacharya et al., 2018). These findings show that CaN is a key molecule not only for LTD modulation, but also for LTP and homeostatic plasticity modulation.

PP2A Modulation of Synaptic Plasticity and Learning

Similarly, other phosphatases have also been implicated on synaptic plasticity. The protein phosphatase 2A (PP2A)

belongs to the serine/threonine protein phosphatase family and have some roles in synaptic plasticity and learning as well. Transgenic mice expressing Simian Virus 40 small-t antigen, which inhibits the PP2A, exhibited a blockade of NMDAR-LTD, as well as deficits in behavioral flexibility (Nicholls et al., 2008). More recently, the intraventricular administration of okadaic acid (OKA), an inhibitor of the protein phosphatase family, attenuated the fEPSP slope and population spike (PS) amplitude of hippocampal dentate gyrus neurons following paired-pulse and HFS (Hamidi et al., 2019). In addition, as mentioned earlier LTP at the PF-Purkinje cell synapses, requires the activation of protein phosphatases as PP1, PP2A, and calcineurin (Belmeguenai and Hansel, 2005; Jörntell and Hansel, 2006; Grasselli and Hansel, 2014). In this regard, several studies have demonstrated that LTD induction requires, or is supported by, the suppression of phosphatases (Ajima and Ito, 1995; Eto et al., 2002; Launey et al., 2004; Kawaguchi and Hirano, 2013). In a recent study, a knock-out for PP2A impaired contextual fear memory extinction, as well as LTD. Surprisingly, the knock-out also disrupted HFS-induced LTP (Wang et al., 2019). Conversely, administration of LB100, a PP2A inhibitor, during conditioning promoted the extinction of methamphetamine induced conditioned place preference in mice (Qian et al., 2020). This evidence suggests that phosphatases of this family may have a broader role in plasticity and learning, however, more experiments are needed to elucidate their participation and possible mechanisms.

LTD and Extinction Modulation by Kinases: CaMKII, AMPK, MET Receptor, and ERK

Growing experimental evidence shows that kinases also play an important role in LTD and extinction. As we mentioned earlier, CaMKII phosphorylates the GluA1-S567 on AMPARs to promote LTD expression (Coultrap et al., 2014; Woolfrey et al., 2018). In addition, the expression of cerebellar LTD depends on the activity of kinases such as PKA (Grasselli and Hansel, 2014). A recent study showed that overactivation of adenosine monophosphate-activated protein kinase (AMPK) before extinction training in the hippocampus promotes fear extinction maintenance (Wang et al., 2020). Moreover, a knock-out for the MET receptor tyrosine kinase exhibited increases in both LTP and LTD in the hippocampus, as well as faster fear-conditioning learning and conditioned fear-extinction (Xia et al., 2021). It also was recently suggested that extracellular-regulated protein kinase (ERK) activity is important for the regulation of extinction learning expression. In this sense, the inhibition of ERK before the extinction training in the amygdala, hippocampus, medial pre-frontal cortex and nucleus accumbens, facilitated extinction and prevented reconsolidation and reinstatement in both inhibitory avoidance and conditioned place preference tasks (Fukushima et al., 2021; Qiao et al., 2021). Taken this evidence, it seems that phosphatases' and kinases differential role in learning and memory processes should be reconsidered.

CALCINEURIN AS A REGULATOR OF EXTINCTION: MOLECULAR MECHANISMS ON BEHAVIOR, HEBBIAN AND HOMEOSTATIC PLASTICITY

As the activity of phosphatases seems to be relevant to extinction learning, knowing their mechanisms of action could lead to a better understanding of the extinction process. For this, we will now focus on the activation and inactivation mechanisms of CaN.

Activation and Inhibition of CaN

In the inactive state of CaN, the AID is located in the catalytic region of CaN-A. As we described before, CaN is activated when there is a low influx of Ca^{2+} (1 μM), which causes Ca^{2+} to bind simultaneously to calmodulin and CaN-B. In turn, the Ca^{2+}-calmodulin complex binds to its domain in CaN-A, promoting the removal of the AID from the catalytic region, thus generating the active form of CaN (Rusnak and Mertz, 2000; **Figure 2B**). Meanwhile, as described above, the active form of CaN dephosphorylates the inhibitor of PP1. Once this happens, the PP1 can dephosphorylate CaMKII, leading to its inhibition. CaN is able to dephosphorylate the AMPA receptors, attaching them through A-kinase anchor proteins (AKAPs), leading to AMPA receptors internalization (Sanderson et al., 2016, 2012). CaN is also capable of directly dephosphorylating NMDAR as well as some transcription factors (e.g., CREB and NFAT), thus altering gene expression (**Figure 3**; Dunning and During, 2003; Groth et al., 2003; Tarasova et al., 2018). All these processes are associated with the induction of LTD, as we mentioned before.

In contrast, the endogenous inhibition of CaN has not been completely understood. Nevertheless, it is known that it occurs at a high intracellular Ca^{2+} concentration (5 μM). It has been described, *in vitro*, that high concentrations of Ca^{2+} activate kinases, such as CaMKII and PKC, which may phosphorylate and inhibit CaN directly (Hashimoto and Soderling, 1989; Martensen et al., 1989). PKA can be activated as well and then phosphorylate the regulating protein of CaN (RCAN1), thus inhibiting this phosphatase (Kim et al., 2015). The high Ca^{2+} concentrations can also activate reactive oxygen species (ROS), which can bind to the zinc region of the CaN catalytic domain leading to its inhibition (Wang et al., 1996). Conversely, Rusnak and Reiter described that CaN directly modulates ROS (Rusnak and Reiter, 2000).

Besides this modulation by Ca^{2+} intracellular levels, endogenous inhibitors of CaN have been described, such as Cain and Cabin (Lai et al., 1998; Sun et al., 1998), as well as CaN-B homologous peptides (Lin et al., 1999; Parry and June, 2003). Cain is a soluble cytosolic protein presenting a pattern of expression in the brain that closely resembles that of calcineurin. Cain binds to both CaN-A and CaN-B, inhibiting calcineurin and suppressing synaptic vesicle endocytosis (Lai et al., 2000, 1998). Cabin 1, on the other hand, is a nuclear protein that requires an hyperphosphorylated state dependent on both calcium signals and PKC activation, to produce a high-affinity interaction with CaN (Sun et al., 1998).

Alternatively, inhibition of CaN can be generated by synthetic inhibitors such as CsA and FK506. Both are immunosuppressants

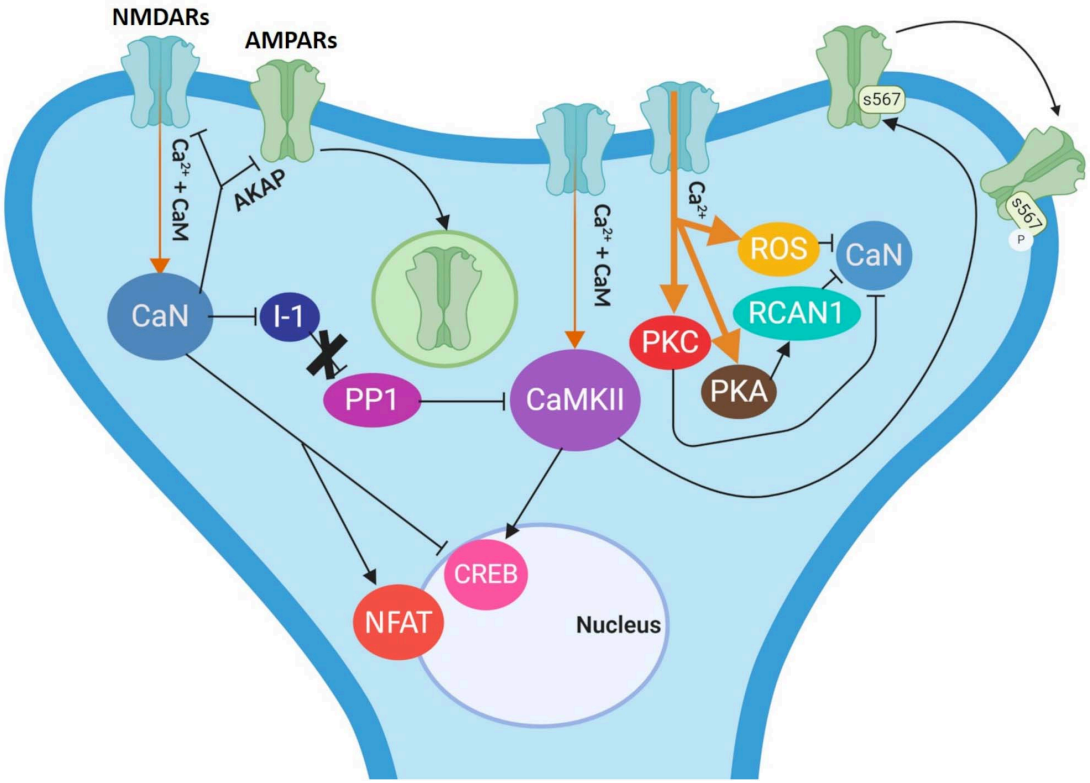

FIGURE 3 | Molecular modulation of extinction. In low concentrations (thin orange arrows), the Ca^{2+}/calmodulin complex activates CaN, which then dephosphorylates the I-1 (inhibitor 1), allowing PP1 to dephosphorylate CaMKII. CaN also dephosphorylates NMDARs (in blue) and AMPARs (in green) through AKAPs leading to AMPARs endocytosis, thus promoting LTD induction. NMDAR stimulation that induce LTD may also activate CaMKII, which elicits the phosphorylation of the AMPARs at residue S567 of the GluA1 subunit, propitiating their removal to the extra-synaptic space. CaN dephosphorylates CREB and the NFAT transcription factors as well. High concentrations of Ca^{2+} (thick orange arrows) activate kinases such as PKC and PKA, ROS and RCAN1 may act as CaN endogenous inhibitors. Arrows indicate activation while T arrows indicate inhibition either by phosphorylation or dephosphorylation. I-1: inhibitor 1; PP1: protein phosphatase 1; NFAT: nuclear factor of activated T-cells; CREB: cAMP response element binding; AKAP: A-Kinase Anchoring Protein; ROS: reactive oxygen species; PKA: protein kinase A; PKC: protein kinase C.

and bind to immunophilins forming complexes, cyclophilin with CsA and the binding protein of FK506 (FKBP12) in the case of FK506. These two complexes inhibit CaN in a very similar fashion, binding the region between CaN-B and CaN-A subunits, eliciting the split of the Ca^{2+}/calmodulin complex. This allows the AID to bind with the catalytic region, once more producing a state of inhibited CaN.

CaN as a Modulator of Receptor Mobility: NMDA and AMPA Receptors Participation in Extinction

We will now focus on the experimental evidence, highlighting the participation of NMDA and AMPA receptors in the extinction process. Hence, the role of CaN as a regulator of the mobility of these receptors, through the mechanisms described above, becomes particularly important during the expression of extinction.

NMDA Receptors

In this sense, the administration of AP5 (an antagonist of the NMDAR) into the amygdala during extinction training blocked the extinction of conditioned fear (Falls et al., 1992). Moreover, the administration of AP5 into the lateral amygdala (LA) also blocked the induction of LTD, preventing the endocytosis of AMPA receptors (Yu et al., 2010). Furthermore, the administration of CPP, another antagonist of NMDAR, during extinction training allowed the acquisition of fear conditioning extinction but blocked its retrieval, which allows to conclude that the consolidation of extinction requires NMDARs (Santini et al., 2001). Sotres-Bayón and collaborators reported that the selective blockade of the NMDARs subunit GluN2B with ifenprodil before extinction training impaired the acquisition and retrieval of fear extinction (Sotres-Bayon et al., 2007). Additionally, it was shown that GluN2A and GluN2B play differential roles in the acquisition and extinction of conditioned fear. The blockade, of GluN2A by NVP-AAM077 before conditioning, impairs acquisition but not extinction, and the blockade of GluN2B, by Ro25-6981, disrupts extinction but not acquisition. These results show a differential role of the NMDARs conformation on conditioning and extinction learning (Dalton et al., 2012). Moreover, the blockade of NMDAR and the inhibition of PKA, CaMKII and MAPK before or after the first test session in a fear-potentiated

startle paradigm impaired the extinction, meaning that these molecules must be part of the underlying mechanisms of extinction learning (Szapiro et al., 2003).

Following this line of ideas, the administration of D-cycloserine (DCS), a partial agonist of the NMDARs, has been reported to facilitate fear extinction (Walker et al., 2002; Ledgerwood et al., 2003, 2005; Woods and Bouton, 2006; Bouton et al., 2008). In addition, DCS administration during extinction training promoted the internalization of the AMPA receptors subunit GluA1 as well as fear-extinction learning (Mao et al., 2008).

More recently, it was shown that D-serine, the endogenous co-agonist of NMDARs, is required for the extinction of cocaine-induced behavioral sensitization and for the establishment of LTD (Liu et al., 2016).

AMPA Receptors

On the other hand, the systemic administration of the AMPA receptor agonist, PEPA {4-[2-(phenylsulfonylamino) ethylthio]-2,6-difluorophenoxyacetamide}, before extinction training led to facilitation of contextual fear extinction. This effect was blocked by the administration of NBQX, an AMPA receptor antagonist (Zushida et al., 2007). Additionally, AMPARs endocytosis blockade during the initial extinction session disrupts both the expression and recall of fear extinction (Dalton et al., 2008). In a series of studies Clementine and Huganir showed that PKA phosphorylation of GluA1 S845 promotes synaptic insertion of GluA1 Ca^{2+}-permeable AMPARs at synapses in the amygdala during fear conditioning to prime subsequent extinction that removes AMPA receptors *via* LTD or depotentiation (Clementine and Huganir, 2010, 2013). Correspondingly, the blockade of GluA2/AMPAR removal in the hippocampus during conditioning prevented the decay of long-term object location memories and impaired depotentiation but not induction of LTP (Migues et al., 2016). In addition, the administration of naltrexone (an antagonist of the μ-opioid receptors) before acquisition promoted AMPAR phosphorylation and its consequent insertion into the membrane, thus protecting memory from extinction (Kibaly et al., 2016).

As we have described, AMPAR trafficking is important for the expression of Hebbian plasticity, but it is also a fundamental process in homeostatic plasticity. For instance, it has been shown that the GluA2 subunit dephosphorylation for both Hebbian and homeostatic plasticity leads to AMPARs internalization, promoting mGluR-LTD and homeostatic synaptic downscaling (Gladding et al., 2009; Cingolani et al., 2019).

These processes occur through different induction mechanisms in the case of mGluR-LTD, mGluR1/5 (group I metabotropic glutamate receptors) are activated by synaptically released glutamate, whereas in the case of homeostatic synaptic downscaling, mGluR1/5 are activated by the immediate early gene Homer1a. The mGluR1/5 signaling then regulates HCN (hyperpolarization-activated, cyclic nucleotide-gated) channel activity, which is also closely involved in the homeostatic plasticity regulation (Cingolani et al., 2019). These mechanisms are still barely explored in relation to extinction. As an example, a recent study showed that mRNA expression of Homer1a in the hippocampus increased after fear extinction (Clifton et al., 2017).

Metaplastic Modulation of Extinction by Kinases/Phosphatases and Neurotrophic Factors

To finish, it is relevant to mention that metaplasticity differential regulation by kinases/phosphatases or neurotrophic factors could also play a role on extinction, e.g., our group reported that the blockade of PKC but not PKA prevented the LTP impairment produced by CTA training (Rodríguez-Durán and Escobar, 2014), thus revealing differential roles of protein kinases on metaplasticity. On the other hand, we have also reported that the infusion of brain-derived neurotrophic factor (BDNF) in the insular cortex promotes CTA-extinction (Rodríguez-Serrano et al., 2014), showing that BDNF is a key regulator and mediator in the extinction process. This metaplastic view of extinction could guide research toward new perspectives that include other processes, factors and modulators potentially involved in extinction.

DISCUSSION

We have described experimental evidence supporting that low Ca^{2+} influx triggers the activation of CaN, which in turn leads to the AMPARs internalization, CaMKII inactivation and CREB repression, thus promoting the expression of LTD, depotentiation and extinction.

Nowadays, the notion still prevails that while kinases preferentially participate in the conditioning process, phosphatases underlie extinction (Pagani and Merlo, 2019). Nevertheless, recent evidence begins to present a nuanced approach of such concepts, since it has been shown that phosphatases are involved in the generation of LTP, while kinases are also involved in the expression of LTD (Coultrap et al., 2014; Sanderson et al., 2016; Woolfrey et al., 2018; Wang et al., 2019; Purkey and Dell'Acqua, 2020; Xia et al., 2021). Likewise, there is some evidence associating LTD to conditioning (Altinbilek and Manahan-Vaughan, 2009; Bilbao et al., 2020; Haley et al., 2020) and the activity of kinases to extinction (Wang et al., 2020; Fukushima et al., 2021; Qiao et al., 2021; Xia et al., 2021). Because of that, synaptic plasticity as a general phenomenon underlying learning and memory might be a more suitable concept than LTP and LTD as the respective underlying mechanisms for conditioning and extinction.

These recent data suggest that the molecular actors of conditioning and extinction may not be dissociated nor distinct. Instead, the same molecular elements could be contributing to both processes, with different targets, probably depending on different cellular conditions (e.g., levels of Ca^{2+}), which must be considered for further explorations.

Furthermore, homeostatic plasticity should also be considered for further research on the extinction memory field. Indeed, we have described some shared mechanisms between Hebbian and homeostatic plasticity that allow similar outcomes, such as AMPARs internalization, depotentiation or LTD. We have

also presented evidence that extinction could lead to metaplastic changes (Rivera-Olvera et al., 2018). With this full view, however, we may also question whether extinction could be considered as a behavioral metaplastic process itself. Although there is evidence that extinction requires the formation of a new engram (Zhang et al., 2020), there is also evidence that extinction could be a synaptic silencing mechanism (Arendt et al., 2013). These findings open new perspectives in the field. In this sense, we should contemplate the participation of homeostatic plasticity, synaptic silencing, as well as the balance between phosphatases and kinases for a broader study of extinction learning.

AUTHOR CONTRIBUTIONS

SR-G: conceptualization, drafting the article, and revising it critically for important intellectual content. ME: conceptualization and design, drafting the article and revising it critically for important intellectual content, and funding acquisition. Both authors contributed to the article and approved the submitted version.

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Activation of Rac1 Has an Opposing Effect on Induction and Maintenance of Long-Term Potentiation in Hippocampus by Acting on Different Kinases

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Rac1 is a small GTPase of the Rho family. A previous study showed that the activation of Rac1 had an opposing effect on induction and maintenance of long-term potentiation (LTP) in the hippocampus. However, the molecular mechanism underlying this opposing effect remains to be addressed. In the present work, we find that the activation of Rac1 during the induction of LTP leads to an activation of PKCι/λ by phosphatidylinositol-3-kinase (PI3K), whereas the activation of Rac1 during the maintenance of LTP leads to the inhibition of PKMζ by LIM_kinase (LIMK) in the hippocampus. This result suggests that during different stages of LTP, the activation of Rac1 can modulate different signaling pathways, which leads to an opposing effect on the induction and maintenance of LTP in the hippocampus.

Keywords: Rac1, long term potentiation, PI3K, LIMK, PKCι/λ, PKMζ

INTRODUCTION

Small GTPases are important signaling molecules in neurons. One of the best characterized subfamilies of the small GTPases is the Rho family, which includes Rac, Cdc42, and Rho (Hall, 2005). Among them, Rac1 has been reported to be involved in morphological plasticity in the hippocampus. Rac1 could induce spine morphogenesis and synapse formation in the hippocampus (Luo, 2000; Tolias et al., 2005). In addition, Rac1 also participates in functional plasticity in the hippocampus. Martinez and Tejada-Simon (2011) reported that the induction of long-term potentiation (LTP) in the hippocampus was coupled with the activation of Rac1 and the inhibition of Rac1 suppressed the induction of LTP in a dose-dependent manner (Martinez and Tejada-Simon, 2011). Interestingly, during the maintenance phase of LTP in the hippocampus, it appeared that the activation of Rac1 had an opposing effect on LTP. Liu et al. reported that the application of an adeno-associated virus that carried transgene to activate Rac1 during the maintenance phase of LTP resulted in an accelerated LTP decay in the hippocampus (Liu et al., 2016). However, the molecular mechanism underlying the opposing effect of the activation of Rac1 on the induction and maintenance of LTP in the hippocampus remains to be addressed.

Typical downstream signal transduction pathway of the activation of Rac1 includes two steps: first, the activation of p21-activated kinase (Pak); second, the Pak-induced activation of LIM-domain-containing protein kinase (LIM-kinase), which subsequently phosphorylates and inhibits cofilin, an actin depolymerization factor, thus inducing actin polymerization (Luo, 2000). However, it is hard to explain the opposing effect of the activation of Rac1 on the induction and maintenance of LTP using this typical downstream signal transduction pathway of Rac1.

It has been known that numerous signaling molecules have been involved in the induction and maintenance of LTP (Baltaci et al., 2019). Among them, Wang et al. (2016) reported that phosphorylated protein kinase C iota/lambda (pPKCι/λ) showed a marked increase during the induction phase of LTP but returned to the control level during the maintenance phase of LTP, whereas PKMζ increased significantly only during the maintenance phase of LTP (Wang et al., 2016). Using a recombinant adeno-associated virus (rAAV2/8) expressing small hairpin RNA (shRNA) that targeted the gene of either PKCι/λ or PKMζ, it was found that the knockdown of PKCι/λ produced a reduction in the early expression of LTP during the induction phase, whereas the knockdown of PKMζ disrupted only the late phase of LTP during the maintenance phase (Wang et al., 2016). These pieces of evidence suggest that the activation of PKCι/λ played an important role in the induction of LTP, while the activation of PKMζ played a key role in the maintenance of LTP. Therefore, we proposed a hypothesis that Rac1 might have an opposing effect on PKCι/λ and PKMζ, thus producing an opposing effect on the induction and maintenance of LTP in the hippocampus. To test this hypothesis, using electrophysiological method combined with the Western blotting and pharmacological approaches, we studied the role of the activation of Rac1 during the induction and maintenance phases of LTP in the hippocampus and further explored the downstream signaling pathways of Rac1 activation during the induction and maintenance phases of LTP.

RESULTS

Activation of Rac1 Has an Opposing Effect on the Induction and Maintenance of LTP in Hippocampal CA1

Long-term potentiation (LTP) is generally divided into at least two distinct phases: the induction phase and the maintenance phase (Baltaci et al., 2019). To evaluate the role of Rac1 in the induction of LTP, we first detected whether Rac1 was activated during the induction phase of LTP. Rats were divided into four groups: one group was the control group where Hippocampal CA1 slices were not given high-frequency stimulation (HFS),and the other three groups were divided into 1-min group, 10-min group, and 30-min group based on the time duration after giving HFS. The result showed that the level of the activation state of Rac1 (Rac1-GTP) was significantly increased at 10 min after HFS (one-way ANOVA, $F_{(3,8)} = 63.24$, control group, 0.19 ± 0.029, $n = 3$; 10-min group, 0.84 ± 0.033, $n = 3$; $P < 0.0001$; **Figure 1A**), but returned to control level at 30 min after HFS (one-way ANOVA, 30-min group, 0.30 ± 0.055, $n = 3$, vs. control group; $P = 0.1974$; **Figure 1A**). This result suggests that LTP induction is associated with a transient activation of Rac1. We then studied the role of Rac1 activation in LTP induction by examining the influence of Rac1-specific inhibitor NSC23766 (Martinez and Tejada-Simon, 2011) on the LTP induction. Rats were divided into two groups: control group where artificial cerebrospinal fluid (ACSF) was applied at 30 min before HFS, and NSC23766 group where NSC23766 (100 μM) was applied at 30 min before HFS. The result showed that after the application of NSC23766, Rac1 activation by HFS at 10 min after HFS was significantly inhibited when compared with the control group (unpaired *t*-test, $t_{(4)} = 4.893$, control group, 0.85 ± 0.068, $n = 3$; NSC23766 group, 0.49 ± 0.029, $n = 3$; $P = 0.0081$; **Figure 1B**), and LTP induction by HFS was also significantly inhibited by NSC23766 (unpaired *t*-test, $t_{(10)} = 5.592$, control group, $146.7 \pm 4.1\%$, $n = 6$; NSC23766 group, $108.3 \pm 8.8\%$, $n = 6$; $P = 0.0027$; **Figure 1C**). These results suggest that Rac1 activation contributes significantly to LTP induction.

We also evaluated the contribution of Rac1 activation to LTP maintenance by examining the influence of Rac1-specific inhibitor NSC23766 on the LTP maintenance. NSC23766 was applied 10 min after the last HFS. Result showed that LTP maintenance was unaffected after the application of NSC23766 (unpaired *t*-test, $t_{(10)} = 0.3290$, control group, $139.0 \pm 5.9\%$, $n = 6$; NSC23766 group, $138.1 \pm 7.7\%$, $n = 6$, $P = 0.7489$; **Figure 2A**). This result suggests that Rac1 activation does not contribute to LTP maintenance. Interestingly, when we applied Rac1 agonist CN04 (Jiang et al., 2016) during the maintenance phase of LTP, CN04 induced an accelerated decay of LTP. The rats were divided into two groups: control group where ACSF was applied and CN04 group where CN04 (424 nM) was applied. The top panel of **Figure 2B** shows that in a normal brain slice containing the CA1 region, 90 min treatment with CN04 could significantly increase the level of Rac1-GTP (unpaired *t*-test, $t_{(4)} = 7.044$, control group, 0.56 ± 0.047, $n = 3$; CN04 group, 1.13 ± 0.065, $n = 3$; $P = 0.0021$; Top panel of **Figure 2B**). The middle and bottom panels of **Figure 2B** show the effect of CN04 on LTP maintenance and baseline of field excitatory post-synaptic potentials (fEPSPs). We could see that CN04 treatment resulted in an accelerated LTP decay during the maintenance phase (unpaired *t*-test, $t_{(10)} = 5.831$, control group, $123.2 \pm 6.4\%$, $n = 6$; CN04 group, $69.1 \pm 6.7\%$, $n = 6$; $P = 0.0002$; The middle panel of **Figure 2B**), whereas the treatment had no influence on baseline of fEPSPs (unpaired *t*-test, $t_{(10)} = 5.592$, 30 min control group, $97.3 \pm 4.5\%$, $n = 6$; 210 min CN04 group, $107.0 \pm 5.8\%$, $n = 6$; $P = 0.2239$; The bottom panel of **Figure 2B**). CN04 is not a specific activator of Rac1 agonist, as it also activates CDC42 and RhoA. So the influence of CN04 on LTP may be due to Rac1, CDC42, or RhoA. To confirm the CN04-induced decrease in LTP maintenance was mediated by Rac1 activation, the Pak1, a specific downstream molecular of Rac1 pathway, was inhibited by IPA-3 before CN04 treatment. The rats were divided into two groups: CN04 group where CN04 (424 nM) was applied, and IPA-3 + CN04 group where the Pak1 inhibitor IPA-3 (100 μM) and CN04 were co-applied. **Supplementary Figure 1** shows that Pak1 inhibitor IPA-3 could reverse CN04-induced decrease in LTP maintenance (unpaired *t*-test, $t_{(10)} = 3.270$, CN04 group,

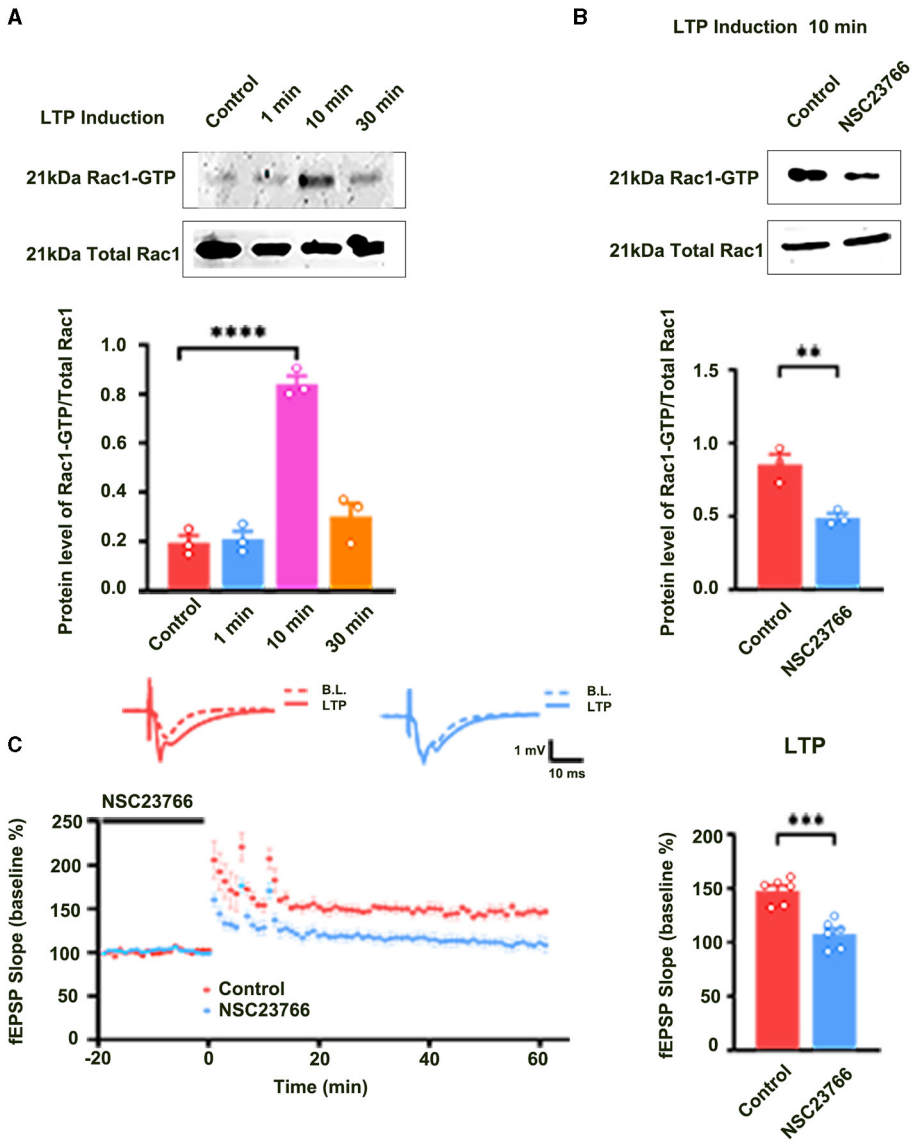

FIGURE 1 | The role of Rac1 in LTP induction. **(A)** Influence of three trains of HFS on Rac1 activation (one-way ANOVA, $^{****}P < 0.0001$, $n = 3$ in each group). **(B)** Influence of Rac1 antagonist NSC23766 (100 μM) on Rac1 activation at 10 min after HFS (unpaired *t*-test, $^{**}P < 0.01$, $n = 3$ in each group). **(C)** Influence of Rac1 antagonist NSC23766 (100 μM) on LTP induction (unpaired *t*-test, $^{***}P < 0.001$, $n = 6$ in each group). Top: Typical fEPSP trace at baseline and during LTP recordings. Left: LTP was recorded for 1 h after three trains of HFS. Right: Average fEPSP slope during the last 15 min of the LTP recording. All data are shown as mean ± SEM.

69.1 ± 6.7%, $n = 6$; IPA-3 + CN04 group, 116.4 ± 7.7%, $n = 6$; $P = 0.0084$; The bottom panel of **Figure 2B**), suggesting that CN04-induced decrease in LTP maintenance was related to Rac1 activation. These results suggest that the activation of Rac1 during the maintenance phase accelerates LTP decay.

Activation of Rac1 During the Induction Phase of LTP Results in Activation of PKCι/λ Through PI3K Pathway in the Hippocampus

Previous study showed that PKCι/λ was activated during the induction phase of LTP and the knockdown of PKCι/λ could inhibit LTP induction (Wang et al., 2016). However, the manner in which PKCι/λ is activated during the induction phase of LTP remains to be addressed. To evaluate whether Rac1 activation was an upstream mechanism of the activation of PKCι/λ during the induction phase of LTP, we examined the influence of Rac1 inhibitor NSC23766 on HFS-induced activation of PKCι/λ. The rats were divided into three groups:one group was the control group where the slices were not given HFS,and the other two groups were divided into 10- min group and 30-min group according to the time duration after giving HFS. **Figure 3A** shows HFS-induced activation of PKCι/λ (one-way ANOVA, $F_{(2,6)} = 19.48$, control group, 0.49 ± 0.041, $n = 3$; 30-min group, 1.06 ± 0.090, $n = 3$; $P = 0.0020$; **Figure 3A**). **Figure 3B**

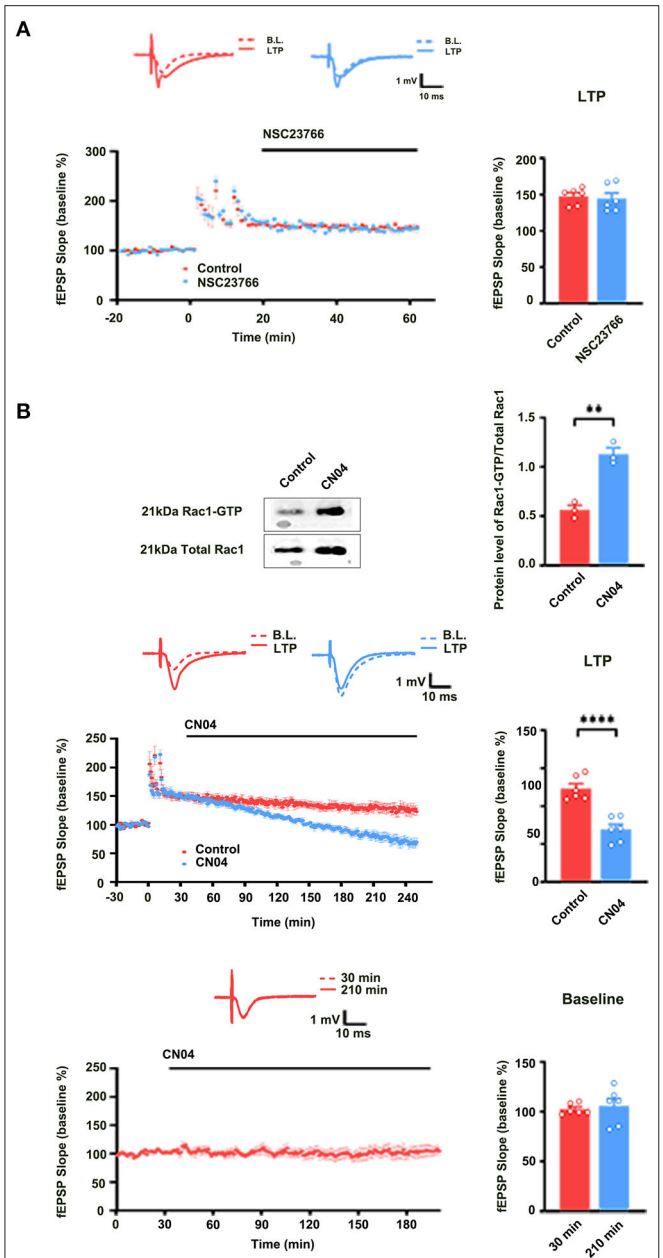

FIGURE 2 | The role of Rac1 in LTP maintenance. **(A)** Influence of Rac1 antagonist NSC23766 (100 μM) on the LTP maintenance (Unpaired *t*-test, $P > 0.05$, $n = 6$ in each group). **(B)** Top: Influence of Rac1 agonist CN04 (424 nM) on Rac1 activation (unpaired *t*-test, $^{**}P < 0.01$, $n = 3$ in each group). Top right: Average fEPSP slope during the last 15 min of the LTP recording. Middle: Influence of Rac1 agonist CN04 (424 nM) on the LTP maintenance (unpaired *t*-test, $^{****}P < 0.0001$, $n = 6$ in each group). Middle right: Average fEPSP slope during the last 15 min of the LTP recording. Bottom: Influence of Rac1 agonist CN04 (424 nM) on the fEPSP baseline (unpaired *t*-test, $P > 0.05$, $n = 6$ in each group). All data are shown as mean ± SEM.

shows the influence of NSC23766 on HFS-induced activation of PKCι/λ. We could see that after the application of NSC23766, HFS-induced activation of PKCι/λ was inhibited (one-way ANOVA, $F_{(2,6)} = 3.02$, control group, 0.54 ± 0.043, $n = 3$; 30-min group, 0.69 ± 0.049, $n = 3$; $P = 0.1233$; **Figure 3B**). This result suggests that Rac1 activation is an upstream mechanism of the activation of PKCι/λ during the induction phase of LTP.

We further studied how Rac1 activation results in the activation of PKCι/λ. We examined the influence of the specific P13K inhibitor LY294002 (Hsueh et al., 2015) on Rac1-induced activation of PKCι/λ. Firstly, we examined Rac1 agonist CN04-induced activation of PKCι/λ. The rats were divided into five groups:one group was control group where ACSF was applied, and the other four groups were divided into 15-min group, 30-min group, 60-min group, and 90-min group according to the duration of the application of CN04 (424 nM). **Figure 4A** shows Rac1 agonist CN04-induced activation of PKCι/λ (one-way ANOVA, $F_{(4,15)} = 5.899$, control group, 0.72 ± 0.014, $n = 4$; 60-min group, 1.03 ± 0.073, $n = 4$, vs. control group; $P = 0.0443$; 90-min group, 1.20 ± 0.140, $n = 4$, vs. control group; $P = 0.0023$; **Figure 4A**). This result suggests that CN04 indeed can induce the activation of PKCι/λ. We then examined the influence of LY294002 on Rac1-induced activation of PKCι/λ. The rats were divided into three groups:control group where ACSF was applied, CN04 group where CN04 (424 nM) was applied, and CN04 + LY294002 group where CN04 (424 nM) and LY294002 (100 μM) were co-applied. **Figure 4B** shows the influence of LY294002 on Rac1-induced activation of PKCι/λ. We could see that after the application of LY294002, Rac1-induced activation of PKCι/λ was inhibited (one-way ANOVA, $F_{(2,6)} = 16.96$, CN04 group, 1.17 ± 0.079, $n = 3$; LY294002 + CN04 group, 0.77 ± 0.033, $n = 3$; $P = 0.0037$; **Figure 4B**), whereas LY294002 treatment had no influence on basis of PKCι/λ activation (unpaired *t*-test, $t_{(4)} = 0.6160$, control group, 0.74 ± 0.031, $n = 3$; LY294002 group, 0.71 ± 0.028, $n = 3$; $P = 0.5712$; **Figure 4C**). These results suggest that Rac1 activation may result in the activation of PKCι/λ through PI3K pathway in the hippocampus.

Activation of Rac1 During the Maintenance of LTP Results in the Inhibition of PKMζ Through LIMK Pathway in the Hippocampus

Previous study showed that PKMζ was a key molecule for the maintenance of LTP (Baltaci et al., 2019). It is unclear whether Rac1-induced LTP decay during the maintenance phase is related to the inhibition of the expression of PKMζ. To address this question, we examined the effect of a specific Rac1 agonist CN04 on increased expression of PKMζ during the maintenance phase of LTP. The rats were divided into three groups:one group was control group where slices were not given HFS,and the other two groups were divided into 60-min group and 120-min group based on the time duration after giving HFS. **Figure 5A** shows that the expression of PKMζ showed a significant increase at 120 min after LTP induction (one-way ANOVA, $F_{(2,6)} = 14.83$, control group, 0.80 ± 0.061, $n = 3$; 120-min group, 1.52 ± 0.088, $n = 3$; $P = 0.0031$; **Figure 5A**). **Figure 5B** shows the influence of CN04 on the increased expression of PKMζ during the maintenance phase of LTP. We could see that after the application of CN04, the expression of PKMζ did not change at 60 and 120 min after LTP

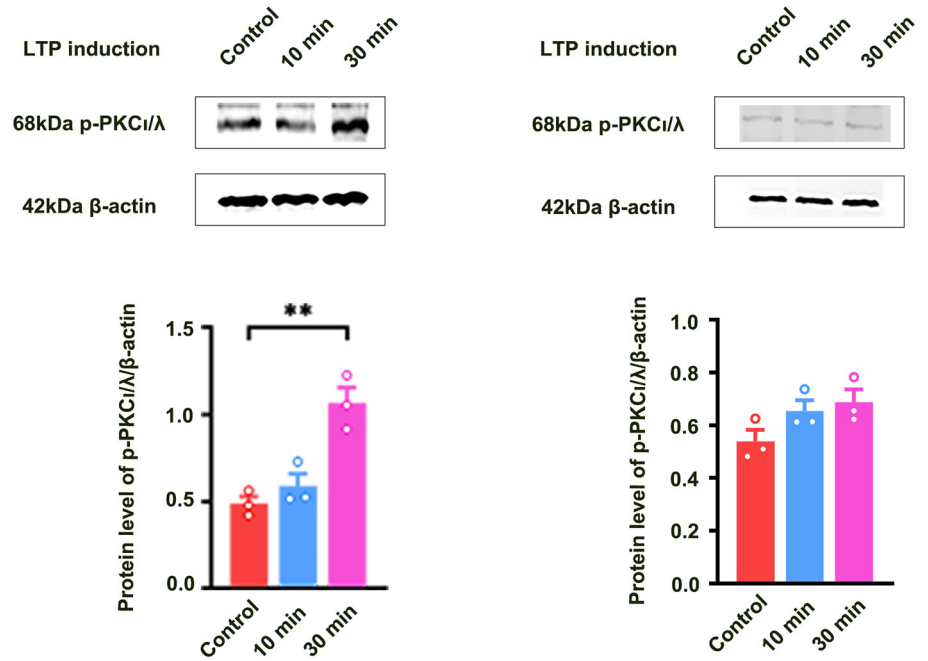

FIGURE 3 | Influence of Rac1 inhibitor NSC23766 on HFS-induced activation of PKCι/λ. **(A)** Influence of three trains of HFS on PKCι/λ activation (one-way ANOVA, $^{**}P < 0.01$, $n = 3$ in each group). **(B)** Influence of Rac1 antagonist NSC23766 (100 μM) on PKCι/λ activation after giving HFS (one-way ANOVA, $P > 0.05$, $n = 3$ in each group). All data are shown as mean ± SEM.

induction, compared with the control group (one-way ANOVA, $F_{(2,6)} = 1.480$, control group, 0.77 ± 0.045, $n = 3$; 120-min group, 0.96 ± 0.082, $n = 3$; $P = 0.3002$; **Figure 5B**). This result suggests that PKMζ is a downstream molecule of Rac1 activation during the maintenance phase of LTP.

We further studied how Rac1 activation results in a decrease in the expression of PKMζ. We examined the influence of a specific LIMK inhibitor BMS-5 (Lunardi et al., 2018) on Rac1-induced decrease in the expression of PKMζ. The rats were divided into three groups:a control group where ACSF was applied, CN04 group where CN04 was applied, and CN04 + BMS-5 group where CN04 and BMS-5 were co-applied. Result showed that after the application of BMS-5, Rac1-induced decrease in the expression of tetanization-induced PKMζ was reversed (one-way ANOVA, $F_{(2,6)} = 60.83$, CN04 group, 24.6 ± 3.5%, $n = 3$; control group, 92.1 ± 5.8%, $n = 3$, vs. CN04 group $P < 0.0001$; BMS-5+CN04 group, 52.8 ± 3.2%, $n = 3$, vs. CN04 group; $P = 0.0067$; **Figure 6** bottom right), whereas the BMS-5 treatment had no influence on the basis of the expression of PKMζ (unpaired *t*-test, $t_{(4)} = 0.2662$, control group, 0.84 ± 0.038, $n = 3$; BMS-5 group, 0.85 ± 0.030, $n = 3$; $P = 0.8033$; **Figure 6B**). This result suggests that Rac1-induced LTP decay during the maintenance phase is related to the inhibition of the expression of PKMζ in the hippocampus.

DISCUSSION

Previous studies have examined the role of the activation of Rac1 in LTP. Martinez and Tejada-Simon (2011) reported that the induction of LTP in the hippocampus was coupled with the activation of Rac1 in area CA1 and the inhibition of Rac1 suppressed the induction of LTP in a dose-dependent manner (Martinez and Tejada-Simon, 2011). This result is consistent with our current conclusion that Rac1 participates in the induction of LTP in the hippocampus. This statement was also supported by the findings that upon induction of structural LTP (sLTP) using uncaging of glutamate on single spines, an activation of Rac1 was observed and the addition of Rac1 inhibitor before sLTP induction effectively inhibited sLTP. However, the statement in this paper that the persistent activation of Rac1 was required for the maintenance of sLTP still lacked the evidence because the activation of Rac1 was observed only for 33 min and not for a longer duration. Moreover, Martinez and Tejada-Simon (2011) reported that the activity of Rac1 showed a transient increase during the induction of LTP, but returned to the control level during the maintenance phase of LTP (Saneyoshi et al., 2019). This result is consistent with our current statement that the level of Rac1 activity is rather low during the maintenance phase of LTP. This statement is also supported by the result

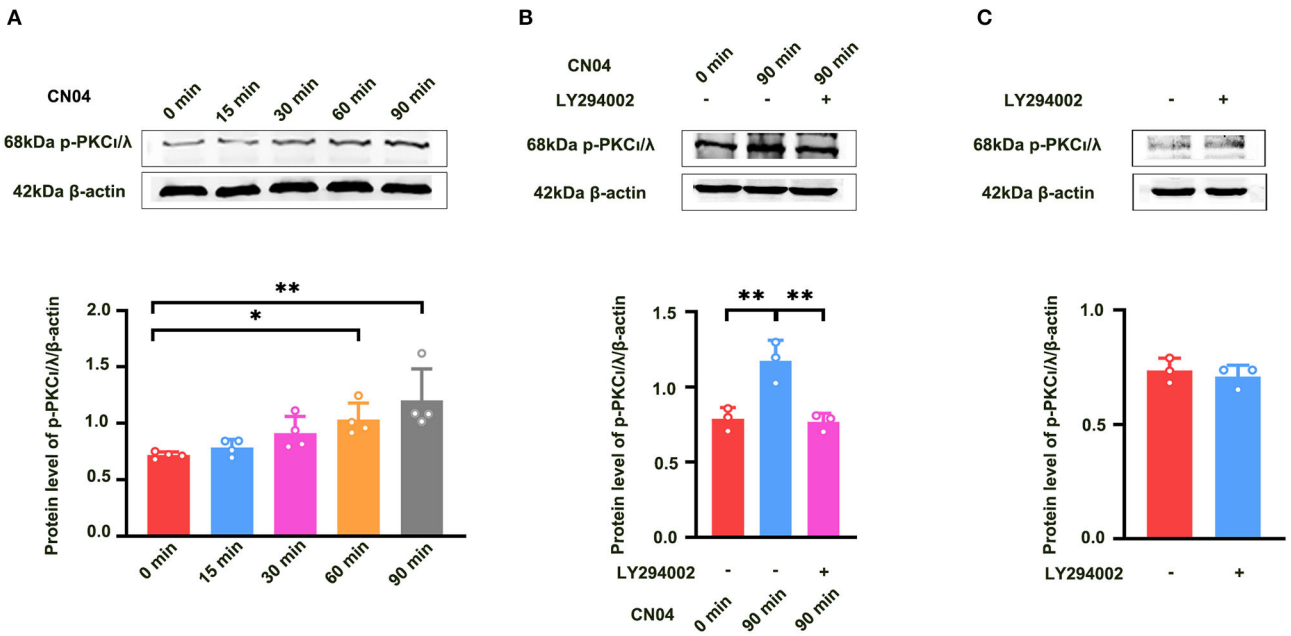

FIGURE 4 | Influence of P13K inhibitor LY294002 on Rac1-induced activation of PKCι/λ. **(A)** Influence of Rac1 agonist CN04 (424 nM) on PKCι/λ activation (one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 4$ in each group). **(B)** Influence of P13K inhibitor LY294002 (100 μM) on the effects of Rac1 agonist CN04 (424 nM) on PKCι/λ activation (one-way ANOVA, $^{**}P < 0.01$, $n = 3$ in each group). **(C)** Influence of P13K inhibitor LY294002 (100 μM) on PKCι/λ activation (unpaired *t*-test, $P > 0.05$, $n = 3$ in each group). All data are shown as mean ± SEM.

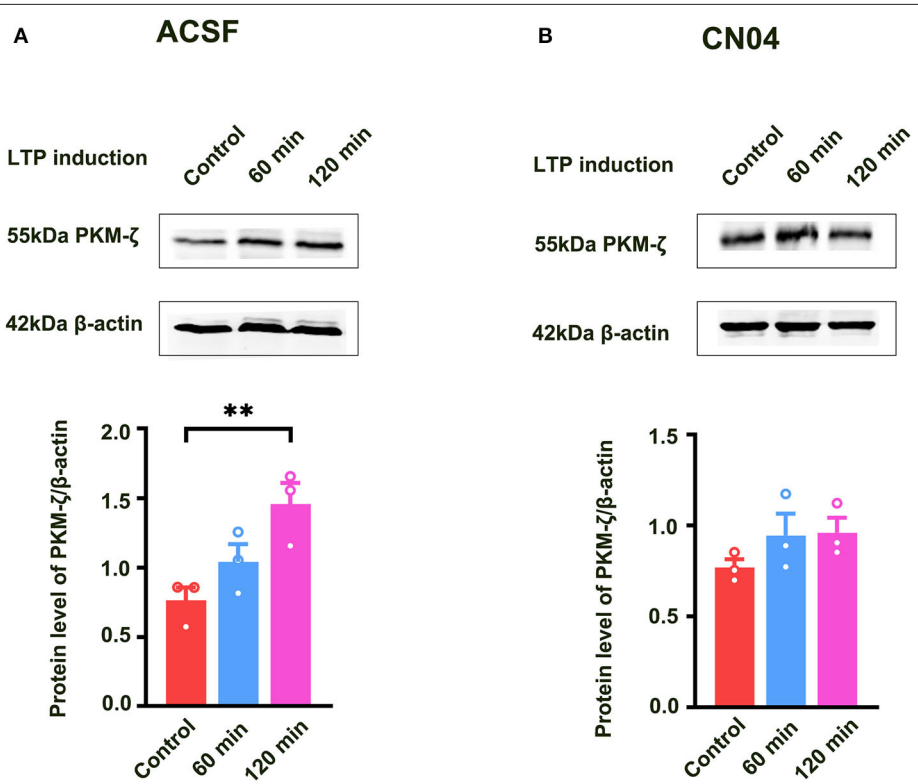

FIGURE 5 | Influence of Rac1 agonist CN04 on HFS-induced activation of PKMζ. **(A)** Influence of three trains of HFS on PKMζ expression (one-way ANOVA, $^{**}P < 0.01$, $n = 3$ in each group). **(B)** Influence of Rac1 agonist CN04 (424 nM) on PKMζ expression after giving HFS (one-way ANOVA, $P > 0.05$, $n = 3$ in each group). All data are shown as mean ± SEM.

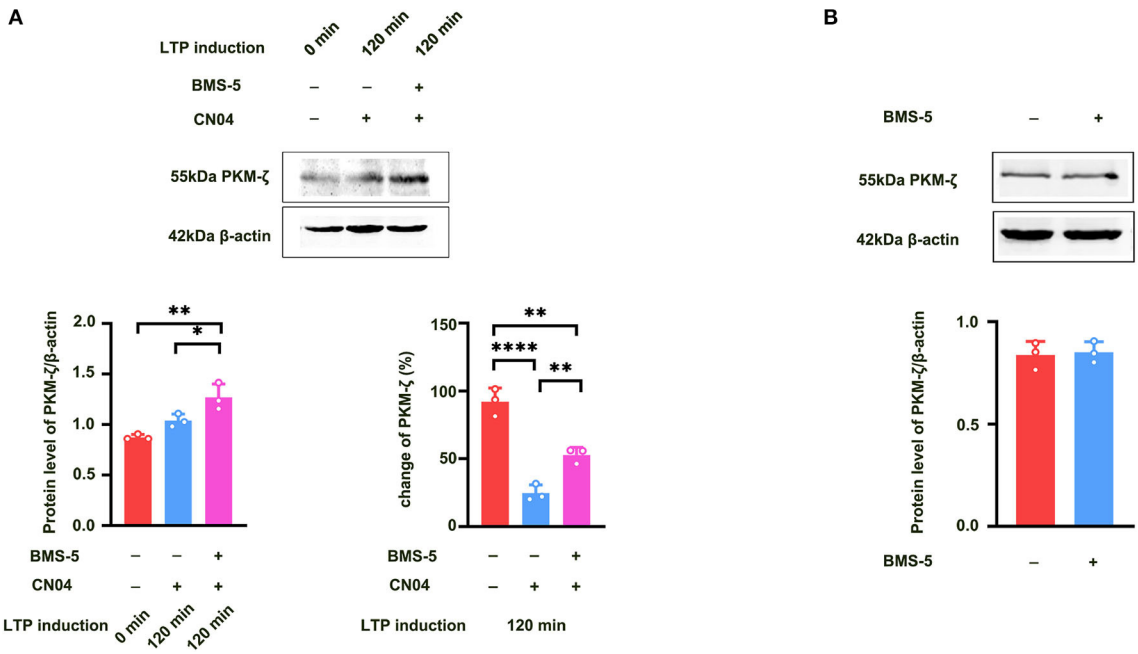

FIGURE 6 | Influence of LIMK inhibitor BMS-5 on Rac1-induced decrease in the expression of PKMζ. **(A)** Influence of LIMK inhibitor BMS-5 (100 μM) on the effects of Rac1 agonist CN04 (424 nM) on PKMζ expression at 120 min after giving HFS (Bottom left: one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 3$ in each group). Bottom right: The bar graph shows the change of PKMζ expression from 0 min to 120 min after LTP induction (one-way ANOVA, $^{**}P < 0.01$, $^{****}P < 0.0001$, $n = 3$ in each group; The control group data is calculated using the data of **Figure 5A**). **(B)** Influence of LIMK inhibitor BMS-5 (100 μM) on PKMζ expression (unpaired *t*-test, $P > 0.05$, $n = 3$ in each group). All data are shown as mean ± SEM.

that Rac1 activity is significantly elevated in the hippocampal tissues of adult mice in response to 7-day social isolation, but decreases to a low level after resocialization (Liu et al., 2018). In contrast, Liu et al. showed that there might be an activation of Rac1 during the maintenance phase of LTP because after the application of adeno-associated viruses (AAVs) that carried transgene to inhibit endogenous Rac1 activity, they found that LTP decay during the maintenance phase significantly decreased (Liu et al., 2016). The reason for the difference in Rac1 activation during the maintenance phase of LTP in different studies remains unknown. However, most of the results from literature (Liu et al., 2016, 2018) and our current study show that the activation of Rac1 during the maintenance phase of LTP in the hippocampus resulted in an accelerated LTP decay. The activator of Rac1 that we used here was CN04 that could also activate CDC42 and RhoA, and hence the influence of CN04 on LTP maintenance may be due to Rac1, CDC42, or RhoA. In order to determine whether CN04-induced decrease in LTP maintenance was related to Rac1 activation, we included an experiment on the influence of the inhibition of specific downstream target Pak1 (Lv et al., 2013) on CN04-induced decrease in LTP maintenance. The result showed that the Pak1 inhibitor IPA-3 could reverse CN04-induced decrease in LTP maintenance, suggesting that CN04-induced decrease in LTP maintenance was related to Rac1 activation.

It is interesting to study the reason behind why the activation of Rac1 during the induction and maintenance phases of LTP has an opposing effect in the hippocampus. In a typical downstream signal transduction pathway of the activation of Rac1, Rac1 first activates Pak, which then activates LIM-kinase, resulting in the phosphorylation of cofilin, thus inducing an actin polymerization. This pathway partly explains the mechanism underlying the participation of Rac1 in the induction of LTP because Rac1-induced actin polymerization results in an enlargement of dendritic spines, which leads to enhanced trapping of AMPA receptors in the postsynaptic membrane and potentiated synaptic transmission (Baltaci et al., 2019). In addition, Tolias et al. (2005) reported that Rac1 could associate with phosphoinositide 3-kinase (PI3K), whereas Ren et al. (2013) reported that LTP induced by PI3K activation could be significantly attenuated by PKCι/λ inhibitor Myr-aPKC-PS and the activation of PI3K could activate PKCι/λ (Ren et al., 2013). Therefore, it is possible that the activation of Rac1 during the induction phase of LTP leads to an activation of PKCι/λ by PI3K pathway in the hippocampus. This hypothesis was confirmed by our present result that the inhibition of PI3K could attenuate Rac1-induced activation of PKCι/λ. Since it has been known that PKCι/λ activation is required for both GluA1 phosphorylation and increased surface expression of AMPA receptors during the induction of LTP, it is possible that in addition to Rac1-induced Pak-LIMK-actin polymerization-enlarged dendritic spines pathway, PI3K-PKCι/λ-GluA1 phosphorylation / increased AMPA receptors may be another downstream signal pathway of the activation of Rac1 to participate in the induction of LTP. In addition, based on the already known upstream signaling pathway of Rac1

(Saneyoshi et al., 2019), the likely core signaling of early-LTP is: NMDAR to Ca2+ to CaMKII to Rac1 to PI3K to PKCι/λ to AMPA receptors. However, it is hard to explain the opposing effect of the activation of Rac1 on the maintenance of LTP using these two Rac1-induced pathways.

Molecules and signaling pathways mediating the maintenance of LTP have been identified in previous sections (Baltaci et al., 2019). Among them, the continuous enzymatic effect of the constitutively active PKMζ is thought to be the key molecule in the maintenance of LTP (Sacktor, 2011). Wang et al. (2016) reported that after 30 min of LTP induction, PKCι/λ significantly increased but then returned to control level 2 h after LTP induction, whereas PKMζ significantly increased after 2 h of LTP induction (Wang et al., 2016). In the meantime, LTP induction-induced increase in the Rac1 activity also returned to control level during the maintenance phase of LTP (Martinez and Tejada-Simon, 2011). Therefore, it appears that Rac1 does not participate in the maintenance of LTP. However, if exogenous activation of Rac1 during the maintenance phase of LTP, the activated Rac1 could inhibit LTP (Liu et al., 2016, 2018 and present result). Obviously, this inhibition is not due to the re-activation of PKCι/λ by Rac1 because the role of the activation of PKCι/λ is to potentiate synaptic transmission. Thus, we proposed that it was possible that the activation of Rac1 during the maintenance phase of LTP inhibited LTP via the suppression of PKMζ. This statement was supported by our present result that the activation of Rac1 during the maintenance phase of LTP could suppress PKMζ.

Positive feedback-like mechanism has been proposed to provide PKMζ required to maintain LTP (Baltaci et al., 2019). The PKMζ mRNA is carried to the dendrites following transcription, but its translation is inhibited by a peptidyl–prolyl isomerase PIN1. Following LTP induction, the activity of PIN1 diminishes and its suppressive effect on PKMζ translation ceases, and then PKMζ performs synthesis during the maintenance phase of LTP. So there are two possible ways by which the activation of Rac1 inhibits PKMζ: one is the inhibition of PKMζ transcription and the other is the inhibition of PKMζ translation. Obviously, the typical downstream signal transduction pathway of Rac1 through LIM-kinase-cofilin-actin cannot explain the inhibitory effect of Rac1 on PKMζ because this pathway is not related to the transcription and translation of proteins. Thus, alternative downstream pathways independent of cofilin-actin of Rac1 should be considered. Yang et al. (2004) reported that LIM-kinase could directly phosphorylate cAMP-responsive element-binding protein (CREB), which led to the stimulation of subsequent gene transcription (Yang et al., 2004) and participated in the maintenance of LTP (Todorovski et al., 2015). Ramos et al. reported that when phospho-CREB was increased in the aged prefrontal cortex, further stimulation of this pathway, even with a very low dose of an activator could exacerbate memory deficits (Ramos et al., 2003). These pieces of evidence suggest that if phospho-CREB already increases, further stimulation of this pathway may accelerate the decay of the maintenance of LTP, which exacerbates memory deficits. This statement was supported by the result that there was an activation of LIM-kinase accompanied by an increase in CREB during the maintenance phase of LTP (Todorovski et al., 2015) and that the further activation of LIM-kinase by Rac1 during the maintenance phase of LTP accelerated the decay of the maintenance of LTP. However, it is still unclear how over-activated CREB inhibits the transcription or translation of PKMζ.

In conclusion, the present results showed that the activation of Rac1 during the induction of LTP leads to an activation of PKCι/λ by PI3K, whereas the activation of Rac1 during the maintenance of LTP leads to the inhibition of PKMζ by LIMK in the hippocampus. These results suggest that during different stages of LTP, the activation of Rac1 can modulate different signaling pathways, which leads to an opposing effect on the induction and maintenance of LTP in the hippocampus.

STAR METHODS

Key Resources Table (KRT)

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-Rac1	Pierce	Cat#16118
anti-β-actin	Santa Cruz	Cat#sc-47778
anti-p-PKCι/λ (Thr555/563)	Abcam	Cat#ab-5813
anti-PKCζ (c-20)	Santa Cruz	Cat#sc-216
Medicine		
NSC23766	Tocris	Cat#2161
Rho/Rac/cdc42 Activator I (CN04)	Cytoskeleton	Cat#CN04-B
LY294002	MedchemExpress	Cat#9901s
IPA-3	Tocris	Cat#3622
BMS-5	MedchemExpress	Cat#HY-18305
Experimental Models: Organisms/Strains		
Wildtype Sprague Dawley rats, male	JSJ Biotech	N/A
Software and Algorithms		
Prism 10.7	GraphPad	https://www.graphpad.com
Adobe Photoshop CS6	Adobe	https://www.adobe.com
Clampfit 10.7	Axon	N/A
Other		
active Rac1 Pull-Down and Detection Kit	Pierce	Cat#16118

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Male adult (6–8 weeks) Sprague-Dawley rats were housed singly in a 12 h light/dark cycle in a temperature- and humidity-controlled environment with food and water freely available. All experimental procedures conformed to Fudan University as well as the international guidelines on the ethical use of animals. All

efforts were made to minimize animal suffering and reduce the number of animals used.

METHOD DETAILS

Slice Preparation and Electrophysiology

Hippocampal slices (400 μm) were prepared from 8-week-old rats using a vibratome (Leica) (Leutgeb et al., 2003). Slices were incubated in 32°C oxygenated artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 3 mM KCl, 1.25 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM glucose (pH 7.2–7.4) for at least 2 h before recording. Slices were placed in a recording chamber and perfused by oxygen-saturated ACSF with a flow rate of 4–5 ml/min. Extracellular field excitatory post-synaptic potentials (fEPSPs) in the Schaffer Collateral pathway were synaptically evoked at 0.017 Hz and recorded in the CA1 region. The fEPSPs were evoked using a stimulation intensity that elicited a 40% maximal response. LTP was induced by three trains of high-frequency stimulation (HFS, 1 s at 100 Hz spaced 5 min apart). The stimulation during the HFS was the same strength as test stimulation. The fEPSPs were recorded with an Axopatch700B amplifier (Axon) connected to a Digidata1440 interface (Axon). Data acquisition and analysis were performed using the Axon software packages Clampfit.

Western Blot Analysis

Four brain slices containing the hippocampal CA1 region were homogenized in a buffer containing 100 mM Tris-HCl (pH=6.7), 1% SDS, 143 mM 2-mercaptoethanol, and 1% protease inhibitor. The lysate was centrifuged at 12,000 rpm for 10 min at 4°C. The samples were treated with the SDS sample buffer at 100°C for 10 min, loaded on a 10% SDS polyacrylamide gel, and blotted to a nitrocellulose (NC) membrane. The membranes were blocked for 1 h at room temperature in a blocking solution (Beyotime, China), followed by incubation overnight at 4°C with various primary antibodies that included anti-Rac1 at a dilution of 1:500; anti-pPKCι/λ, anti-PKMζ, and anti-β-actin at a dilution of 1:1,000. Afterward, the membranes were rinsed with 1 × TBST (Sangon, China) for three times (5 min for each wash), followed by incubation respectively with IRDye 680 LT goat anti-rabbit secondary antibody (1:10,000) and IRDye 800 CW goat anti-mouse secondary antibody (1:10,000) for 1 h at room temperature. Finally, after rinsing the membranes for three times (5 min for each wash) with 1 × TBST, we acquired the images with LI-COR Odyssey system.

Assay for GTPase Activity

Active Rac1 pull-down was performed as described by the commercial active Rac1 Pull-Down and the Detection Kit protocol (Pierce, catalog #16118). Briefly, lysates of the rat hippocampal CA1 tissue were centrifuged at 16,000 *g* at 4°C for 15 min, and then the supernatants were transferred to a new tube, and GTPYS or GDP was added and incubated at 30°C for 15 min under the condition of constant agitation. The mixtures were then incubated with glutathione resin beads and glutathione S-transferase-fused Rac-binding domain of p21-activated kinase (Pak) at 4°C for 1 h; the beads had been washed several times previously to remove nonspecific binding. The beads and proteins bound to the fusion protein were washed three times with wash buffer at 4°C, eluted in SDS sample buffer, and analyzed for bound Rac1 by Western blotting using anti-Rac1.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical significance was determined using unpaired *t*-test for comparisons between two groups or ANOVAs for comparisons among three or more groups. All of the statistical details of the experiments can be found in the results. In all cases, *n* refers to the number of animals. Graphpad Prism 8.4 was used to process and analyze data and make statistical graphs. Data are presented as mean ± SEM.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Care and Use Committee of Shanghai Medical College of Fudan University.

AUTHOR CONTRIBUTIONS

DC was responsible for conception and design, acquisition of data, analysis, interpretation of data, and drafting or revising the article. XJ, MC, HS, DS, LY, XG, and YW were responsible for the acquisition of data and analysis and interpretation of data. BL was responsible for conception and design and analysis and interpretation of data. PZ was responsible for conception and design, analysis and interpretation of data, and drafting or revising the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2021.720371/full#supplementary-material

Supplementary Figure 1 | Influence of Pak1 inhibitor IPA-3 on CN04-induced decrease of LTP maintenance.Influence of adding Pak1 inhibitor IPA-3 (100 μM) before the application of CN04 (424 nM) on the CN04-induced decrease of LTP maintenance (unpaired *t*-test, $^{**}P < 0.01$, $n = 6$ in each group). All data are shown as mean ± SEM.

Supplementary Figure 2 | The full-length immunoblot.

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ORIGINAL RESEARCH
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Transcranial Laser Photobiomodulation Improves Intracellular Signaling Linked to Cell Survival, Memory and Glucose Metabolism in the Aged Brain: A Preliminary Study

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Aging is often accompanied by exacerbated activation of cell death-related signaling pathways and decreased energy metabolism. We hypothesized that transcranial near-infrared laser may increase intracellular signaling pathways beneficial to aging brains, such as those that regulate brain cell proliferation, apoptosis, and energy metabolism. To test this hypothesis, we investigated the expression and activation of intracellular signaling proteins in the cerebral cortex and hippocampus of aged rats (20 months old) treated with the transcranial near-infrared laser for 58 consecutive days. As compared to sham controls, transcranial laser treatment increased intracellular signaling proteins related to cell proliferation and cell survival, such as signal transducer and activator of transcription 3 (STAT3), extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), p70 ribosomal protein S6 kinase (p70S6K) and protein kinase B (PKB), also known as Akt that is linked to glucose metabolism. In addition, ERK is linked to memory, while ERK and JNK signaling pathways regulate glucose metabolism. Specifically, the laser treatment caused the activation of STAT3, ERK, and JNK signaling proteins in the cerebral cortex. In the hippocampus, the laser treatment increased the expression of p70S6K and STAT3 and the activation of Akt. Taken together, the data support the hypothesis that transcranial laser photobiomodulation improves intracellular signaling pathways linked to cell survival, memory, and glucose metabolism in the brain of aged rats.

Keywords: photobiomodulation, low-level laser therapy, brain, aging, intracellular signaling proteins

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INTRODUCTION

In the world, the number of people aged 60 and over has been growing faster than any age group (Desa, 2015). However, aging is accompanied by a cognitive impairment, which has been one of the socio-economic concerns in developed and developing countries (Attia and Ahmed, 2020). Among the various theories to interpret this damage, intracellular signaling proteins have gained special attention (Kosik et al., 2012). For example, the expression and activation of signaling proteins linked to cell growth, proliferation, and survival, as Akt, p70S6K and extracellular signal-regulated protein kinase (ERK), are decreased in the brain of aged rats, including the hippocampal formation, a brain region linked to certain mnemonic processes (Jin et al., 2008). Importantly, activation and expression of intracellular signaling proteins linked to apoptosis, such as c-Jun N-terminal kinase (JNK) and p38, are increased in the hippocampus of aged rats (O'Donnell et al., 2000). Current knowledge points to a crucial role of ERK and JNK signaling pathways in regulating glucose metabolism (Papa et al., 2019). In addition, Akt facilitates glucose metabolism by increasing the translocation of glucose transporters to the plasma membrane (Nicholson and Anderson, 2002). After glucose is transported inside cells, it can fuel mitochondrial respiration for ATP production during oxidative energy metabolism (Gonzalez-Lima et al., 2014). In this sense, the search for new therapies for the treatment or prevention of age-related harm is necessary for the medical society.

Transcranial stimulation with near-infrared light is a type of low–level laser therapy or photobiomodulation (PBM; Anders et al., 2015), which has been used recently as a non-pharmacological tool for the treatment of brain damage and age-related cognitive decline (Naeser et al., 2014; Vargas et al., 2017; Chan et al., 2019). For example, Naeser and collaborators (Naeser et al., 2014) demonstrated that 6 weeks of red/near-infrared light-emitting diode treatment improved the executive function and verbal memory of elderly people with chronic mild traumatic brain injury. Vargas and collaborators (Vargas et al., 2017) showed that a laser session with 1,064 nm wavelength and fluence of 60 J/cm^2 per week for 5 weeks improved the cognitive function and EEG rhythms of older adults with memory complaint. Using D-galactose-induced aging mice, Salehpour and collaborators (Salehpour et al., 2017) noted that red (660 nm) and near-infrared (810 nm) laser for 6 weeks attenuated the impairment in spatial and episodic-like memories. One hypothesis to explain these effects may be related to the capacity of PBM to regulate neuronal functions (Eells et al., 2003; Rojas et al., 2008; Freitas and Hamblin, 2016), including cell proliferation (Shefer et al., 2002; Gao et al., 2006), DNA and protein synthesis (Feng et al., 2012), and oxidative energy metabolism (Wang et al., 2017b).

Interestingly, some studies have shown that PBM can alter the expression of intracellular signaling proteins in several conditions (Yip et al., 2011; Meng et al., 2013; Zhang et al., 2020). For example, Yip and collaborators (Yip et al., 2011) noted that only one laser session was able to increase the Akt activation in mice after transient cerebral ischemia. Zhang and collaborators (Zhang et al., 2020) observed an increase in activation of PKA and SIRT1 in beta amyloid protein precursor transgenic mice. Based on this, this study investigated the cellular signaling proteins in the cortex and hippocampus of aged rats (20 months old) subjected to either a PBM protocol or a placebo/sham control for 58 consecutive days. For testing brain PBM effects, we analyzed the activation and expression of intracellular signaling proteins, such as Akt, p70S6K, signal transducer and activator of transcription 3 (STAT3), signal transducer and activator of transcription 5 (STAT5), ERK, JNK, and p38.

MATERIALS AND METHODS

Animals

Eleven male Wistar rats, aged (20 months old) were used in this study. The colony room was maintained at 21 ± 2°C with a 12 h light/ dark schedule (light: 7 am until 7 pm), and food and water were provided *ad libitum* throughout the experimental period. All experimental protocols were approved by the ethics committee of the University of Mogi das Cruzes (UMC; # 016/2017) and all efforts were made to minimize animal suffering in accordance with the proposals of the International Ethical Guideline for Biomedical Research (Council for International Organizations of Medical Sciences, 1985).

Laser and Control Protocols

The rats were randomly distributed into two groups: laser (n = 6) and control (n = 5). One week before the treatment protocol, the animals were adapted to manual handling (with no anesthesia). This procedure was adopted to minimize discomfort during treatment. After this, the animals from the laser group were manually immobilized and received treatment with a laser diode of 810 nm wavelength and 100 mW power for 30 s (3 Joules of energy/point) by approximation at each of the 5 irradiation points of application (point 1 = AP +4.20 mm and ML 0.00 mm; point 2 = AP −3.00 mm and ML −6.60 mm; point 3 = AP −3.00 mm and ML +6.60 mm; point 4 = AP 0.00 mm and ML 0.00 mm; point 5 = AP −5.52 mm and ML 0.00 mm). The daily laser treatment total was 15 Joules of energy, 150 s of irradiation, and fluency of 535.7 Joules/cm^2. Also, no difference in scalp temperature measured in the animals was noted with a non-contact thermometer during the treatment protocol, corroborating with the results of Wang and collaborators (Wang et al., 2017a). Animals were exposed to the transcranial low-level laser over 58 consecutive days. Our rationale was to investigate the therapeutic capacity of chronic treatment with laser. Animals from the control group received the same procedure as the laser group, but as placebo sham control (laser off). The parameters used in the present study were based on our previous studies with different animal models (Haslerud et al., 2017; Naterstad et al., 2018).

List of Laser Parameters

Center wavelength (nm): 810
Operating mode: CW
Average radiant power (W): 0.1

Aperture diameter (cm): 0.6
Irradiance at aperture (W/cm^2): 3.571
Beam shape: Circular
Beam spot size (cm^2): 0.028
Irradiance at target (W/cm^2): 3.571
Exposure duration/point (s): 30
Radiant exposure (J/cm^2) per session: 535.7
Number of points irradiated: Five
Delivery mode: contact mode
Number and frequency of sessions: one session/day for 58 consecutive days.
Total radiant energy (J) per head: 15

Analysis of Intracellular Proteins Levels

Tissue Preparation

Twenty-four hours after the final laser/placebo session (59th day of the experiment), the rats were euthanized by decapitation and their cerebral cortex (all cortical tissue) and hippocampus (Ammon's horn and dentate gyrus) were immediately collected and frozen. The tissue samples were homogenized in ice-cold RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS) with freshly added protease (Cat# M222–1ml; Lot# 1295C056; Amresco) and phosphatase (Cat# B15001-A and B; Lot# 510011; Biotool) inhibitors. Homogenates were centrifuged at 10,000× *g* for 10 min at 4°C and supernatants were transferred to a new tube.

Intracellular Proteins Measurements

The expression and activation (phosphorylated/total) of intracellular proteins in the brain samples were quantified using the Milliplex® MAP kits magnetic bead panel assay (48-680MAG and 48-681MAG, Merck Millipore) following the manufacturer's specifications. This multiplex immunoassay allows the simultaneous quantification of the expression and activation of cortical and hippocampal proteins: Akt, p70S6K, STAT3, STAT5, ERK, JNK, and p38. The plates were read on a Luminex™ Magpix™ instrument and results were analyzed with the Milliplex Analyst 5.1 Software using a Logistic 5P Weighted regression formula to calculate sample concentrations from the standard curves.

Statistical Analysis

Statistical procedures were conducted using the Mann-Whitney U-Test for independent group comparisons of nonparametric data. The Z-score was used to remove outlier values (−/+2 SD). In the cortex, we removed one outlier of p-ERK/ ERK from the control group and one outlier of p-p38/p38 from the laser group. In the hippocampus, we removed one outlier of STAT3 and STAT5 from the control group and one outlier of p-ERK/ERK from the laser group. All analyses were performed using the Statistical Package for the Social Science (SPSS Inc, IBM, version 221.0, Chicago, IL, USA). A statistical difference was considered significant when the two-tailed *P*-value was lower than 0.05. All plots were acquired using the Graph Pad Prism (6.0). Data are presented as individual points to show dispersion and as mean and standard error of the mean (±SEM).

RESULTS

The expression and activation of intracellular signaling proteins (Akt, p70S6K, STAT3, STAT5, ERK, JNK, and p38) were investigated in the cerebral cortex and hippocampus of rats from the laser and control groups. The cortical and hippocampal expression and activation are presented in (**Figures 1**, **2**). The figures showed that measures from laser-treated rats had less data dispersion than for the control rats for most proteins. However, data dispersion appeared similar for both cortex and hippocampus.

Cortical Expression and Activation of Intracellular Signaling Pathways

No significant difference between the groups studied was observed in the cortical expression of Akt ($U = 8.000$; $p = 0.201$), p70S6K ($U = 12.000$, $p = 0.584$), STAT3 ($U = 11.000$; $p = 0.465$), STAT5 ($U = 12.000$; $p = 0.584$), ERK ($U = 13.000$; $p = 0.715$), JNK ($U = 9.000$; $p = 0.273$) and p38 ($U = 5.000$; $p = 0.068$). With regard to the activation of signaling proteins (phosphorylated/total), no significant difference was observed between the groups studied in the cortical activation of Akt ($U = 12.000$; $p = 0.584$), p70S6K ($U = 10.000$; $p = 0.361$), STAT5 ($U = 10.000$; $p = 0.361$) and p38 ($U = 10.000$; $p = 0.602$). However, PBM significantly increased the cortical activation of STAT3 ($U = 2.000$; $p = 0.018$), ERK ($U = 1.000$; $p = 0.019$) and JNK ($U = 2.000$; $p = 0.018$; **Figure 1**).

Hippocampal Expression and Activation of Intracellular Signaling Pathways

No significant difference between the groups studied was observed in the hippocampal expression of Akt ($U = 11.000$; $p = 0.754$), STAT5 ($U = 4.000$; $p = 0.140$), ERK ($U = 10.000$; $p = 0.602$), JNK ($U = 10.000$; $p = 0.602$) and p38 ($U = 12.500$; $p = 1.000$). However, PBM significantly increased the hippocampal expression of p70S6K ($U = 2.000$; $p = 0.028$) and STAT3 ($U = 1.000$; $p = 0.027$; **Figure 2**). In relation to the activation of signaling proteins, no significant difference was observed between the groups studied in the hippocampal activation of p70S6K ($U = 12.000$; $p = 0.917$), STAT3 $U = 12.000$; $p = 0.917$), STAT5 ($U = 4.000$; $p = 0.142$) ERK ($U = 8.000$; $p = 0.624$), JNK ($U = 6.000$; $p = 0.175$) and p38 ($U = 5.000$; $p = 0.117$). However, PBM significantly increased the hippocampal activation of Akt ($U = 2.000$; $p = 0.028$; **Figure 2**). Taken together, these data show that PBM changes the expression and activation of intracellular signaling proteins in the brain of aged rats.

DISCUSSION

The purpose of our study was to investigate the cellular signaling proteins in the cortex and hippocampus of aged rats subjected to a PBM protocol with a laser of 810 nm wavelength and 100 mW power over 58 consecutive days.

In this study, our laser treatment increased the cortical activation of JNK in aged rats. However, we expected that the laser could reduce the JNK activation, as found in the aging mice study conducted by Salehpour and collaborators

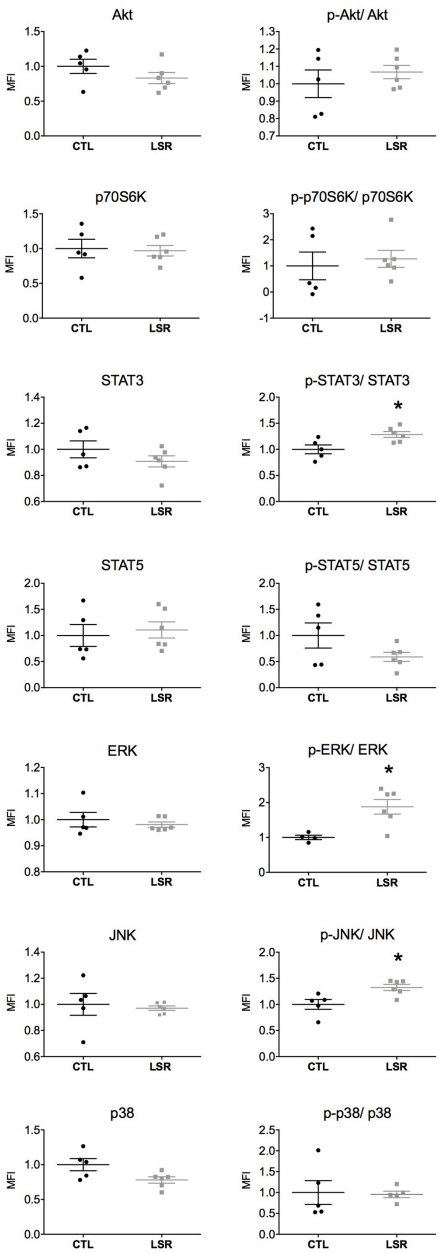

FIGURE 1 | Cortical expression and activation (phosphorylated/total protein) of intracellular signaling pathways in rats from the laser (LSR) and control (CTL) groups. Data are presented as individual points to show dispersion and as mean and standard error of the mean (±SEM). Significant increases in the activation of STAT3, ERK, and JNK were found in the laser group when compared to the control group (*). Data were normalized to the mean fluorescence intensity (MFI) of the control group ($p < 0.05$; Mann-Whitney U-Test).

(Salehpour et al., 2019). This difference may be related to the different treatment protocols. They used a laser treatment protocol of 810 nm wavelength for five consecutive days (with a fluence of 33.3 J/cm^2), while our protocol consisted of 58 consecutive days of laser treatment with 810 nm wavelength and fluence of 535.7 J/cm^2. Therefore, our laser treatment was

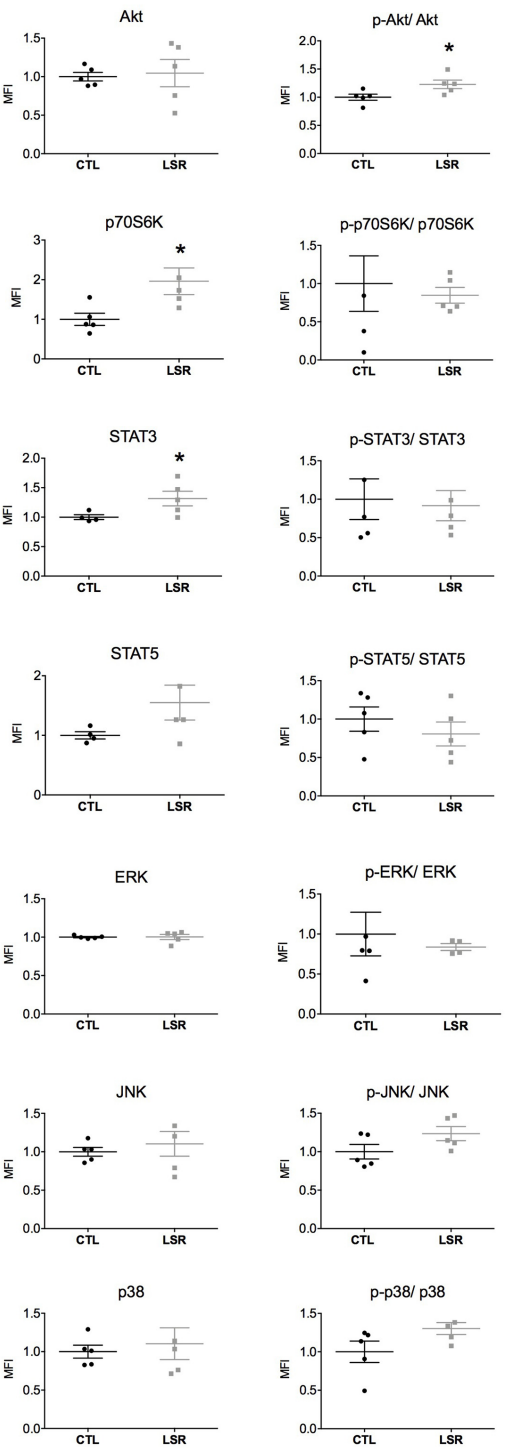

FIGURE 2 | Hippocampal expression and activation (phosphorylated/total protein) of intracellular signaling pathways in rats from the laser (LSR) and control (CTL) groups. Data are presented as individual points to show dispersion and as mean and standard error of the mean (±SEM). Significant increases in the expression of p70S6K and STAT3 and Akt activation were found in the laser group when compared to the control group (*). Data were normalized to the mean fluorescence intensity (MFI) of the control group ($p < 0.05$; Mann-Whitney U-Test).

10 times greater. PBM is well known to show hormetic dose-responses, with opposite effects at lower and higher doses (Rojas and Gonzalez-Lima, 2011; Xuan et al., 2013).

Furthermore, our laser treatment protocol also increased the cortical activation of ERK in aged rats. Activation of ERK and JNK signaling pathways play critical roles in regulating glucose metabolism (Papa et al., 2019). Improvement of glucose metabolism by PBM would be particularly beneficial to aging brains that are characterized by energy hypometabolism and memory decline (Gonzalez-Lima et al., 2014). Our results are consistent with the findings of Meng and collaborators (Meng et al., 2013). They found an increase in ERK activation after a 632.8 nm laser treatment protocol with fluences of 0.5, 1, 2, and 4 J/cm^2 in neuronal cells. This finding is important since the activation of ERK is involved in cellular proliferation, differentiation, and migration (Kim and Choi, 2010). In addition, the reduction of the ERK signaling pathway has been associated with long-term potentiation deficits and memory impairment in aged rats (English and Sweatt, 1997; Jin et al., 2008). Possibly, this is because ERK activation occurs when Thr183 and Tyr185 residues are phosphorylated by MAPKK or MEK1 (Anderson et al., 1990; Crews et al., 1992). After its activation, ERK activates regulatory proteins and kinases, as well as transcription factors (Fox et al., 1998). For this reason, ERKs are presumably present in processes of memory and synaptic plasticity (Sweatt, 2004).

Our results show that PBM increased the cortical activation of STAT3. STAT3 can modulate the expression of the anti-apoptotic genes of the Bcl-2 family by regulating neuronal survival (Fukada et al., 1996). In addition, JAK2/STAT3 signaling is critical for increasing neuronal proliferation and differentiation in IL-6 treated cells (Müller et al., 2009; Cheng et al., 2011). During aging, a decrease in STAT3 levels is observed (Bazhanova and Anisimov, 2016).

In the hippocampus, our results showed that PBM increased the expression of p70S6K and STAT3 and activation of Akt in aged rats. Our data regarding activation of Akt corroborates the findings of Yip and collaborators (Yip et al., 2011) in mice after transient cerebral ischemia exposed to a laser treatment session with three different fluences (2.64, 13.20, or 26.40 J/cm^2). These results suggest a neuroprotective effect of PBM since these proteins are linked to cell survival, apoptosis, and protein synthesis (Vara et al., 2004; Harrington et al., 2004; Wang and Proud, 2006), and in turn, they are decreased in the hippocampus of aged rats (Jin et al., 2008). For example, phosphatidylinositol 3-phosphate kinase (PI3K) activates Akt (Sheppard et al., 2012). Activated Akt is recruited to the cell membrane, promoting the phosphorylation of the residues: treonine-308 (Thr-308) and serine-473 (Ser-473; Alessi et al., 1996). Thus, Akt inhibits the pro-apoptotic pathways of the cell, such as the activity of the pro-apoptotic protein of the family Bcl-2 (Bad; Vara et al., 2004). Also, p70S6K is capable of inhibiting Bad protein (Harada et al., 2001). p70S6K signaling is linked to the activation of transcription and translation processes of essential genes for protein synthesis (Harrington et al., 2004; Wang and Proud, 2006). For example, p70S6K promotes an increase in synaptic transmission-related proteins, such as postsynaptic density protein-95 (PSD-95) and a-amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid receptor (AMPAR; Lee et al., 2005; Hoeffer and Klann, 2010), contributing to the maturation of excitatory synapses (El-Husseini et al., 2000) and improving synaptic transmission and plasticity (Migaud et al., 1998; Ehrlich and Malinow, 2004).

Finally, PBM-induced activation of Akt can also contribute to enhancing the PBM-induced activation of ERK and JNK signaling pathways, which are crucial in programing glucose metabolism (Papa et al., 2019). That is because Akt increases the translocation of glucose transporters to the plasma membrane (Nicholson and Anderson, 2002), suggesting that PBM can promote energy metabolism in the aged brain *via* these intracellular signaling pathways.

A limitation of this study was the lack of a group of young animals to compare with aged animals. It would be interesting to investigate the effects of age on the expression and activation of intracellular signaling proteins investigated in this study. Also, we would be able to analyze whether the effects of laser treatment are different in young and aged rats. These analyses would provide more information on the therapeutic potential of PBM in age-related disorders. Despite this, the current study is the first to investigate the effects of PBM on intracellular signaling proteins in the brain of healthy aged rats. Recently, many authors have investigated the therapeutic potential of laser treatment in diseases related to brain aging (Meng et al., 2013; El Massri et al., 2017; Zhang et al., 2020). However, little is known about the effects of PBM on the old brain without the disease. Another important point is the inter-individual variability observed during aging (Nyberg, 2017; Myrum et al., 2019). However, our results corroborate studies using laser treatment in other tissues (Kim et al., 2017; Tani et al., 2018; Wang et al., 2021). Other limitations are the small number of rats used and the low output power and the very small spot size of the laser device used. Nevertheless, our research group has found interesting results in various tissues using this same laser device with similar numbers of rats (Haslerud et al., 2017; Naterstad et al., 2018; Cardoso et al., 2021).

Our data indicate that transcranial PBM improves intracellular signaling pathways linked to cell survival, memory, and glucose metabolism in the brain of aged rats.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study and all experimental protocols were reviewed and approved by the ethics committee of the University of Mogi das Cruzes (UMC; # 016/2017).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: FC, FM, and SG. Performed the experiments: FC and FM. Analyzed the data:

FC and SG. Contributed reagents, materials, and analysis tools: FC and SG. Wrote the manuscript: FC, RL-M, FG-L, and SG. Approved the final version of the manuscript: FC, FM, RL-M, FG-L, and SG.

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Calcium and Spike Timing-Dependent Plasticity

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Since its discovery, spike timing-dependent synaptic plasticity (STDP) has been thought to be a primary mechanism underlying the brain's ability to learn and to form new memories. However, despite the enormous interest in both the experimental and theoretical neuroscience communities in activity-dependent plasticity, it is still unclear whether plasticity rules inferred from *in vitro* experiments apply to *in vivo* conditions. Among the multiple reasons why plasticity rules *in vivo* might differ significantly from *in vitro* studies is that extracellular calcium concentration use in most studies is higher than concentrations estimated *in vivo*. STDP, like many forms of long-term synaptic plasticity, strongly depends on intracellular calcium influx for its induction. Here, we discuss the importance of considering physiological levels of extracellular calcium concentration to study functional plasticity.

Keywords: synaptic plasticity, synapse, STDP, hippocampus, learning, memory, calcium

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INTRODUCTION

Spike timing-dependent plasticity (STDP) is a form of long-term synaptic modification thought to constitute a mechanism underlying formation of new memories. The polarity of synaptic modifications is controlled by the relative timing between pre- and post-synaptic action potentials (APs; Dan and Poo, 2004; Feldman, 2012). Following the Konorski-Hebb principle (Konorski, 1948; Hebb, 1949), timing-dependent long-term synaptic potentiation (t-LTP) in hippocampal and neocortical pyramidal neurons, results from the temporal conjunction of synaptic activity followed by one or more backpropagating APs in the post-synaptic cell (Gustafsson and Wigström, 1986; Markram et al., 1997; Bi and Poo, 1998; Debanne et al., 1998; Feldman, 2000). In contrast, following Stent principle (Stent, 1973), timing-dependent synaptic depression (t-LTD) is induced when synaptic activity is repeatedly preceded by one of more backpropagating action potentials (Debanne et al., 1994, 1996a, 1998; Markram et al., 1997; Bi and Poo, 1998; Feldman, 2000). It is important to note that t-LTP and t-LTD have been reported in early studies when two synaptic inputs, namely a weak input producing a subthreshold response and a strong input producing an action potential were paired with positive or negative delays (Baranyi and Fehér, 1981; Levy and Steward, 1983; Stanton and Sejnowski, 1989).

STDP AND CALCIUM

At excitatory synapses, the amplitude of post-synaptic calcium influx determines the orientation of plasticity towards synaptic potentiation or depression (Artola et al., 1990). The better

demonstration for that is provided by the fact that buffering post-synaptic calcium with BAPTA prevents the induction of both LTP and LTD (Debanne et al., 1994; Nevian and Sakmann, 2006). In a similar way, uncaging of calcium in CA1 pyramidal cells selectively induces LTP or LTD depending on the magnitude of calcium influx (Yang et al., 1999). In Hebbian STDP, the correlation between an EPSP and the backpropagated action potential (bAP) corresponding to a pre-before-post pairing that leads to t-LTP, induces large calcium entry (Koester and Sakmann, 1998). In contrast, post-before-pre pairing that induces t-LTD, comparatively produces a weaker calcium entry (Koester and Sakmann, 1998). Induction of t-LTP involves several mechanisms: (1) removal of the magnesium block from the NMDA receptor (Kampa et al., 2004); (2) inactivation of A-type current and activation of sodium channels to improve signal propagation (Hoffman et al., 1997; Stuart and Häusser, 2001); and (3) AMPA receptor depolarization to boost NMDA receptor calcium signal (Fuenzalida et al., 2010; Holbro et al., 2010). However, the NMDA receptor is the major player as its blockade (by perfusion of MK801 in the post-synaptic neuron for example) prevents LTP induction. Expression of t-LTP requires kinases activation such as CaMKII to phosphorylate AMPA and NMDA receptors thereby increasing their conductance (Otmakhova et al., 2002; Lisman et al., 2012) as well as the insertion of new AMPA receptors (Malinow and Malenka, 2002). In t-LTD, low calcium entry leads to inactivation of NMDA receptors by activation of phosphatases (Rosenmund et al., 1995). These two forms of plasticity which are dependent on post-synaptic NMDA receptors are found in the hippocampus at CA3-CA1 synapses of rodents (Nishiyama et al., 2000; Andrade-Talavera et al., 2016) or in the layer II/III of the cortex of rodents (Froemke et al., 2006). Some forms of LTD are also dependent on the presynaptic NMDA receptor. In the cortex or hippocampus, perfusion of MK801 in the pre-synaptic neurons prevents LTD but not LTP (Sjöström et al., 2003; Rodríguez-Moreno and Paulsen, 2008; Banerjee et al., 2009; Andrade-Talavera et al., 2016). Other forms of NMDA receptor-independent LTD expressed at hippocampal CA3-CA1 and cortical L4-L2/3 synapses, requires metabotropic glutamate receptors (mGluRs), voltage-dependent calcium channels, cannabinoid receptors and astrocytic signaling (Normann et al., 2000; Bender, 2006). In these forms of LTD, production of a retrograde messenger, the endocannabinoids (eCBs), will decrease the probability of pre-synaptic release (Bender, 2006; Chevaleyre et al., 2006). However, this simplistic view is now challenged by several studies that do not necessarily observe a correlation between calcium entry and plasticity. In layer II/III of the cortex, the same rise in post-synaptic calcium can lead to LTP or LTD (Nevian and Sakmann, 2006) and a broadening of the action potential that induce a larger calcium influx may surprisingly facilitate LTD (Zhou et al., 2005).

NMDA SPIKES

STDP relies heavily on the bAP which cannot propagate too deeply into the dendritic tree (Spruston, 2008). As a result, distal synapses require a local source of depolarization for t-LTP. Thus, dendritic NMDA receptors are an important source of calcium (Schiller and Schiller, 2001). Several studies have now shown that they are necessary for the induction of t-LTP. In the hippocampus, at mossy fibers-CA3 synapses, t-LTP can only be induced when NMDA spikes are triggered (Brandalise et al., 2016). In the cortex, at layer II/III-V distal synapses, dendritic depolarization can switch plasticity between LTD and LTP (Sjöström and Häusser, 2006). In addition, distal synapses can be cooperative. In CA1 pyramidal cells, synaptic cooperativity is observed at distal but not proximal dendritic locations following repetitive subthreshold activation of small spine clusters (Weber et al., 2016). Recently, in layer 5 pyramidal neurons, it was shown that synaptic cooperativity disrupts t-LTD and extends the temporal window for the induction of t-LTP (Tazerart et al., 2020).

MATHEMATICAL MODELS OF STDP BASED ON CALCIUM

Synaptic plasticity models have been first theorized by John Lisman in 1989. According to this pioneering work, high post-synaptic calcium influx represents a condition favorable to LTP induction because protein kinases are preferentially activated whereas low to moderate calcium influx induces LTD because protein phosphatases are selectively activated. Most recent mathematical models of STDP incorporate the biochemical pathways described above to link post-synaptic calcium and plasticity (Karmarkar and Buonomano, 2002; Shouval et al., 2002; Graupner and Brunel, 2010). One of the first models to investigate the role of extracellular calcium in STDP was the one developed by Graupner and Brunel (2012). By variation of the extracellular calcium, and therefore postsynaptic calcium entry, they showed that a multitude of STDP curves could be obtained in response to a simple pre-post (1:1) or post-pre protocol (1:1; **Figure 1**). In the most extreme cases, only LTD or LTP could be observed respectively for a very weak or very strong calcium influx. For intermediate influx, the classical curve was found with a window of t-LTD ($\Delta t \leq 0$ ms), a window of t-LTP ($\Delta t \geq 0$ ms) and sometimes a second window of depression for longer delays ($\Delta t = 20–30$ ms). The concentration of extracellular calcium is consequently important for the orientation of plasticity but also for its maintenance. Time scales of memory maintenance can be extended by lowering extracellular calcium concentration to *in vivo* levels (Higgins et al., 2014). Spontaneous synaptic activity is known to erase memory induced by STDP in the tadpole visual system (Zhou et al., 2003). Similar behavior has been reproduced *in silico* with a calcium-based model (Higgins et al., 2014). In the presence of background synaptic noise, synaptic changes induced by STDP disappear in a few minutes with a high concentration of calcium, whereas they last 1 hour with a physiological concentration.

ROLE OF EXTERNAL CALCIUM LEVEL

Most, if not all, *in vitro* studies of STDP have used non-physiological extracellular calcium concentrations

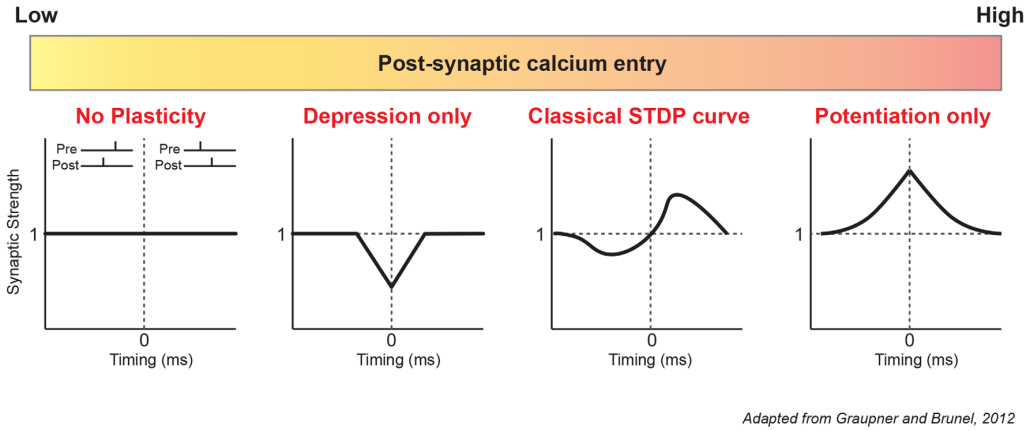

FIGURE 1 | Diversity of modeled STDP curves based on calcium. According to the modeling of Graupner and Brunel (2012), post-synaptic calcium is a major determinant of plasticity. From left to right: low calcium entry causes no plasticity or only depression (*t-LTD*). Intermediate calcium entry produces the classic STDP curve (*Hebbian STDP*), with a depression window (*t-LTD)* for negative delays and a potentiation window (*t-LTP*) for positive delays. High calcium entry results in only potentiation for all delays. Note that these results were obtained with a simple 1:1 protocol (1 presynaptic, 1 postsynaptic stimulation) with a low pairing frequency of 1 Hz and 60 repetitions. STDP, Spike Timing-Dependent Plasticity.

TABLE 1 | Selected publications on Spike Timing-Dependent Plasticity.

Reference	Synapses	Induction protocol	Plasticity observed	$[Ca^{2+}]_e$
Debanne et al. (1994)	CA3-CA1	50–100 pairings @ 0.3 Hz + Postsynaptic burst	LTD ($\Delta t < 0$ ms)	2.8 mM
Bi and Poo (1998)	Hippocampal neurons	60 pairings @ 1 Hz + Postsynaptic depolarization	LTP ($\Delta t > 0$ ms) and LTD ($\Delta t < 0$ ms)	3 mM
Markram et al. (1997)	L5-L5	10 pairings @ 20 Hz (2:2 or 5:5 or 10:10)	LTP ($\Delta t > 0$ ms)	2 mM
Sjöström et al. (2001)	L5-L5	15 pairings @ 40 Hz (1:5)	LTP ($\Delta t > 0$ ms)	2.5 mM
Froemke et al. (2006)	L2/3	60–100 pairings @ 0.2 Hz (1:1)	LTP ($\Delta t > 0$ ms) and LTD ($\Delta t < 0$ ms	2.5 mM
Froemke et al. (2006)	L2/3	30–40 pairings @ 0.2 Hz (5:5)	No Plasticity, LTD or LTP depending on burst frequency	2.5 mM
Wittenberg and Wang (2006)	CA3-CA1	70–100 pairings @ 0.1–0.5 Hz (1 :1)	LTD only ($\Delta t > 0$ ms and $\Delta t < 0$ ms)	2 mM
Wittenberg and Wang (2006)	CA3-CA1	100 pairings @ 5 Hz (1:2)	LTD ($\Delta t < 0$ ms) and LTP ($\Delta t > 0$ ms)	2 mM
Nishiyama et al. (2000)	CA3-CA1	Train of stimuli at 5 Hz + postsynaptic Spike	LTP ($\Delta t > 0$ ms) and LTD ($\Delta t < 0$ ms)*	2–2.6 mM
Campanac and Debanne (2008)	CA3-CA1	100 pairings @ 0.3 Hz for LTP 150 pairings @ 0.3 Hz for LTD (1:1)	LTD ($\Delta t < 0$ ms) and LTP ($\Delta t > 0$ ms)	3 mM
Pawlak and Kerr (2008)	Corticostriatal pathway	60 pairings @ 0.1 Hz (1:1)	LTD ($\Delta t < 0$ ms) and LTP ($\Delta t > 0$ ms)	2.5 mM
Mishra et al. (2016)	CA3-CA3	300 pairings @ 1 Hz (1:1)	LTP only ($\Delta t > 0$ ms and $\Delta t < 0$ ms)	2 mM
Inglebert et al. (2020)	CA3-CA1	100 pairings @ 0.3 Hz for LTP 150 pairing @ 0.3 Hz for LTD (1:1)	No Plasticity or LTD only	1.3–1.8 mM

*This table summarizes the plasticity observed in several studies as a function of extracellular calcium concentration. The brain region studied and the induction protocol used are also indicated. The number of repetitions, the pairing frequency and number of pre/post stimulation are specified. For example, 5:5 indicates five presynaptic and five postsynaptic stimulations. *A second LTD window is visible around Δt = 20 ms. STDP, Spike Timing-Dependent Plasticity.*

($[Ca^{2+}]_e$), commonly between 2 and 3 mM (**Table 1**). But physiological $[Ca^{2+}]_e$ is about 2 to 3 times lower, i.e., ranging from 1.3–1.8 mM in young rodent (Jones and Keep, 1988; Silver and Erecińska, 1990; Ding et al., 2016). In fact, past studies already showed discrepancies regarding STDP induced *in vitro*. The same protocol in the hippocampus could lead to a radically different STDP curve (Wittenberg and Wang, 2006; Campanac and Debanne, 2008) and the only obvious difference was the concentration of extracellular calcium used (2 vs. 3 mM). Recently, it has been confirmed experimentally that the overall calcium concentration has a significant effect on plasticity (Inglebert et al., 2020). At CA3-CA1 synapses, while the Ca^{2+}/Mg^{2+} ratio is kept unchanged, Hebbian STDP is found for $[Ca^{2+}]_e$ = 3 mM while no plasticity is observed for $[Ca^{2+}]_e$ = 1.3 mM and only LTD for positive and negative timing for $[Ca^{2+}]_e$ = 1.8 mM (**Figure 2**). But adjusting the protocol by increasing the pairing frequency from 0.3 to 5–10 Hz, or the number of postsynaptic APs from 1 to 3–4, allows to restore classical Hebbian STDP (Inglebert et al., 2020). These results obtained in young animals need to be confirmed by further studies in the adult and encourage a reexamination of STDP under physiological conditions.

REEVALUATION OF STDP RULES

Multiple STDP Rules

The findings of Inglebert et al. invite a reexamination of plasticity at many synapses. For example, at CA3-CA3 synapses

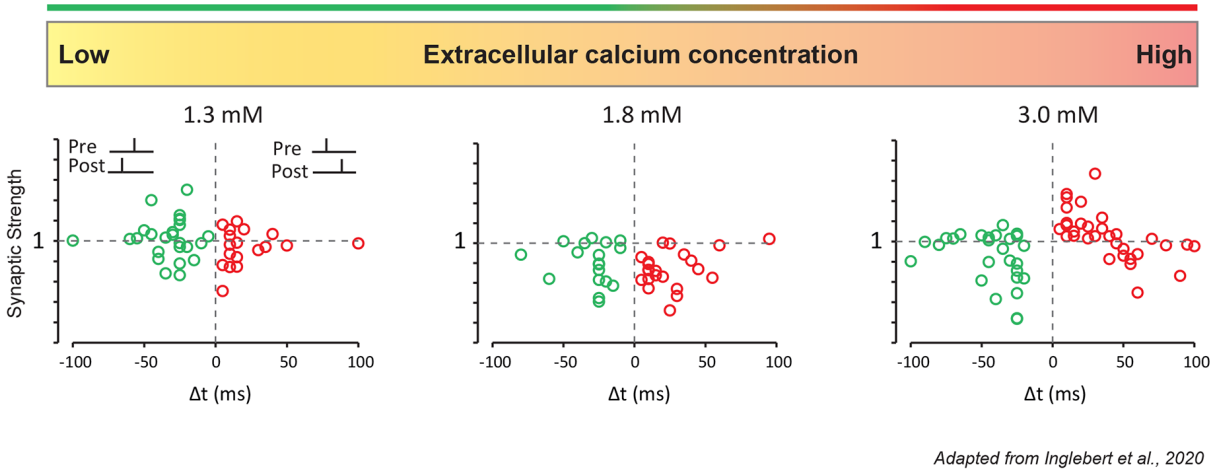

FIGURE 2 | STDP under various external calcium concentrations. CA1 pyramidal neurons were recorded in a whole-cell configuration. Pre-synaptic stimulation was evoked by a stimulation electrode placed in the Schaffer collaterals. These results were obtained with a simple 1:1 protocol (1 presynaptic, 1 postsynaptic stimulation) with a low pairing frequency of 0.3 Hz and 100 repetitions for t-LTP, and 150 repetitions for t-LTD. In 1.3 mM extracellular calcium, no plasticity is induced. In 1.8 mM extracellular calcium, pre-post and post-pre protocols leads to t-LTD. In 3 mM extracellular calcium, pre-post protocol leads to t-LTP and post-pre protocol leads to t-LTD.

where only LTP is observed (Mishra et al., 2016), it is likely that this would not be the same in physiological calcium. Hebbian STDP (t-LTP for positive timings and t-LTD for negative timings) is found predominantly at excitatory synapses. Despite being ubiquitous, STDP curve can take many different shapes. Anti-Hebbian STDP (t-LTP for negative timings or t-LTD for positive timings) is found largely at inhibitory synapses. In particular, the striatum, which is composed principally of inhibitory neurons, has many different STDP curves depending on the neuron considered (Fino and Venance, 2010). For example, interneurons that expressed Nitric Oxide synthase, present a window of LTP for a positive timing around Δt = +50 ms. In the dorsal cochlear nucleus (DCN), pre-before-post protocol induced LTD in cartwheel cells (Tzounopoulos et al., 2004). Would all these results persist in physiological calcium or plasticity has been overestimated?

Dendritic Calcium Spikes in Physiological Calcium

Among the factors that could be potentially affected by physiological calcium, the occurrence of dendritic calcium spikes or NMDA spikes that have been shown to be critical in LTP induction (Kampa et al., 2006; Brandalise et al., 2016) could be greatly reduced. A possibility is that in physiological calcium, a larger number of synaptic inputs would be required to trigger an NMDA spike. Alternatively, the spatial extent of the NMDA spike could be reduced in physiological calcium rending induction of plasticity more difficult. Further studies will be required to test these possibilities.

Calcium Micro-Domains in Physiological Calcium

Calcium micro-domains are supposed to play a critical role in STDP (Mihalas, 2011). A major consequence of reducing external calcium concentration to physiological values is a great reduction of the size of calcium micro-domains. As a consequence, the calcium-sensitive effector might be disconnected from the source of calcium, thus accounting for the observed reduction in plasticity (Inglebert et al., 2020). Additional studies will be, however, required to test further these hypotheses.

Presynaptic Aspects of STDP in Physiological Calcium

Neocortical STDP relies heavily on presynaptic glutamate release and presynaptic firing rate (Markram et al., 1997; Sjöström et al., 2001, 2003). However, it is now clear that spontaneously released vesicles and evoked transmission use distinct mechanisms (Kavalali, 2015; Abrahamsson et al., 2017). Therefore, physiological calcium could have distinct consequences on evoked and spontaneous release. At many synapses, evoked glutamate release is controlled by pre-synaptic Ca^{2+} entry through voltage-dependent calcium channels (VDCC) and $[Ca^{2+}]_e$ (Südhof, 2012). Reduced $[Ca^{2+}]_e$ is associated with decreased synaptic transmission (Borst and Sakmann, 1996; Debanne et al., 1996b; Hardingham et al., 2006). On the contrary, spontaneous release is poorly sensitive to fluctuations in $[Ca^{2+}]_e$ and is not triggered by Ca^{2+} entry *via* VDCC (Scanziani et al., 1992; Vyleta and Smith, 2011). Interestingly, neocortical presynaptic NMDA receptors regulate both spontaneous and evoked

release by distinct molecular pathways (Abrahamsson et al., 2017; Bouvier et al., 2018), are required for neocortical t-LTD (Sjöström et al., 2003) and for hippocampal t-LTD (Andrade-Talavera et al., 2016). The work of Inglebert et al. (2020) focused on the post-synaptic calcium hypothesis but further studies are needed to explore pre-synaptic long-term plasticity.

Towards Standardization of Induction Protocols

All studies use different protocols (**Table 1**). The number of repetitions, the pairing frequency, the somato-dendritic distance of the inputs or the number of postsynaptic potentials are different among studies. Therefore, it is often difficult to compare the different results obtained by each study. In physiological calcium (i.e 1.3 mM), it appears that t-LTP and t-LTD requires a greater frequency of pairing (>5 Hz) or a greater number of postsynaptic APs (>3) even with a large number of repetitions (100 or 150; Inglebert et al., 2020). A better understanding of the rules of STDP induction *in vitro* under physiological conditions will allow a more robust application *in vivo*.

Implication for *In vivo* Exploration of STDP

In opposition to *in vitro* studies, and by definition, *in vivo* studies are inherently in a physiological calcium concentration. First demonstration of STDP *in vivo* was performed in the retinotectal pathway of Xenopus (Zhang et al., 1998). t-LTP and t-LTD were observed but were not robust and easily abolished by hyperpolarizations or spontaneous activities, a limitation recently highlighted by a biophysical model of STDP (Higgins et al., 2014). The use of a non-physiological concentration of calcium may lead to an underestimation of the time scales of memory maintenance as background activities is an important factor for limiting plasticity. Furthermore, most *in vitro* studies are performed in juvenile rodent while *in vivo* studies are performed in older animals. This may constitute an additional limitation to the transposition of the results observed *in vitro*. Several studies suggest that the capacity to induce t-LTD decreases with age (Banerjee et al., 2009; Verhoog et al., 2013) although a recent study has shown t-LTD at cortical layer V synapses in adult mice following pre-before-post protocol (Louth et al., 2021). Similarly, induction of t-LTD by a STDP protocol in the somato-sensory cortex of adult rats by pairing postsynaptic spikes and subthreshold whisker deflection is relatively frequent whereas induction of t-LTP in the same preparation is rare (Jacob et al., 2007). t-LTD disappeared rapidly after a few minutes (5–10 min) and t-LTP was sporadic following pre-before-post pairings (Jacob et al., 2007). As already suggested, STDP in older animals may require protocols that produce stronger depolarization (Meredith et al., 2003). In concordance with this idea, electrical stimulation of afferent input at high frequency paired with post-synaptic burst produced robust t-LTP in cat visual cortex (Frégnac et al., 2010). It is important to note that most of the results observed *in vivo* are obtained in the anesthetized animal. Although there are variations in extracellular calcium concentration of about 0.2 mM between awake and anesthetized animals (Ding et al., 2016), they are not sufficient to produce a major effect on the induction and maintenance of plasticity. An attractive explanation could be that in anesthetized animals, neuromodulation is largely depressed.

Importance of Neuromodulation

As demonstrated by Inglebert et al. fine tuning of pre- and postsynaptic activity can restore t-LTD and t-LTP in physiological extracellular calcium condition. But would it be possible to restore classic plasticity rules under regular patterns of activity? The key component could be neuromodulation. Interestingly, replay of activity from place-cells with overlapping firing field in hippocampal slices induced t-LTP only in the presence of Carbachol, a cholinergic agonist (Isaac et al., 2009). Many studies have now shown the effects of various neuromodulators on STDP. One of the most explored is dopamine (DA). It is involved in learning and reward processes (Schultz, 1997; Suri and Schultz, 1999). The activation of the D1 receptor (D1-R) has been shown to increase temporal window for t-LTP and to allow induction of t-LTP with fewer spike pairs at glutamatergic synapses of hippocampal neurons (Zhang et al., 2009). At CA3-CA1 synapses, D1-R activation switches t-LTD into t-LTP (Brzosko et al., 2015). In the prefrontal cortex, DA application allow t-LTP induction (He et al., 2015). The effects of the D1-R are widespread (Neve et al., 2004), but many of them could explain the reasons for promoting LTP. They could facilitate signal propagation by inhibiting A-type current (Hamilton et al., 2010; Edelmann and Lessmann, 2011; Yang and Dani, 2014) or simply increased intracellular calcium (Lezcano and Bergson, 2002). D2 receptors (D2-R) are also involved in t-LTP and t-LTD. In lateral amygdala, D2-R gates LTP induction by suppressing feedforward inhibition (Bissière et al., 2003). In some cases, a synergy is observed between the two receptors. In layer V of the prefrontal cortex, D1-R and D2-R co-activation enables the induction of t-LTP at extended timing interval (Xu and Yao, 2010). Noradrenaline (NA) is also involved in memory formation. Activation of β-adrenergic receptors (β-R) increases intracellular calcium (Seol et al., 2007) and facilitate bAP by inhibition of A-type current (Yuan et al., 2002) or SK channels (Faber et al., 2008). At CA3-CA1 synapses, β-R activation increases the temporal window induction for t-LTP (Lin et al., 2003). In layer II/III of visual cortex, co-activation of β-R and α-adrenergic receptor (α-R) are required for bidirectional STDP in fast-spiking and somatostatin interneurons. β-R activation promoted t-LTP whereas α-R activation induced t-LTD (Huang et al., 2013, 2014). Neuromodulation could be seen as the necessary factor for the induction of t-LTP and t-LTD in physiological calcium without tuning pre- and postsynaptic activity.

CONCLUSION

Use of Physiological Calcium Levels for Studying Short-Term Synaptic Plasticity

The use of physiological external calcium concentration not only modulates the learning rules for long-term synaptic plasticity

but it also enhances context-dependent synaptic plasticity. Analog-digital modulation of action potential-evoked synaptic transmission lies on modification of spike shape, by either broadening the axonal spike (Shu et al., 2006; Kole et al., 2007) or by modulating its amplitude (Rama et al., 2015; Zbili et al., 2020). Because transmitter release is almost maximal in high calcium, conditions that enhance release are somehow difficult to reach. Indeed, switching to physiological calcium concentration (i.e., 1.3 mM) was found to significantly enhance spike amplitude-dependent synaptic plasticity (Rama et al., 2015; Zbili et al., 2020).

Use of Physiological Calcium Levels for Studying Intrinsic Plasticity

Hebbian plasticity and Intrinsic plasticity are closely linked and are synergistically modified (Debanne et al., 2019). Generally, t-LTP is associated with an increase in excitability and t-LTD with a reduced excitability (Ganguly et al., 2000; Li et al., 2004). Interestingly, recordings using physiological calcium show a significant increase excitability of CA1 pyramidal neurons: lowered firing threshold, increased spontaneous firing and more depolarized resting membrane potential (Bjorefeldt et al., 2018). Dendritic integration or EPSP-Spike coupling is also modified with synaptic changes (Campanac and Debanne, 2008). Generally, LTP is accompanied by an increase in the probability of emitting an AP for the same synaptic input (EPSP-spike potentiation) whereas LTD is accompanied by a decrease in the probability of emitting an AP (EPSP-spike depression). This plasticity of the output, without modification of the input, result partially from postsynaptic changes in voltage dependent channels such as I_H or I_A (Daoudal and Debanne, 2003). EPSP-Spike coupling modification is also conditioned by changes in inhibitory synaptic transmission. Intriguingly, recording in physiological calcium in hippocampus have revealed that excitatory-inhibitory balance was disrupted and disynaptic inhibition was strongly decreased (Aivar et al., 2014).

AUTHOR CONTRIBUTIONS

YI and DD wrote the article and YI built the figures. All authors contributed to the article and approved the submitted version.

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REVIEW
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Neuromodulation of Hippocampal-Prefrontal Cortical Synaptic Plasticity and Functional Connectivity: Implications for Neuropsychiatric Disorders

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The hippocampus-prefrontal cortex (HPC-PFC) pathway plays a fundamental role in executive and emotional functions. Neurophysiological studies have begun to unveil the dynamics of HPC-PFC interaction in both immediate demands and long-term adaptations. Disruptions in HPC-PFC functional connectivity can contribute to neuropsychiatric symptoms observed in mental illnesses and neurological conditions, such as schizophrenia, depression, anxiety disorders, and Alzheimer's disease. Given the role in functional and dysfunctional physiology, it is crucial to understand the mechanisms that modulate the dynamics of HPC-PFC communication. Two of the main mechanisms that regulate HPC-PFC interactions are synaptic plasticity and modulatory neurotransmission. Synaptic plasticity can be investigated inducing long-term potentiation or long-term depression, while spontaneous functional connectivity can be inferred by statistical dependencies between the local field potentials of both regions. In turn, several neurotransmitters, such as acetylcholine, dopamine, serotonin, noradrenaline, and endocannabinoids, can regulate the fine-tuning of HPC-PFC connectivity. Despite experimental evidence, the effects of neuromodulation on HPC-PFC neuronal dynamics from cellular to behavioral levels are not fully understood. The current literature lacks a review that focuses on the main neurotransmitter interactions with HPC-PFC activity. Here we reviewed studies showing the effects of the main neurotransmitter systems in long- and short-term HPC-PFC synaptic plasticity. We also looked for the neuromodulatory effects on HPC-PFC oscillatory coordination. Finally, we review the implications of HPC-PFC disruption in synaptic plasticity and functional connectivity on cognition and neuropsychiatric disorders. The comprehensive overview of these impairments could help better understand the role of neuromodulation in HPC-PFC communication and generate insights into the etiology and physiopathology of clinical conditions.

Keywords: hippocampus, prefrontal cortex, long-term potentiation (LTP), long-term depression (LTD), local field potential (LFP), acetylcholine, monoamines, cannabinoids

INTRODUCTION

A remarkable diversity of neural circuits in the mammalian brain provides a substrate for adaptive and maladaptive behavioral responses (Deisseroth, 2014). The HPC-PFC circuit plays a fundamental role in cognitive functions, such as short-term and long-term memory, attention, and decision-making, which are affected by several neurological diseases and psychiatric disorders (Kovner et al., 2019). In rodents, the anatomical description of the hippocampal-prefrontal pathway has been combined with long-range and local electrophysiological measures to investigate the processing of neural information (Laroche et al., 2000; Takita et al., 2013). Noteworthy, the adjustment and perpetuation of the information in the hippocampal-prefrontal pathway occur through short-term plasticity and long-term plasticity, respectively (Citri and Malenka, 2008). The HPC-PFC pathway is neuromodulated by several neurotransmitter systems: cholinergic, monoaminergic, and endocannabinoid (Goto et al., 2010; Puig and Gener, 2015; Ruggiero et al., 2017; Ranjbar-Slamloo and Fazlali, 2020). The neuromodulation of the HPC-PFC pathway is essential to a fine-tune regulation of the circuit, affecting the synaptic efficacy, synaptic plasticity, and oscillatory patterns implicated in behavioral and circuit alterations related to neuropsychiatric conditions (Godsil et al., 2013).

This review outlines the neuromodulation of the synaptic plasticity and network coupling in the hippocampal-prefrontal pathway underlying relevant aspects of the neuroanatomy and electrophysiological measures in rodents. We also examine the disruption of these circuits related to mal-adaptive impairments and provide a critical discussion for new potential developments in the field.

HIPPOCAMPUS-PREFRONTAL CORTEX ANATOMICAL PROJECTION

The hippocampus is probably one of the most studied brain regions and is well known to exert a critical role in semantic memory formation and spatial learning. The hippocampal formation is localized in the temporal lobe and is constituted by the *Cornu Ammonis* (CA) fields (CA1, CA2, and CA3), the dentate gyrus, the subicular complex, and the entorhinal cortex (Andersen et al., 2009). In rodents, the hippocampal formation extends in a C-shaped manner through a dorsal (septal) to a ventral (temporal) axis, corresponding to the posterior to the anterior axis in humans (Andersen et al., 2009; Strange et al., 2014). Lesion and connectivity studies indicate a functional distinction in the hippocampus, with the dorsal part mediating cognitive aspects (especially spatial memory) and the ventral region modulating emotional processes (Groenewegen et al., 1987; Van Groen and Wyss, 1990; Moser et al., 1993; Risold and Swanson, 1996). More recently, genetic expression domain studies have shown a tripartite profile that divides the dentate gyrus and CA1 into dorsal, intermediate, and ventral portions (Lein et al., 2007). Furthermore, extrinsic connectivity shows a topographical representation - both from the neocortex to the hippocampus and from the hippocampus to subcortical structures - presenting a gradual transition along the septo-temporal axis (Strange et al., 2014).

The PFC is a brain region that presents marked differences during phylogenetic development. Compared to other mammal species, the PFC of primates shows a dramatic volume increase and differentiation (Fuster, 2008). Several authors discuss if the murine species used in neuroscience research present a prefrontal cortex (Carlén, 2017; Laubach et al., 2018). The classical definition of the PFC (Rose and Woolsey, 1948) is the cortical projection area of the mediodorsal thalamic nucleus. This broad definition included all mammals as possessing a frontal region equivalent to the primate frontal granular cortex (Carlén, 2017) and motivated initial studies of functional similarity between the PFC of rodents and the higher cognitive dorsolateral prefrontal cortex (dlPFC) of primates (Laubach et al., 2018). However, more recent literature indicates notable differences in the functional aspects of the PFC and the dlPFC of humans (see Carlén, 2017; Laubach et al., 2018 for a review). Indeed, the granular dorsolateral PFC is considered to be unique to primates (Wise, 2008), and cytoarchitecture evidence indicates that the rodent PFC is homologous to the human anterior cingulate cortex (ACC) (Vogt et al., 2013; Vogt and Paxinos, 2014).

The mPFC has a central role in regulating cognitive processes, both in humans and other mammals. Recent studies using perturbation of the mPFC activity in mice have shown that mPFC is involved in sensory processing, motor planning, emotional regulation, reward, attention, working memory, decision making, long and short-term memory, and social behaviors (Le Merre et al., 2021). Such diverse sets of functions are sustained by a dense pattern of reciprocal connectivity with other cortices, thalamus, subcortical and brainstem regions. Indeed, a recent study in mice found that it is possible to differentiate the mPFC from other cortices based on corticocortical and thalamic connectivity patterns. The mPFC has the highest proportion of feedback projections (Harris et al., 2019).

The HPC and mPFC are connected by both polysynaptic (indirect) and monosynaptic (direct) projections (Jin and Maren, 2015). This pathway seems to be conserved throughout different mammalian groups, as shown by studies in rats (Jay et al., 1989, 1996; Hoover and Vertes, 2007), mice (Tripathi et al., 2016), cats (Irle and Markowitsch, 1982; Cavada et al., 1983), and monkeys (Rosene and Van Hoesen, 1977). The HPC afferents originate in the intermediate and ventral CA1 and in the proximal limb of the subiculum. It then courses through the alveus, following dorsal and rostral by the ipsilateral fimbria and fornix. The fibers continue rostroventrally through the medial part of the lateral septum and nucleus accumbens, reaching the infralimbic (IL), prelimbic (PL) the anterior cingulate cortices (Jay et al., 1989; Jay and Witter, 1991). The IL, PL and ACC form the rodent medial prefrontal cortex.

The hippocampal innervation to the PFC presents differential patterns between the ventral and dorsal PL region. While the ventral portion receives dense projections in the layers II-VI, the dorsal portion receives less dense inputs, mainly present in the layers V-VI (Jay and Witter, 1991; Thierry et al., 2000). Interestingly, ventral HPC inputs make a similar connection onto cortico-cortical and cortico-amygdalar pyramidal neurons

in superficial layers of the IL cortex, but make a preferential connection onto cortico-cortical over cortico-pontine neurons in deep layers of IL and PL (Spellman et al., 2021). It has also been demonstrated that HPC targets different types of interneurons in the PFC, presenting differential axon collateral projections. Part of the HPC neurons that project to somatostatin positive interneurons also project to the contralateral CA1, while HPC principal cells that target parvalbumin neurons tend to send projections also to the nucleus accumbens (NAc) (Sun et al., 2019). Furthermore, it is also proposed that differences in the microcircuitry of the target interneurons could explain differential electrophysiological properties of the intermediate (iHPC) and ventral (vHPC) routes (see section 4). It is also clear from the anatomical studies a differentiation between the projections of the dorsal, intermediate, and ventral thirds of the HPC. While there is no projection from the dorsal HPC, the intermediate third of the HPC projects moderately to the infralimbic area, light projection to the prelimbic, and scarce projections to the anterior cingulate area. The ventral part of CA1 projects moderately to the dorsal infralimbic, dense projections to the prelimbic area, and moderate projections to the anterior cingulate area (Jay and Witter, 1991; Cenquizca and Swanson, 2007). Based on this differential projection, electrophysiological patterns, and the aforementioned molecular profile of the hippocampal septo-temporal axis, we adopted the nomenclature dividing the HPC into the dorsal hippocampus (dHPC), iHPC, and vHPC regions (**Figure 1A**).

HIPPOCAMPUS-PREFRONTAL CORTEX SYNAPTIC EFFICACY

Synaptic efficacy can be measured by recording postsynaptic potentials (PSP) or postsynaptic currents (PSC) from neurons in culture or in the brain tissue. In *in vivo* and freely behaving preparations, field PSPs (fPSP) are the primary electrophysiological measurement used to investigate synaptic efficacy of a given pathway (Manahan-Vaughan, 2018a,b). The fPSP is evoked when a stimulus in the presynaptic population of neurons generates a depolarization of postsynaptic targets, which can be detected if sufficient extracellular current flows to the recording electrode referenced to an isoelectric ground (Manahan-Vaughan, 2018a). Although field postsynaptic amplitude responses usually relate to the synchronization and spiking activity of target neurons, fPSPs result from the contribution of all synaptic currents flowing through the extracellular space (Manahan-Vaughan, 2018a).

Field PSPs in the mPFC induced by HPC stimulation were first described by Laroche et al. (1990). The authors originally described that CA1 stimulation elicited a characteristic biphasic potential recorded extracellularly in the prelimbic area of mPFC and an excitatory response in single-unit spike recordings (50/120 units). The HPC-mPFC fPSP consists of an initial positive wave followed by a large negative wave between 15 and 22 ms interval latency (Laroche et al., 1990; Takita et al., 1999; Izaki et al., 2003b; Takita et al., 2010; Lopes-Aguiar et al., 2013; Bueno-Junior et al., 2017; Esteves et al., 2017). The negative wave is associated with the synchronous discharge of mPFC neurons since excitatory single–unit responses coincide with the negative wave component of fPSP (Laroche et al., 1990). Indeed, the long latency response is compatible with the estimated slow conduction velocity of fibers (0.6 ms^{-1}) and the latency of antidromic stimulation of HPC-mPFC (Ferino et al., 1987).

HIPPOCAMPUS-PREFRONTAL CORTEX SYNAPTIC PLASTICITY

Synaptic plasticity is the ability of nerve cells to modify the efficacy of synaptic transmission, which can be induced either by direct electrical stimulation or by environmental experience (Ho et al., 2011). In general, synaptic plasticity can be divided into two categories time-related: short-term plasticity and long-term plasticity, which can last from milliseconds to minutes or hours to days, respectively (Citri and Malenka, 2008). While short-term synaptic plasticity is related to transient behavioral changes, such as short-term memory and adaptations in sensory pathways, long-term synaptic plasticity is associated with long-lasting behaviors, such as long-term memory, sleep-wake cycle, or even maladaptive behaviors (Romcy-Pereira and Pavlides, 2004; Romcy-Pereira et al., 2009; Godsil et al., 2013).

Experimentally, short-term synaptic plasticity in the HPC-mPFC pathway can be assessed by two pulse stimuli in the presynaptic terminal, which can induce paired-pulse facilitation (PPF) or paired-pulse inhibition (PPI). PPF occurs when two pulse stimuli in the presynaptic terminal enhance the second fPSP amplitude, while PPI represents the reduction of second fPSP (Zucker and Regehr, 2002). Long-term potentiation (LTP) and long-term depression (LTD) are the most extensively studied forms of long-term plasticity in the HPC-mPFC pathway and reflect an increased or decreased neural response, respectively (Laroche et al., 2000). Commonly, the protocol required to induce LTP in the HPC-mPFC projection consists of high-frequency stimulation, while the LTD protocol consists of low-frequency stimulation (Manahan-Vaughan, 2018b). HPC-mPFC LTP and PPF *in vivo* were first described by Laroche et al. (1990), who observed a significant and persistent potentiation of fPSPs for hours (Laroche et al., 1990; Jay et al., 1996; Esteves et al., 2017). More recently, Abraham's group showed the persistence of HPC-mPFC LTP up to 20 days (Taylor et al., 2016; **Figure 1B**).

Interestingly, short-term and long-term plasticities in the HPC-mPFC pathway depend on the origin of the HPC projections (Takita et al., 2013). Projections from the vHPC show PPF and PPI peak latencies shorter when compared to projections from iHPC (Izaki et al., 2001, 2002). Moreover, iHPC-mPFC PPF responses are stronger than vHPC-mPFC PPF responses for similar paired-pulse stimulation intervals (Kawashima et al., 2006). The exact mechanism of different functional activities between both pathways is not fully understood. However, Takita et al. (2013) proposed that heterosynaptic circuits in mPFC could provide an explanation to these differences in short-term plasticity (Takita et al., 2007, 2013). Regarding long-term plasticity, iHPC-mPFC is more malleable to long-term plasticity protocols than vHPC-mPFC

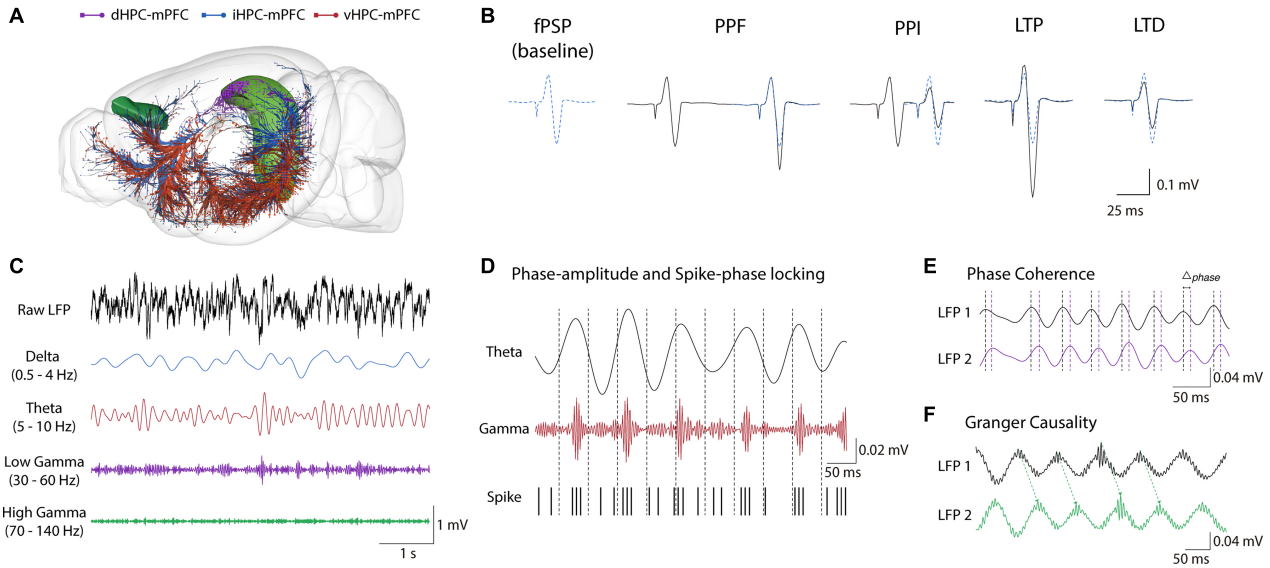

FIGURE 1 | Hippocampus-mPFC anatomical and electrophysiological measures. **(A)** Distinct HPC projections along the septo-temporal axis. Purple: dHPC; Blue: iHPC; Red: vHPC (brain sites adapted from the Brain Explorer, Allen Institute). **(B)** Electrophysiological measures of synaptic plasticity in HPC-mPFC pathway. From Left to right: typical fPSP baseline of the iHPC-mPFC pathway, short-term plasticity (PPF and PPI), and long-term plasticity (LTP and LTD) representatives. Paired-pulse stimulations facilitate or inhibit fPSPs response in longer and shorter interval stimulus, respectively. High-frequency stimulation induces LTP while low frequency typically induces LTD. **(C)** Representative traces of LFPs. Top to down: Raw LFP followed by band-filtered signal in the oscillatory activity commonly investigated in HPC-mPFC (Delta: 0.5–4 Hz, Theta: 5–10 Hz, Low-Gamma: 30-60 Hz, High-Gamma: 70-140 Hz). **(D)** Phase-amplitude and Spike-phase locking. In the example: Theta coupling to Gamma amplitude and neuronal spikes. These methods measure how the amplitude of a fast activity or the firing rate in one region is modulated by phase of the oscillatory activity in another region **(E)** Synchrony measures. In the example: Phase difference (Δ_{phase}) between theta oscillatory activity across time. Synchrony methods such as spectral and phase coherence measure the consistency of Δ_{phase} between two signals over time. **(F)** Granger Causality. Granger Causality is inferred when the future values of a signal are predicted using prior values of another signal. fPSP, field post-synaptic potential; PPF, paired-pulse facilitation; PPI, paired-pulse inhibition; LTP, long-term potentiation; LTD, long-term depression; LFP, local field potential.

(Takita et al., 2013). For example, in the iHPC-mPFC pathway, LTP induction is more susceptible to stimulus intensity variation, and previously induced LTP prevents LTD induction, and vice versa (Izaki et al., 2003a; Takita et al., 2010, 2013). Similarly, LTP induction in vHPC-mPFC increases a previously weak LTP and PPF response in iHPC-mPFC. Furthermore, electrolytic lesions in vHPC-mPFC attenuate iHPC-mPFC LTP (Kawashima et al., 2006). These results indicate that iHPC-mPFC and vHPC-mPFC provide distinct but convergent inputs to the mPFC long-term plasticity (Kawashima et al., 2006). Remarkably, HPC-mPFC pathways are related to different behavioral aspects, especially in working memory (Laroche et al., 2000). While bilateral lesions of iHPC interfered with working memory in a delayed alternation task on the order of a few seconds, bilateral lesions of vHPC did not (Izaki et al., 2008). However, vHPC-mPFC is important for longer aspects of working memory (Floresco et al., 1997; Wang and Cai, 2006). In fact, gamma power elevation in mPFC required for working memory is related to LTD induction in vHPC-PFC (Izaki et al., 2004).

HIPPOCAMPUS-PREFRONTAL CORTEX FUNCTIONAL CONNECTIVITY

Functional connectivity between the HPC and mPFC can be inferred from electrophysiological recordings of spike activity and local field potentials (LFP) during spontaneous behaviors. There are several methods to measure inter-area interaction (**Figures 1C-F**). Generally, these methods use electrophysiological time-series of different regions to quantify the statistical dependencies of neuronal activity over time (Buzsáki et al., 2012; Sigurdsson and Duvarci, 2016).

It is hypothesized that slow oscillations synchrony, such as delta and theta, provides a mechanism to coordinate network activity. These oscillations are transmitted with minimum phase delays between distant brain regions, allowing the coordination of neuronal spikes and local fast oscillatory activity (Roy et al., 2017). During active and exploratory behavior, HPC activity is dominated by theta oscillation (4-12 Hz) (Buzsáki, 2002), which is generated in the HPC-medial septum network but is a global rhythm recorded in various brain regions, including the mPFC. To date, however, there is no clear evidence whether the mPFC theta is a local oscillation of prefrontal neurons entrained by the HPC or is a measure of HPC volume conduction. Phase-locking of prefrontal spikes and gamma activity to theta oscillation and reduction of this phase-locking under inhibition of HPC projections indicate that theta oscillation can be driven by the hippocampus (O'Neill et al., 2013). In addition, theta coherence is attenuated monotonically as a function of distance from the hippocampus and there is no precise current source density estimate detected in the theta band in the parietal area overlying the hippocampus, suggesting that theta can be volume conducted

to cortical areas (Sirota et al., 2008). Despite the controversy, theta oscillation is essential for HPC-mPFC communication. Theta synchrony in the HPC-PFC is dynamically modulated during spatial working memory tasks, and phase locking of PFC units to hippocampal theta and hippocampal gamma oscillations is increased during correct choices in a spatial working memory task (Benchenane et al., 2010). Although the majority of studies investigated theta synchrony between the dHPC and the mPFC, theta synchrony is more robust between the mPFC and vHPC, which is consistent with the anatomical pathways (Adhikari et al., 2010; O'Neill et al., 2013) and has been related to fear and anxiety behaviors.

Synchrony between LFP rhythms in the HPC-PFC pathway has also been described in the gamma frequency (30-80 Hz) and it is thought to support the formation of neuronal assemblies coordinating excitatory spike activity into gamma cycles (Jung and Carlén, 2021). It has been postulated that inter-area functional connectivity and transfer of information can be coordinated by gamma oscillations (Fries, 2015). Gamma coherence in the dHPC-PFC, for example, increases with the learning of spatial reference memory (Yamamoto et al., 2014). Inter-areal brain connectivity can also be measured by cross-frequency coupling (CFC), which quantifies the interaction between oscillatory activities in different frequency bands. Phase-amplitude coupling (PAC) is a measure of CFC that estimates the statistical dependencies of the phase of a slower oscillation and the amplitude of a faster rhythm (Nandi et al., 2019). This oscillatory coupling is proposed to be a mechanism of brain coordination across regions. In the HPC-PFC pathway, it has been described that the HPC theta modulates the envelope of PFC gamma (Sirota et al., 2008; Tamura et al., 2017) and the HPC gamma (> 60Hz, reflecting population spike activity) induces postsynaptic membrane fluctuations in the theta frequency (Nandi et al., 2019). Theta-gamma PAC in the PFC is also related to improved cognitive performance and spatial working memory (Tamura et al., 2016, 2017).

Theta and gamma activity dominates the HPC-PFC interactions during active behavior. However, distinct oscillatory patterns coordinate communication between the HPC and neocortex during passive behaviors, especially during sleep (Jung and Carlén, 2021). According to the two-stage model of memory consolidation (Buzsáki, 1989), recently encoded representations are gradually transferred from the hippocampus to cortical regions, such as the PFC, during offline behavioral states (i.e. resting or sleeping) (Buzsáki, 2015). Recent studies demonstrated that fine temporal coordination between distinct hippocampal-cortical oscillatory patterns occurs during non-rapid-eye movement (NREM) sleep. Particularly, the coordination between hippocampal sharp-wave ripples (SWR), a well-described high-frequency oscillation registered in CA1 (Buzsáki, 2015), thalamic-cortical spindles, and slow cortical oscillations seems to be critical for this process (Maingret et al., 2016; Latchoumane et al., 2017). Accordingly, depolarization of mPFC neurons was observed synchronously to hippocampal place-cells reactivation during SWR events (Nishimura et al., 2021). Although there is plenty of evidence supporting that ripples trigger the propagation of memory traces from the hippocampus towards the neocortex, the idea of a unidirectional modulation was challenged by recent findings in animal models (Abadchi et al., 2020) and humans (Helfrich et al., 2019). Furthermore, the HPC-PFC dialog during SWRs is accompanied by the inhibition of many diencephalic, midbrain, and brainstem regions. This suggests a possible prioritization of the HPC-PFC interaction by silencing subcortical inputs (Logothetis et al., 2012). There is evidence suggesting that SWRs are endogenous candidates for promoting both LTP (Sadowski et al., 2016) and synaptic depression (Norimoto et al., 2018), but it remains unclear how they regulate the HPC-PFC communication. Particularly, during awake, SWR occurs in periods of post-consummatory behavior with reward-associated dopaminergic activity. The stimulation of hippocampal dopaminergic fibers from the midbrain increases reactivation during SWRs (McNamara et al., 2014). Dopamine also induces the facilitation of SWRs and is thought to reorganize cell assemblies during these events (Miyawaki et al., 2014). During sleep, SWRs are usually detected when cholinergic and noradrenergic levels are reduced. Indeed, the activation of septal-hippocampal cholinergic neurons suppresses SWR (Vandecasteele et al., 2014) and norepinephrine modulates its induction (Ul Haq et al., 2012). Although these neurotransmitters modulate SWR, there is no clear understanding of how they affect the HPC-PFC coordination.

In summary, the interactions between HPC and mPFC are dynamically modulated in different temporal scales, according to environmental and cognitive demands. Neuromodulatory systems play an essential role in regulating both synaptic plasticity and network coupling, enabling an adequate dynamical communication between hippocampal and cortical circuits. In the following sections, we will review studies demonstrating the effects of the main neurotransmitter systems in long- and short-term HPC-PFC synaptic plasticity and its functional connectivity.

NEUROMODULATION

Acetylcholine

Acetylcholine (ACh) is one of the main neuromodulators of the central nervous system playing an essential role in attention, regulation of the sleep-wake cycle, learning, and memory (Dannenberg et al., 2017). The brain has two major cholinergic projections: the basal forebrain and the brainstem cholinergic system. The basal forebrain cholinergic system includes the nucleus basalis of Meynert (nucleus basalis magnocellularis in rodents), substantia innominata (NB/SI), the medial septal nucleus, and the horizontal and vertical limbs of the diagonal band of Broca. These regions modulate learning, memory, synaptic plasticity, arousal, and attention (McCormick, 1993; Leanza et al., 1996; Villano et al., 2017). The brainstem cholinergic system comprises the peduncolopontine nucleus and the laterodorsal pontine tegmental nucleus, which has been described as part of the ascending reticular activating system. This system is implicated in the regulation of rapid eye movement (REM) sleep, wakefulness, and vigilance (Shouse and Siegel, 1992; Datta and Siwek, 1997). In rodents, HPC receives cholinergic inputs from the septum-diagonal band complex, whose fibers

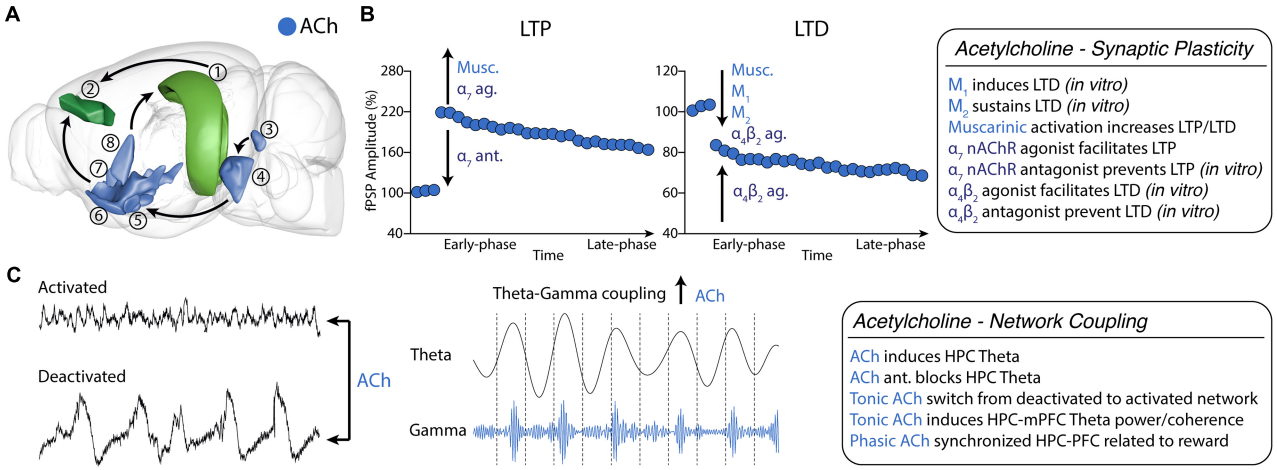

FIGURE 2 | Cholinergic neuromodulation of HPC-mPFC communication. **(A)** HPC-mPFC pathway and cholinergic circuits. (1) Hippocampus CA1/Subiculum; (2) Prelimbic prefrontal cortex; (3) Laterodorsal tegmental nucleus; (4) Pontine reticular nucleus; (5) Magnocellular nucleus; (6) *Substantia innominata*; (7) Diagonal band nucleus; (8) Medial septal nucleus. Blue: main cholinergic anatomical structures (brain sites adapted from the Brain Explorer, Allen Institute). **(B)** Muscarinic and nicotinic receptor activation bidirectionally affects long-term synaptic plasticity, increasing LTP and LTD induction. **(C)** Tonic cholinergic modulation is essential to switch from a deactivated state to an activated state. In addition, modulation of muscarinic activation promotes theta-gamma PAC in HPC-mPFC. Ach, Acetylcholine; LTP, long-term potentiation; LTD, long-term depression; ag, agonist; ant., antagonist.

project to hippocampal subfields and most cell types, including pyramidal cells, granule cells, interneurons, and hilar neurons (Frotscher and Léránth, 1985; Woolf, 1991). The ventral regions of the mPFC (prelimbic and infralimbic), on the other hand, receive strong projections from the horizontal and diagonal band of Broca and only a few projections from the nucleus basalis (Chaves-Coira et al., 2018; **Figure 2A**).

ACh acts primarily by activating two types of membrane receptors, the G protein-coupled muscarinic acetylcholine receptors (mAChRs) and the ligand-gated nicotinic acetylcholine receptors (nAChRs). Muscarinic acetylcholine receptors (mAChRs) are divided into five subtypes (M_1-M_5). Of these receptors, M_1, M_3, and M_5 are excitatory and are coupled with the $G_{q/11}$ family of G proteins, while M_2 and M_4 are inhibitory and are coupled with the $G_{i/o}$ family (Volpicelli and Levey, 2004). The M_1 and M_2 subtypes are the most abundant mAChRs in the brain (Volpicelli and Levey, 2004). While the M_1 receptor is localized post-synaptically and is expressed abundantly in the cerebral cortex and HPC, the M_2 mAChR is an inhibitory autoreceptor localized mainly on large cholinergic interneurons (Levey et al., 1991; Crook et al., 2001). In contrast, nAChRs belong to the family of the ligand-gated ion channels. There are 16 nAChRs subunits identified (α_1-α_{10}, β_1-β_4, γ, δ, ε), which can combine in multiple forms of receptors according to the brain region, neuronal subtype and animal developmental stage. The homomeric α_7 nAChRs and the heteromeric $\alpha_4\beta_2$ are the most abundant in the adult mammalian brain and are highly expressed in the HPC and mPFC (Gotti et al., 2007).

Synaptic Plasticity

Our understanding of the cholinergic modulation of synaptic plasticity in the HPC-mPFC pathway comes mostly from animal studies performed *in vitro* and *in vivo*. The work of Parent et al. (2010) describing a new preparation that allowed the identification and stimulation of pathway-specific ventral hippocampal inputs to neurons of the prelimbic cortex was essential for studying the HPC-mPFC pathway *in vitro*. Using this preparation, Wang and Yuan described that bath application of carbachol (an unspecific AChRs agonist) resulted in a pronounced decay of evoked fPSP (acute phase) that returned to baseline after ∼40 min in cortico-cortical stimulation. However, in vHPC, fPSP was maintained in 75% of the baseline response, characterizing an LTD induction (Wang and Yuan, 2009). They also verified that M_1 and M_2 antagonists contributed to the acute phase of synaptic depression, while M_2 was more related to long-term suppression. Caruana et al. (2011) replicated those findings and showed that a specific prolonged (450 pulses) low-frequency stimulation (1 Hz) induced an LTD form that is dependent on the M_1 receptor and does not depend on NDMAr.

In a series of elegant experiments, Maksymetz et al. (2019) investigated specific regional inputs to the prefrontal cortex using *in vitro* electrophysiology and optogenetics. First, they reproduced the previous carbachol data using a more specific muscarinic agonist to induce LTD in layer II/III to layer V fPSPs in the prelimbic cortex (Ghoshal et al., 2016). Following, they used an optogenetic approach to stimulate specific afferent projections. Interestingly, the M_1-induced LTD was specific to the basolateral amygdala or the vHPC projections and produced only mild effects in the mediodorsal nucleus of thalamus projections to the prelimbic cortex. Also, using a viral-mediated selective deletion of M_1 receptors from glutamatergic pyramidal neurons in the mPFC, they demonstrated that the muscarinic induced LTD in the vHPC-mPFC is dependent on the postsynaptic expression of M_1 receptors.

Our group pioneered the *in vivo* investigation of the cholinergic modulation of the iHPC-mPFC synaptic plasticity.

We first demonstrated that intraperitoneal administration of the M_1 preferential agonist pilocarpine (Pilo) potentiated the late-phase of iHPC-mPFC LTP (Lopes Aguiar et al., 2008). Following, we investigated the effects of Pilo on iHPC-mPFC LTD. We demonstrated that previous administration of pilocarpine converted a transient cortical depression into a robust and stable LTD. Importantly, we demonstrated that iHPC-mPFC LTD induction is dependent on NMDA receptors since the selective antagonist AP7 blocked the pilocarpine-induced LTD conversion and the induction of a strong LTD using a supra-threshold protocol (Lopes-Aguiar et al., 2013). The effects of muscarinic activation on iHPC-mPFC synaptic plasticity were confirmed in a later study using intracerebroventricular (i.c.v.) administration of Pilo. However, i.c.v. Pilo administration mainly potentiated LTP induction (Ruggiero et al., 2018). In the same work, we showed that lithium - a drug that interacts with downstream targets of M_1 signaling - dampened the muscarinic effects of Pilo on LTP, but enhanced on LTD (Ruggiero et al., 2018). Taken together, these results show a bidirectional modulation of iHPC-mPFC synaptic plasticity by M_1 activation. Importantly, in neither of these studies, cholinergic activation altered basal synaptic transmission, indicating that *in vivo* effects are mainly mediated by downstream targets related to synaptic plasticity.

nAChRs also modulate synaptic plasticity in brain areas intrinsically related to reward and addiction, such as the ventral tegmental area (Mansvelder and McGehee, 2000; McKay et al., 2007). Particularly, in the vHPC-mPFC pathway of intact animals under urethane anesthesia α_7 receptor agonism facilitates LTP at lower doses and blocks LTP at higher doses (Stoiljkovic et al., 2016). A recent *in vitro* study further elucidated the role of nAChRs on the vHPC-mPFC pathway, using a spike-timing-dependent plasticity (STDP) protocol that induces only a transient increase in excitatory postsynaptic current (Sabec et al., 2018). The authors observed LTP induction or blockade in the presence of α_7 nAChRs agonist or α_7 nAChRs antagonist, respectively. On the other hand, STDP induced LTD in the presence of a $\alpha_4\beta_2$ receptor agonist and was prevented by co-application of a $\alpha_4\beta_2$ antagonist. In addition, $\alpha_4\beta_2$ nAChRs LTD was blocked by gabazine, which suggests that GABAergic neurons might intermediate the cholinergic action. Interestingly, neither α_7 nor $\alpha_4\beta_2$ nAChRs agonists affected synaptic transmission by themselves (**Figure 2B**).

Functional Connectivity

The cholinergic modulation of theta rhythm is well known for decades. Numerous studies have demonstrated that acetylcholine, cholinesterase inhibitors, and cholinergic agonists can induce hippocampal theta oscillation while antagonists block it (reviewed by Nuñez and Buño, 2021). Cholinergic induction of theta is largely mediated by the activation of M_1 mAChRs in hippocampal pyramidal neurons (Ovsepian et al., 2004), although nAChRs may also play a role through the basal forebrain cholinergic projections to hippocampal interneurons (Griguoli et al., 2010). In the neocortex, cholinergic inputs exert an activation effect, bringing membrane polarization closer to firing threshold, probabilistically increasing neurotransmission and neuronal responses that are critical for higher arousal states such as awake and REM sleep (Steriade, 2004). This activation is done directly through the NB/SI projections or indirectly through the brainstem cholinergic system afferents to the thalamus and the NB (Steriade, 2004). Activation of both the brainstem or forebrain cholinergic systems disrupts the main oscillatory rhythms of the non-REM sleep (delta oscillation, slow oscillation and spindles) and induces the occurrence of fast oscillatory activity (20-60 Hz) that are prominent during awake and REM sleep and involves thalamo-cortical and cortical circuits (Steriade, 2004).

Under urethane anesthesia, rodents show two distinct spontaneous oscillatory patterns (states): a deactivated state characterized by delta and slow oscillation activity; and an activated state, described by fast oscillatory activity and hippocampal theta (Clement et al., 2008; Pagliardini et al., 2013a,b). Clement et al. (2008) showed that this brain state alternation depends on cholinergic and muscarinic activation. Inactivation of the forebrain cholinergic system disrupted spontaneous alternation (i.e., prolonged the deactivated states), while stimulation of the brainstem cholinergic system immediately produced activated patterns (Clement et al., 2008). In addition, we have recently shown that distinct coupling occurs between the HPC-mPFC during each state. In the deactivated state, we observed a coherence peak in the 0.5-2 Hz band, with the mPFC leading the HPC, while in the activated state, there was a peak in theta with the HPC leading the mPFC and a time lag consistent to data of freely-moving animals (Lopes-Aguiar et al., 2020). We also showed that mAChR activation by pilocarpine dose-dependently increased activated over deactivated states (Lopes-Aguiar et al., 2013). Activated states could also be induced by i.c.v. administration of nicotine. In this case, the increase observed in gamma and beta rhythms were lower compared to muscarinic activation (Bueno-Junior et al., 2012). Similar effects, showing decrease in delta and increase in theta and gamma bands were observed in various cortical areas in freely moving animals after cholinergic modulation (Cape et al., 2000). Interestingly, we further showed an increase in iHPC-mPFC gamma coherence following muscarinic activation (Lopes-Aguiar et al., 2013; Ruggiero et al., 2018). Muscarinic modulation was also shown to promote PAC between HPC theta and mPFC low-gamma activity (Ruggiero et al., 2018). Theta-gamma coupling is also produced in the PFC following phasic acetylcholine release induced by the detection of environmental cues (**Figure 2C**). Finally, M1 blockade disrupted theta-low gamma coupling in the mPFC (Howe et al., 2017).

Dopamine

Dopamine (DA) is the most extensively studied modulator of HPC-mPFC synaptic plasticity. Dopaminergic transmission is involved in numerous cognitive, emotional, and motor functions (Tritsch and Sabatini, 2012; Speranza et al., 2021), and impairments of this system are implicated in neuropsychiatric disorders, such as schizophrenia and major depression (Goto et al., 2010; Grace, 2016).

Dopamine is produced in midbrain and hypothalamic neurons. In the midbrain, the ventral tegmental area (VTA) is the primary source of afferents to limbic and cortical structures.

There are five subtypes of dopamine receptors ($D_{1\text{-}5}$) identified to date. They are grouped into two main classes: D_1-like and D_2-like. All DA receptors are G-protein coupled receptors (GPCR) which generally activate (via Gs/olf: D_1-like) or inhibit (via Gi/o: D_2-like) adenylate cyclase and protein kinase A (PKA) pathway (Beaulieu and Gainetdinov, 2011). The mPFC receives dense projections from the VTA, while the HPC receives more sparse terminals (Santana and Artigas, 2017; Edelmann and Lessmann, 2018). The expression of D_1 receptors in the mPFC is more densely concentrated in the deep layers (V-VI), which also receives limbic excitatory afferents. In contrast, the expression of D_2 receptors is preferentially localized in the superficial cortical layers (I-III), largely comprising intracortical connections (Berger et al., 1991; Gaspar et al., 1995; Santana and Artigas, 2017). In the HPC, there is a gradient of D_1 and D_2 expression across the dorsoventral axis, in which the ventral region aspect shows greater expression than the dorsal part (for a review, see Edelmann and Lessmann, 2018). Remarkably, VTA terminals in the mPFC occur in both dendritic shafts and spines and present proximity with HPC excitatory afferents forming synaptic triads (Carr and Sesack, 1996). This structural characteristic intriguingly suggests a relationship between DA, HPC, and mPFC.

Although *in vitro* studies demonstrate the dependence of PFC plasticity on DA, they do not reveal such a clear picture of how this modulation occurs (reviewed in Goto et al., 2010) as it has been observed in the intact brain (Jay et al., 2004). For this reason, we will review only studies that preserved the HPC-mPFC and mesolimbic circuits (**Figure 3A**). Jay et al. (1995) showed that electrical stimulation of the VTA, known to induce cortical DA release, inhibits the neural response of most (73%) mPFC neurons responsive to vHPC stimulation. This finding provided clear electrophysiological evidence for the convergence between vHPC and VTA afferents onto the mPFC, corroborating previous morphological studies. Additionally, they reported that electrical stimulation of the NAc induced antidromic activation of vHPC-responsive mPFC neurons (Jay et al., 1995). This work also suggested a circuit where vHPC controls DA release from the VTA through the PFC and NAc. Other studies confirmed that vHPC stimulation increases DA levels (Blaha, 1997; Floresco et al., 2001). However, the subsequent studies showed that the vHPC could control the VTA even when the PFC is inhibited by tetrodotoxin (Floresco et al., 2001). The control of the HPC over the VTA release is reviewed in Lisman and Grace (2005).

Synaptic Plasticity

Gurden et al. (1999) showed that the lesion of DA neurons in the VTA by local injection of 6-hydroxydopamine suppresses LTP in the vHPC-mPFC pathway. The same work showed a strong positive correlation between DA levels in the mPFC and vHPC-mPFC LTP, demonstrating the critical importance of the mesolimbic system and DA in the vHPC-mPFC long-term plasticity. In another study, Gurden et al. (2000) showed that a D_1 antagonist abolishes vHPC-mPFC LTP, while a D_1 agonist enhances it. In turn, both D_2 agonist and antagonist did not affect HPC-mPFC LTP. The dependence of vHPC-mPFC LTP on D_1-mediated signaling has been replicated several times and is likely the best-known mechanism of modulation of the vHPC-mPFC long-term synaptic plasticity (Jay, 2003; Goto and Grace, 2008; Xu et al., 2016). Importantly, DA seems to modulate both the early and late phases of LTP in the HPC-mPFC synapses, suggesting a role in modulating short-term plasticity. Goto and Grace (2008) showed that theta-burst stimulations (TBS) induced short-term potentiation of the dHPC-mPFC fPSP and that D_1, but not D_2, antagonists could block this induction. Together, these findings suggest that D_1-mediated signaling is critical for short-term and long-term synaptic facilitation of the HPC-mPFC pathway.

Another finding of Gurden et al. (2000) was that D_1 overstimulation could worsen LTP. Therefore, indicating that D_1 modulation of HPC-mPFC plasticity follows an inverted U-shape relationship, where low levels of D_1 activation (e.g., D_1 antagonism or VTA lesion) suppresses LTP, then an optimal level of DA is associated with increased LTP, but high doses would impair LTP again. Remarkably, the same inverted U-shape association of D_1 and PFC function has been well established for *in vitro* plasticity and for working memory performance (Goldman-Rakic et al., 2000; Seamans and Yang, 2004; Williams and Castner, 2006; Goto et al., 2007; Kolomiets et al., 2009; **Figure 3B**).

Functional Connectivity

Despite showing greater concentrations onto the PFC and striatum, DA afferents are widespread throughout the brain, suggesting a role in modulating network activities. DA levels are enhanced during working memory tasks (Phillips et al., 2004; Robbins and Arnsten, 2009). Benchenane et al. (2010) showed an increase in iHPC-mPFC theta coherence throughout a spatial working memory task, especially during delayed periods. Next, they showed in the anesthetized rat that the local infusion of DA in the mPFC mimicked the electrophysiological effects observed during memory performance, such as iHPC-mPFC theta coherence and phase-locking of mPFC neurons to iHPC theta field. This finding is remarkable because it suggests that DA facilitation of HPC inputs promotes greater synchrony in the theta band, which comprises an oscillatory activity generated in the hippocampus, but is essential for many PFC-associated behavioral functions (Benchenane et al., 2011). In support, Xu et al. (2016) reported that i.c.v. injection of a D_1 antagonist does not change vHPC and mPFC theta power but decreases both vHPC-mPFC theta coherence, directionality, and theta-slow gamma coupling (Xu et al., 2016). Dzirasa et al. (2009), using transgenic mice knockout for the DA transporter, which presents hyperdopaminergia, reported increased gamma coherence between dHPC and mPFC. Recently, Gener et al. (2019) showed in freely moving mice that D_2 agonism reduced dHPC-mPFC theta power and synchrony but increased them in delta. They also showed decreases in beta and gamma power, all paralleled to reduced locomotion. Then, D_2 antagonism with haloperidol reversed the effects on beta, gamma and theta power and theta synchrony (**Figure 3C**).

Noradrenaline

Noradrenaline (NA) is produced in the brainstem in specific locus coeruleus (LC) neurons. It has widespread terminals onto

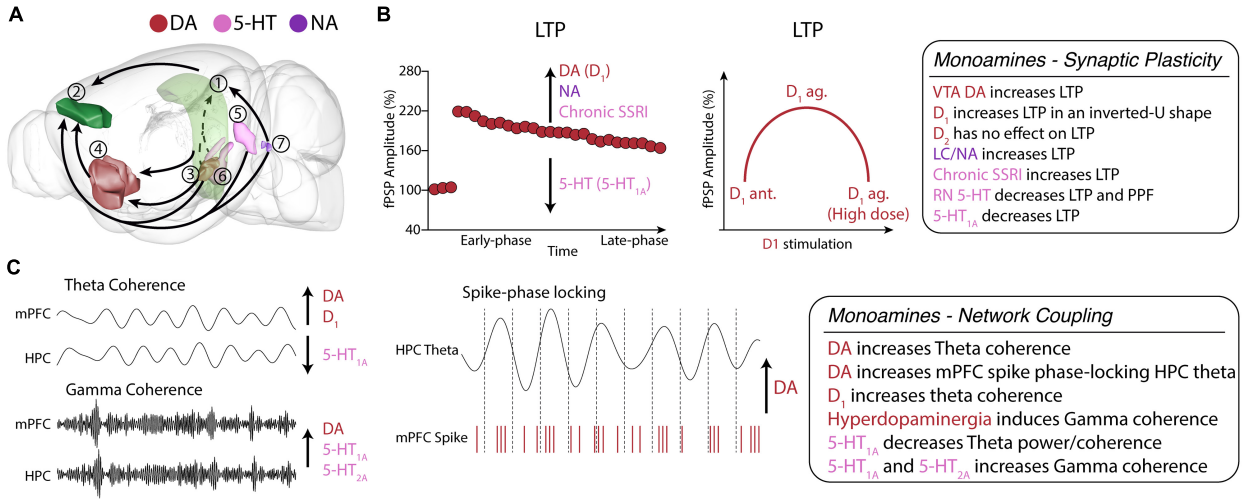

FIGURE 3 | Monoamine neuromodulation of HPC-mPFC communication. **(A)** HPC-mPFC pathway and monoaminergic circuits. (1) Hippocampus CA1; (2) Prelimbic prefrontal cortex; (3) Ventral tegmental area; (4) Nucleus accumbens; (5) Dorsal raphe nuclei; (6) Median raphe nuclei; (7) *Locus coeruleus*. Red: main dopaminergic anatomical structures; Purple: main noradrenergic anatomical structures; Pink: main serotoninergic anatomical structures (brain sites adapted from the Brain Explorer, Allen Institute). **(B)** HPC-mPFC LTP is positively modulated by DA, NA, and chronic SSRI, while 5-HT decreases LTP. In particular, D_1 activation affects LTP in an inverted U-shaped dose dependence, providing a fine-tune functional regulation in the HPC-mPFC pathway. **(C)** In HPC-mPFC communication, D_1 and 5-HT_{1A} receptors induce opposite effects in theta coherence, however, both increase gamma coherence. DA, dopamine; 5-HT, serotonin; NA, noradrenaline; SSRI, selective serotonin reuptake inhibitor; LTP, long-term potentiation; LTD, long-term depression.

the forebrain, and it is proposed to be one of the most important brain systems to modulate arousal and attention (Sara, 2009). NA has three main types of receptors, α_1 (Gq GPCR), α_2 (Gi/o GPCR), and β (Gs GPCR), which are subdivided into three subtypes (Marzo et al., 2009). Importantly, NA receptors also have an affinity with epinephrine, a hormone released in the body during stress, suggesting that NA transmission is associated with stress-related modulation of brain function (Ross and Van Bockstaele, 2021). The PFC expresses NA receptors less densely than DA and in a more distributed manner across layers (Santana and Artigas, 2017). The HPC, in turn, expresses more adrenergic receptors than DA, where it is critical for LTP, especially in the Schaffer collateral pathway (CA3-CA1) (Nguyen and Connor, 2019). NA modulation of synaptic plasticity, including at the HPC and PFC, is reviewed in Marzo et al. (2009) (**Figure 3A**).

Lim et al. (2010) performed a comprehensive investigation demonstrating a positive link between NA and enhancement of vHPC-mPFC LTP. LC electrical stimulation just before bursts of HFS increased vHPC-mPFC LTP. In contrast, inhibition of the LC by local lidocaine injection decreased LTP. Recently, Takeuchi et al. (2016) showed that DA rather than NA released from the LC might play a more important role in plasticity and memory within the HPC. Nevertheless, Lim et al. (2010) showed that a NA reuptake inhibitor and systemic administration of an α2 antagonist, which disinhibits NA release, also increased LTP. In contrast, α2 agonist and partial lesion of NAergic LC neurons by DSP-4 decreased LTP. Noteworthy, no studies manipulated NA transmission directly in the PFC, and we still do not know the roles of specific adrenergic receptors at the mPFC in the facilitatory effect of NA on HPC-PFC plasticity. Nevertheless, NA exerts positive effects on mPFC-related cognitive functions and HPC plasticity via β-mediated signaling, which generally acts through the PKA pathway (Chamberlain and Robbins, 2013; Nguyen and Connor, 2019), while it modulates HPC and intracortical mPFC LTD mainly via α1 (Marzo et al., 2010). In addition, Lim et al. (2017) showed that NA indirectly modulates vHPC-PFC plasticity via the amygdala, a key region for processing fear and stress. NA-mediated activation and inhibition of the amygdala positively and negatively modulated vHPC-PFC LTP, respectively. Besides the role in stress, Bhardwaj et al. (2014) investigated the effects of neonatal lesion of vHPC, which is described as a model of schizophrenia, and reported a disruption of NA modulation of mPFC corticocortical plasticity via alterations of α1 signaling. Notably, NA and DA interact (Xing et al., 2016; **Figure 3B**). Therefore another possible mechanism of NA modulation could be indirectly by influencing DA transmission. NA manipulations can increase DA levels, and LC stimulation can activate the VTA (Grenhoff et al., 1993). The interaction between these signaling pathways has been investigated in the HPC and the mPFC (Xing et al., 2016) but still not in the HPC-PFC pathway.

Serotonin

Serotonin (5-hydroxytryptamine; 5-HT) is produced in specific neurons of the midbrain that comprise the raphe nuclei (RN). The RN has widespread innervations to all the forebrain, and the HPC and PFC are principal targets of these innervations (Azmitia and Jacobs, 1992). The most relevant nuclei regarding HPC and PFC function are the dorsal raphe nucleus (DRN) and the median raphe nucleus (MRN) (Puig and Gener, 2015). The DRN has widespread terminals in the forebrain and is the primary source of serotonin to the PFC, to whom it has reciprocal

connections (Puig and Gulledge, 2011; Pollak Dorocic et al., 2014). In turn, the MRN has more specific connections, and it is the primary source of 5-HT to the septohippocampal system (Vertes and Kocsis, 1997). There are seven families of 5-HT receptors (5-HT_{1-7}) divided into fourteen subtypes. All subtypes are GPCR, except for the 5-HT_3, which is ionotropic. The most expressed receptors in both structures are the 5-HT_{1A} (Gi/o GPCR) and the 5-HT_{2A} (Gs GPCR). There is a specific profile of expression of the distinct 5-HT receptor throughout neurons. In both PFC and HPC, 5-HT_{1A} is preferentially expressed in the somata and axons, while the 5-HT_{2A} is on the apical dendrites of pyramidal cells (Puig and Gener, 2015). PFC pyramidal neurons show co-expression of both receptors in the same neurons (Amargós-Bosch et al., 2004). Therefore, 5-HT has a significant inhibitory effect on the firing rate but promotes excitatory effects on field potentials (Puig and Gulledge, 2011; **Figure 3A**).

Synaptic Plasticity

The first implication of the 5-HT system in the modulation of HPC-mPFC synaptic function showed that both single and chronic applications fluvoxamine, an antidepressant that acts as a selective serotonin reuptake inhibitor (SSRI), could increase vHPC-mPFC fPSP (Ohashi et al., 2002). Also, the fPSP increases occurred in a dose-dependent manner by acute injection. However, they observed that only the chronic, but not acute, treatment increased vHPC-mPFC LTP (Ohashi et al., 2002). It is well established that SSRI antidepressants only promote their therapeutic effects against depression through chronic treatment, which can begin to manifest up to two weeks after treatment (Kraus et al., 2017). Thus, the distinct modulation of LTP by chronic and acute administration of SSRI may represent a manifestation of long-term mechanisms equivalent to those underlying the clinical effects of antidepressants rather than acute effects of 5-HT receptors activation.

In another study, Ohashi et al. (2003) investigated the effects of the lesion of 5-HTergic neurons by administration of 5,7-dihydroxytryptamine. They showed that 5-HT depletion increased vHPC-mPFC PPF and produced a remarkable increase of LTP, which showed a negative correlation with mPFC 5-HT levels (Ohashi et al., 2003). Little is known about the role of specific 5-HT receptors in the modulation of HPC-mPFC plasticity. Xu et al. (2016) administered an i.c.v. injection of a 5-HT_{1A} agonist and reported increased vHPC-mPFC LTP. This finding is discussed in the scenario where 5-HT_{1A} agonism acts on RN presynaptic terminals inhibiting 5-HT release, which supports the role of 5-HT in suppressing this LTP. However, the drug could also be acting directly on the PFC to some degree, so further studies should address this issue (**Figure 3B**).

Functional Connectivity

5-HTergic neurons project densely to the HPC and PFC and present widespread innervations to the forebrain (Azmitia and Jacobs, 1992). Early studies demonstrated that MRN 5-HT transmission is the main regulator of HPC theta oscillations, the most prominent network activity of this structure (Vertes and Kocsis, 1997; Vertes et al., 2004). Lesion of the MRN and 5-HT inhibition enhances theta, while stimulation decreases it (reviewed in Vertes and Kocsis, 1997). 5-HT has also been shown to decrease HPC gamma *in vitro* (Krause and Jia, 2005; Johnston et al., 2014). 5-HT_{1A} knockout mice exhibit greater HPC and PFC theta power in anxiogenic environments (Gordon, 2005; Adhikari et al., 2010). More recently, the role of 5-HT transmission was investigated in the PFC. Puig et al. (2010) showed that 5-HT modulates fast-spiking interneurons, which are critical for generating brain rhythms. 5-HT infusion in the PFC in anesthetized rats decreased firing rates but increased the frequency of UP states, which are rhythmic periods of excitability. These UP states carry gamma oscillations, so 5-HT increased them consequently. The effects of 5-HT on the HPC and PFC network activities are reviewed in Puig and Gener (2015).

Only recently, the modulation of 5-HT on HPC-PFC network synchrony has been examined. In anesthetized rats, i.c.v. injection of a 5-HT_{1A} agonist increased PFC, but not vHPC, theta power and decreased both slow and fast gamma in the HPC and mPFC. No effects were observed in theta coherence, but they reported an increase in unidirectional gamma coherence from vHPC to mPFC, but not the other way around. 5-HT_{1A} agonism also increased vHPC-mPFC fast gamma coherence but decreased theta-fast gamma coupling. Interestingly, these findings indicate that 5-HT and DA modulate theta coupling to distinct gamma frequencies (Xu et al., 2016). In alert rats, particularly during awake immobility states, 5-HT_{1A} activation decreased power in a broad range of oscillatory bands including theta, beta, slow gamma, and fast gamma activities in both dHPC and PFC, as well as induced a profound decrease in dHPC-mPFC theta coherence. Noteworthy, this study also showed that subsequent administration of 5-HT_{1A} antagonist reversed all of these effects. In turn, 5-HT_{2A} activation produced an enhancement of PFC high-gamma power and HPC-PFC high-gamma coherence. 5-HT_{2A} antagonist profoundly decreased HPC-PFC power of theta, slow and high gamma, and decreased theta coherence, while it dramatically increased HPC-PFC delta power (Gener et al., 2019). Interestingly, the effects of PFC delta power may reflect antipsychotic properties since both 5-HT2A and D2 antagonists increase it while 5-HT2A agonists with hallucinogenic effects decrease it. Also, Kjaerby et al. (2016) showed that presynaptic activation of 5-HT_{1B} in the mPFC suppressed vHPC inputs *in vitro* and reduced both mPFC theta power and anxiety measures in the elevated plus maze (**Figure 3B**).

Endocannabinoids

The endocannabinoid (eCB) system is a complex, widespread neuromodulatory pathway in the mammalian brain regulating multiple neural functions involved in cognitive, sensory, and emotional processes (Piomelli, 2003). The eCB system includes cannabinoid receptors, eCB neurotransmitters, and enzymes related to the synthesis and degradation of eCBs (Lu and MacKie, 2016).

The main eCB receptors are the CB_1 and CB_2 receptors, primarily expressed in the central nervous system and the peripheral immune cells, respectively (Chen et al., 2017; Kendall and Yudowski, 2017). The CB_1 and CB_2 receptors are

Gi/Go-coupled receptors which inhibits adenylyl cyclases and voltage-dependent calcium channels, while activating MAP kinases and potassium channels, reducing neurotransmitter release (Howlett et al., 2002). The most abundant eCB neurotransmitters are 2-arachidonoylglycerol (2-AG) and anandamide (AEA), which are post-synaptically, synthesized "on-demand" by 2-arachidonoyl-containing phospholipids and N-arachidonoyl phosphatidyl ethanol, respectively (Pacher et al., 2006). In turn, monoacylglycerol lipase degrades 2-AG, and fatty acid amide hydrolase (FAAH) degrades AEA (Di Marzo et al., 1994; Dinh et al., 2002). In the central nervous system, CB_1 receptors are fundamentally pre-synaptically expressed both in excitatory and inhibitory synapses in mesocorticolimbic circuits, mediating complex retrograde signaling (Ohno-Shosaku and Kano, 2014). Not surprisingly, the eCB is a fundamental regulator in synaptic plasticity and functional connectivity in the HPC-mPFC pathway (Heifets and Castillo, 2009; Katona and Freund, 2012; **Figure 4A**).

Synaptic Plasticity

In vitro CB_1 activation of mPFC pyramidal neurons decreases baseline excitatory postsynaptic currents, increasing the proportion of cells exhibiting LTD and decreasing the proportion of cells exhibiting LTP. In contrast, CB_1 antagonism induces the opposite effect (Auclair et al., 2000; Riedel and Davies, 2005). However, little is known about the HPC-mPFC eCB modulation *in vivo* and in freely moving animals (Augustin and Lovinger, 2018). CB_1 agonists, when administered during adolescence, affect short-term and long-term plasticity in the vHPC-mPFC pathway in adults (Raver et al., 2013; Cass et al., 2014; Renard et al., 2016, 2017a,b). Chronic exposure to CB1 agonist in early adolescent rats facilitates fPSP response in PPI-like protocols susceptible to GABAergic modulation (Thomases et al., 2013; Cass et al., 2014). Indeed, CB_1 agonist exposure during adolescence reduces mPFC GABAergic transmission *in vivo*, and intra-mPFC GABA-Aα1 receptor positive allosteric modulator restores mPFC transmission (Cass et al., 2014). During adolescence, CB_1 agonist treatment also reduced the dendritic arborization in pyramidal neurons and postsynaptic levels of PSD95 in mPFC, which is related to impairments of vHPC-mPFC LTP induction (Renard et al., 2016). These results seem to emerge from a developmental impairment of mPFC GABAergic transmission by overactivation of the CB_1 receptor (Raver et al., 2013; Caballero et al., 2014; Renard et al., 2016, 2017b,a; **Figure 4B**).

Functional Connectivity

Δ9-tetrahydrocannabinol (THC) is the main psychoactive constituent of *Cannabis sativa* (Connor et al., 2021). THC is a partial agonist of CB_1 and CB_2 receptors and is an essential pharmacological tool for understanding drug abuse effects and eCB neuromodulation (Connor et al., 2021). Low acute doses of THC (1 mg/kg) decreased the average firing rate of mPFC cells and increased vHPC-mPFC delta (0.3-4 Hz) coherence, without effects on mPFC and vHPC power (Aguilar et al., 2016). However, high THC doses (5 mg/kg) decreased gamma (30-55 Hz) power in mPFC and overall spectral power in the vHPC (Robbe et al., 2006; Nelong et al., 2019). Interestingly, THC chronic exposure during adolescence increases mPFC gamma oscillations *in vitro* and *in vivo* permanently in adult rats while reduces the expression levels of GAD67 in the mPFC (Raver et al., 2013; Renard et al., 2017b).

Like THC effects, acute intraperitoneal treatment with CP55940 (synthetic full CB_1 agonist) in awake rats decreased firing rates in vHPC and burst activity in mPFC (Robbe et al., 2006; Kucewicz et al., 2011). Moreover, CP55940 decreased the overall power spectrum of vHPC LFP in anesthetized and freely moving rats, mainly on theta, high gamma (62-90 Hz), and ripple oscillations (100-200 Hz) (Robbe et al., 2006; Hajós et al., 2008). Interestingly, the CP55940 effects on vHPC power cannot be explained by firing rate changes. There was no correlation between LFP power and firing rates in vHPC, regardless of CP55940 dose (Robbe et al., 2006). However, CP55940 selectively decreased the incidence of short interspike intervals, which was positively correlated with LFP power changes, suggesting a role of interneurons in vHPC oscillations. Also, CP55940 reduced temporal synchronicity of previously simultaneous recorded cell pairs in vHPC, demonstrating decreased spike coactivation (Robbe et al., 2006).

The CB_1 agonist effects on HPC activity are associated with mPFC dysfunction as well. The theta power reduction in iHPC induced by CP55940 treatment reduces mPFC gamma (30-100 Hz) power, which could also be related to CB_1 effects on interneurons (Kucewicz et al., 2011). Indeed, CP55940 reduced firing rates of mPFC in 10-100 ms interspike intervals associated with decreases in gamma oscillations (Kucewicz et al., 2011). Accordingly, CP55940 treatment significantly impaired spatial working memory performance associated with iHPC theta power and gamma (30-60 Hz) reductions (Robbe et al., 2006; Kucewicz et al., 2011). Additionally, CP55940 disrupted iHPC-mPFC theta coherence during the spatial working memory task and impaired spike-locking of mPFC single-units to the local gamma oscillation (Kucewicz et al., 2011). Taken together, these results suggest that CB_1 agonism effects on HPC-mPFC communication are associated with abolishing the temporal synchrony essential for working memory. However, the complexity of eCB neuromodulation does not allow generalizations about its CB_1 agonists.

Differently from THC low acute dose treatment, the enhancement of AEA signal by URB597 (FAAH inhibitor) increased mPFC low gamma power (30-55 Hz) without increasing local firing rate (Aguilar et al., 2016). The AEA enhancement reduced vHPC delta power and vHPC-mPFC delta coherence, while THC in low doses increased vHPC-mPFC delta coherence (Aguilar et al., 2016). The mechanisms of the distinct effects of THC and AEA in the HPC-mPFC pathway have not been fully understood, but it is reasonably associated these effects to distinct effects occurs regional specificity of eCB system. URB597 only acts in regions with FAAH activity and will augment AEA levels on-demand, whereas THC widely activates CB_1 and CB_2 receptors (Egertová et al., 2003). Besides, the AEA signal affects other receptors, such as transient receptor potential vanilloid 1 (TRPV1) (Di Marzo et al., 2002; **Figure 4C**).

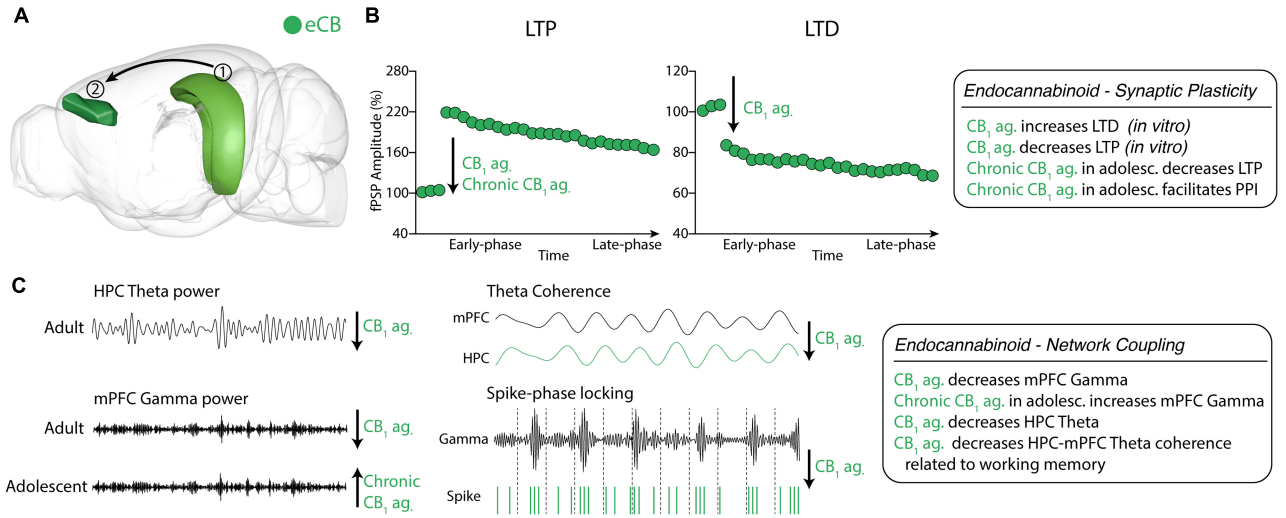

FIGURE 4 | Endocannabinoid neuromodulation of HPC-mPFC communication **(A)** eCB system in HPC and mPFC areas. (1) Hippocampus CA1; (2) Prelimbic prefrontal cortex. Green: eCB neurotransmission in HPC and mPFC (brain sites adapted from the Brain Explorer, Allen Institute). **(B)** CB_1 activation decreased LTP maintenance in HPC-mPFC pathway. **(C)** CB_1 agonism reduces HPC theta power and decreases mPFC Gamma power in adults, while chronic CB_1 agonist treatment during adolescence increased mPFC Gamma power in adults. Also, CB_1 agonism decreases HPC-mPFC theta coherence during a spatial working memory task and spike-gamma band phase locking in mPFC. eCB, endocannabinoid; LTP, CB_1, cannabinoid receptor 1; long-term potentiation; LTD, long-term depression. ag, agonist; ant., antagonist.

HIPPOCAMPUS-PREFRONTAL CORTEX NEUROMODULATION IN COGNITION AND ANIMAL MODELS OF NEUROPSYCHIATRIC DISORDERS

Hippocampus-prefrontal cortex communication underlies cognitive and behavioral functions that are critically impacted in neuropsychiatric conditions such as schizophrenia, major depression, anxiety disorders, and Alzheimer's disease (Godsil et al., 2013; Liu et al., 2016; Padilla-Coreano et al., 2016; Sampath et al., 2017). Therefore, a deeper understanding of the HPC-PFC functional connectivity, its regulatory dynamics, and neuromodulatory mechanisms are relevant for advancing towards a better treatment to these conditions.

Cognition

Several studies report the role of the HPC-mPFC pathway in cognitive processes, such as spatial working memory and memory consolidation (Laroche et al., 2000; Eichenbaum, 2017). It has been argued that the ventral and intermediate HPC-mPFC projections are more related to working memory tasks, while the dorsal HPC is related to memory consolidation. Optogenetic inhibition of vHPC terminals in the mPFC during the encoding phase of a T-maze working memory task results in memory impairments (Tamura et al., 2017). Further, CNO injection into mPFC of subjects expressing hM4D(Gi) DREADD in HPC inactivated HPC-mPFC communication and disrupted memory consolidation (Ye et al., 2017). Similar approaches have been employed to investigate the mechanisms of plasticity in the HPC-PFC circuit. For example, the firing responses in the mPFC after LTP induction in the iHPC-mPFC projections were attenuated by optogenetically controlling the paraventricular/mediodorsal thalamic area (Bueno-Junior et al., 2018). Functionally, several studies report increases in HPC-mPFC oscillatory synchrony during spatial working memory tasks (Jones and Wilson, 2005; Benchenane et al., 2011; Yu and Frank, 2015) and memory consolidation (Paz et al., 2008; Binder et al., 2019). Despite the few works that directly investigated neuromodulation of HPC-mPFC during these cognitive processes, cholinergic neuromodulation of HPC-mPFC seems to be fundamental.

Microdialysis experiments demonstrated that the cholinergic drive increases in the neocortex and hippocampus during cognitive processes (Pepeu and Giovannini, 2004). This indirect evidence may lead us to conjecture that cholinergic tone can modulate the synchronization dynamics between HPC-mPFC during cognitive tasks. Recent evidence has shown that this interaction is more complex, depending on brain state and cognitive demand. First, the traditional view of the acetylcholine neurotransmission as primarily spatially diffuse and slow-acting, in the scales of minutes, has been challenged (Sarter et al., 2009; Sarter and Lustig, 2020). A recent study using *in vivo* amperometry and LFP recordings has helped elucidate the dHPC-mPFC acetylcholine release dynamics showing that tonic and phasic ACh release occurs in both regions, occur in different timescales, and conduct different functions (Teles-Grilo Ruivo et al., 2017).

Interestingly, both modes of transmission occur in a coordinated manner in the HPC and PFC. Tonic ACh release mode is strongly associated with a transition to a high-arousal state and the switch from deactivated to activated. On the other hand, phasic ACh release occurred only during a performance in a cognitive task and was strongly related to reward occurrence. In addition, Maharjan et al. (2018) used viral vector-mediated expression of muscarinic M_4 receptor coupled to Gi protein, hM4D(Gi) DREADD, to inactivate excitatory neurons in the

dHPC and mPFC in a W-track spatial alternation task that tested both working and spatial memory. Their results demonstrated that contralateral dHPC-PFC inactivation with CNO impaired working but not spatial memory in the W-track test. Taken together, these results demonstrate that while coordinated HPC-PFC tonic ACh release is associated with arousal and deactivated states, creating a purposeful context to dynamical coordination between HPC-PFC during cognitive tasks may involve other neurotransmitters (Benchenane et al., 2010). Synaptic plasticity and functional effects of muscarinic stimulation in iHPC-mPFC are associated with enhancement of dopamine release in mPFC (Lopes Aguiar et al., 2008). In addition, D_1 receptor modulation in HPC-mPFC is critical to performance spatial working memory (Seamans and Yang, 2004). However, as seen before, D_1 modulation in HPC-mPFC synaptic plasticity occurs in an inverted U-shaped dose dependence, and DA facilitates theta oscillation HPC inputs to mPFC engagement (Gurden et al., 2000; Benchenane et al., 2010). Thus, dopaminergic stimulation plays a role in fine-tuning the information flow in HPC-mPFC.

Schizophrenia

The HPC-PFC synaptic plasticity and functional connectivity have also been implicated in several animal models of schizophrenia. Goto and Grace (2006) observed an aberrant increase of vHPC-mPFC LTP in the neurodevelopmental model of prenatal exposure to methylazoxymethanol acetate (MAM). In a genetic mouse model of schizophrenia, Sigurdsson et al. (2010) observed a decrease of HPC-PFC theta synchrony correlated with poor spatial working memory performance. Dickerson et al. (2010) found a similar reduction of synchrony in the maternal immune activation (MIA) neurodevelopmental model. Ultimately, Spellman et al. (2015) showed an augmented HPC theta-mPFC slow gamma coupling in wild-type mice correlated to spatial working memory task difficulty, which was even higher in a genetic model of schizophrenia that presented an impaired performance on the task. Similarly, pharmacological animal models of schizophrenia using NMDA receptor antagonists also indicate HPC-mPFC synaptic disruption. Ketamine, an NMDA receptor antagonist, reduced vHPC-mPFC LTP induction in rats, while co-administration of the atypical antipsychotic clozapine counteracted these effects (Rame et al., 2017). Alvarez et al. (2020) showed that early ablation of NMDA receptors in corticolimbic parvalbumin-expressing interneurons of mice impacts vHPC-mPFC connectivity before and after adolescence. While juvenile mice exhibited elevated cortical excitability in an uncoordinated manner relative to the HPC, adult mice showed weaker evoked potentials in mPFC and increased LTD, suggesting impaired functional connectivity during adulthood. In iHPC-mPFC, ketamine-induced aberrant oscillatory coupling in mPFC and alterations in the HPC-PFC synaptic efficacy (Lopes-Aguiar et al., 2020). The authors demonstrated that prior induction of LTP in the CA1-mPFC attenuates these effects, highlighting the importance of high-frequency stimulation as a non-pharmacological alternative strategy to investigate HPC-PFC modulation and help in the prevention or attenuation of cognitive impairments in schizophrenia.

Remarkably, dopaminergic and eCB neuromodulation in HPC-mPFC are associated with the impairments in animal models of schizophrenia. D_2 agonism or D_1 antagonism revert aberrant LTP enhancement in the MAM model (Goto and Grace, 2006). However, there is still no evidence for the D_2 attenuating effect of LTP in a non-pathological brain state. Although most studies focused on the positive modulation of LTP by D_1 and showed the independence of this phenomenon on D_2, recent evidence implicates D_2 signaling in the occurrence of LTD. Banks et al. (2015) applied a TBS protocol to induce LTD in the HPC-PFC *in vitro*. Then, they showed that applying a D_2 antagonist, but not D_1, blocked LTD induction. Strikingly, they showed that LTD induction impaired NMDAr, but not AMPAr, synaptic transmission in this pathway. This finding is intriguing because it provides a mechanism that gathers exaggerated DA, hypofunction of NMDAr, and treatment by D_2 antagonism, which comprises critical neurobiological elements of schizophrenia (Grace, 2016). There is also a complex relationship between eCB neuromodulation and HPC-mPFC (Aguilar et al., 2016; Ruggiero et al., 2017). URB597, a FAAH inhibitor that leads to AEA accumulation, increases mPFC firing rate only in rats submitted to subchronic phencyclidine (PCP) model, while THC does not affect mPFC firing rate following PCP model but decreases firing rate in the control group (Aguilar et al., 2016). The exact mechanism of these effects remains to be elucidated but it is likely related to disruption in other neurotransmission systems (i.e., dopaminergic and GABAergic) induced by PCP, which differently influence eCB effects (Laviolette and Grace, 2006; Volk and Lewis, 2016).

Major Depression and Anxiety

Alterations in synaptic plasticity are among the main effects of a deleterious experience of stress (Pittenger and Duman, 2008). It is proposed that antidepressants, such as SSRIs, counteract the effects of stress on plasticity across the brain (Pittenger and Duman, 2008; Duman, 2014). This pattern also occurs in the HPC-mPFC pathway. Rocher et al. (2004) exposed rats to an unbalanced elevated platform, which induced a lasting increase in stress-related glucocorticoids, and they observed a marked impairment of vHPC-mPFC LTP. Then, they observed that acute systemic injection of fluoxetine, an SSRI, and the atypical antidepressant tianeptine restored LTP. Interestingly, both drugs did not affect LTP in non-stressed animals. Also remarkable, tianeptine presented a more significant effect than fluoxetine (Rocher et al., 2004). Another study applying the same stress procedure compared the effects of tianeptine and imipramine, a general monoamine reuptake blocker, and observed no significant effect of imipramine on restoring stress-induced effects on LTP (Qi et al., 2009). Nevertheless, although these findings have significant implications for the link between HPC-mPFC plasticity and depression etiology and treatment, tianeptine does not have a specific mechanism of action, so it hinders interpretation. Additionally, there are reports that fluoxetine increases the release of both DA and NA in the cortex (Bymaster et al., 2002), which could also be the actual modulators of plasticity. Moreover, recent evidence indicates that antidepressants may bind directly to tyrosine kinase receptors

(TrkB) (Casarotto et al., 2021), which are usually activated by the brain-derived neurotrophic factor (BDNF), which is a pivotal regulator of neural plasticity (Castrén and Antila, 2017). In conclusion, 5-HT modulates HPC-PFC LTP negatively, but antidepressants, including SSRIs, facilitate plasticity, possibly through serotonin-independent pathways.

Among the best studied behavioral functions of HPC-PFC transmission is anxiety. A series of studies using the elevated plus-maze showed that mPFC synchronizes preferentially with the vHPC rather than dorsal parts, especially in the theta frequencies at the environment's safer zones (closed arms) (Adhikari et al., 2010). Then, they also showed that the mPFC neurons that encode aversion-related information, such as the location in the closed or open arms, are strongly phase-locked to vHPC theta (Adhikari et al., 2011). Similar results were found in the open field. Optogenetic inhibition of vHPC-mPFC terminals decreased both theta synchrony in the anxious environment and expression of anxiety-related behaviors (Padilla-Coreano et al., 2016). More recently, it was shown that sinusoidal optogenetic stimulation of vHPC-PFC terminals at the theta frequency, which mimic more precisely endogenous LFP, induced behavioral avoidance to the safer zones more robustly than by applying single pulses (Padilla-Coreano et al., 2019). Anxiety-related behaviors are also linked to serotonin neuromodulation in the vHPC-mPFC circuit. Knockout mice for 5-HT_{1A} exhibited increases in both anxiety and mPFC theta power, while 5-HT_{1B} agonist in the mPFC reduced both anxiety and theta power in the elevated plus maze (Adhikari et al., 2010; Kjaerby et al., 2016).

Impairments of HPC-PFC connectivity have also been associated with depression. Zheng and Zhang (2015) investigated the effects of LTP induction on the oscillatory coupling between vCA1 and mPFC in the chronic unpredictable stress (CUS) model of depression in rats. Remarkably, they showed that CUS rats showed both a weaker LTP induction and reduced theta phase synchrony. Furthermore, they reported a positive correlation between the two measures, suggesting a relationship between these two estimates of neural connectivity. Jia et al. (2019) investigated the oscillatory and antidepressant effects of electrical LFS or HFS in the mPFC. They showed that CUS promoted a reduction of HPC-PFC beta and gamma powers, but electrical stimulations promoted both antidepressant responses and increases in beta and gamma HPC-PFC coherence. Carreno et al. (2016) used optogenetics and chemogenetics to show that the selective activation of the vHPC-mPFC ascending pathway also produces an antidepressant-like response comparable to that observed with sub-anesthetic doses of ketamine. Finally, Marques et al. (2019) investigated iHPC-PFC network dynamics during exposure to controllable or uncontrollable stress. They identified a pattern of increased HPC-PFC theta connectivity that accurately predicted resistant animals but was absent in helpless subjects, suggesting a role of HPC-PFC communication in adaptive stress coping.

Alzheimer's Disease

Recent studies in animal models for Alzheimer's disease (AD) have provided further evidence on HPC-mPFC disruption. Optogenetic activation of hippocampal cells involved in memory formation resulted in long-term memory retrieval in a genetic model of AD, suggesting that retrieval, rather than storage, is impaired in the disease (Roy et al., 2016). Specifically in the HPC-mPFC, alterations in proteins related to AD, such as the decay of calcium-independent phospholipase A2 (iPLA2) and the increase of amyloid-β peptide (Aβ), decreased prefrontal gamma oscillation engagement during spatial working memory (Liu et al., 2016) and abolished CA1-mPFC LTP induction related to spatial working memory deficits, respectively (Shalini et al., 2014). In addition, Bazzigaluppi et al. (2018) showed that the TgF344 genetic rat model of AD, presents lower HPC-mPFC low-gamma power, decreased theta-gamma PAC, and weaker gamma coherence compared to non-transgenic rats. The same frequency bands were analyzed during a stimuli association task performed by adult rats overexpressing the tau protein in the entorhinal cortex (Tanninen et al., 2017). In this case, tau-expressing rats showed attenuated theta-gamma phase-phase and amplitude-amplitude coupling between dHPC and mPFC. Interestingly, these oscillatory activities are also cholinergically modulated, which is impaired in AD (Ballinger et al., 2016; Ferreira-Vieira et al., 2016; Al-Onaizi et al., 2017). In fact, recent evidence has contributed to the advance of new AD treatment through cholinergic neuromodulation. Esteves et al. (2017) showed that chronic nicotine treatment prevented novel object recognition memory deficits and disruption of iHPC-mPFC LTP in the streptozotocin animal model of AD. However, further research is necessary to evaluate the effects of cholinergic neuromodulation on functional connectivity in HPC-mPFC in AD.

CONCLUDING REMARKS

Acetylcholine plays a key role in regulating brain-wide state transition (Huang and Hsu, 2010). The tonic cholinergic drive is fundamental to cortical change from a deactivated to an activated state, allowing HPC-PFC theta synchrony as a form of hippocampal inputs to influence prefrontal activity in time (Teles-Grilo Ruivo et al., 2017). Interestingly, *in vivo* experiments show that cholinergic activation modulates synaptic plasticity bidirectionally, potentiating LTP and facilitating LTD (Lopes Aguiar et al., 2008; Lopes-Aguiar et al., 2013). Also, experimental evidence suggests that nAChR activation can facilitate both forms of synaptic plasticity (α_7 facilitates LTP and α4β2 LTD) (Sabec et al., 2018). Taken together, these results indicate that ACh enables a specific type of communication to the HPC-PFC pathway (measured as theta rhythm). Once this interaction is established (or because this interaction is established), the cholinergic drive can facilitate activity-dependent synaptic strengthening or weakening. The occurrence of this bidirectional modulation is thought to be essential to regulate excitatory/inhibitory balance and learning.

It has been well established that monoamines provide a fine-tuning functional regulation in HPC-mPFC communication. Through their main signaling pathways in the mPFC, catecholamines (DA and NA) are positive modulators of LTP, while 5-HT is negative (Gurden et al., 2000; Ohashi et al., 2003; Lim et al., 2010). However, D_1-mediated modulation occurs in an inverted U-shaped dose dependence (Gurden et al., 2000), NA indirectly exerts detrimental effects via amygdala

(Lim et al., 2017), and chronic treatment with SSRI can reverse negative impacts of stress (Ohashi et al., 2002; Rocher et al., 2004). Remarkably, we found a qualitative relationship between monoamine modulation of HPC-mPFC synaptic plasticity and network synchrony. Pharmacological manipulations that increase LTP also increase LFP coupling (e.g., Gurden et al., 1999; Benchenane et al., 2010), while negative modulators of LTP decrease it (Zheng and Zhang, 2015; Xu et al., 2016), particularly in theta oscillations. This finding strengthens a link between distinct scales of functional connectivity.

The eCB system is critical to temporal coordination between HPC and mPFC activity. CB_1 stimulation abolishes spike timing coordination in both HPC and mPFC, which is related to disruption of slow oscillating in the HPC (theta) and fast oscillations in the mPFC (gamma) (Robbe et al., 2006; Kucewicz et al., 2011). In addition, these impairments decrease the synchronicity in HPC-mPFC, and ultimately, behaviors related to vHPC-mPFC communication, such as working memory (Kucewicz et al., 2011). In turn, chronic CB_1 stimulation impacts short-term and long-term synaptic plasticity in the vHPC-mPFC pathway by promoting an inhibitory imbalance and probably disrupting the vHPC -mPFC information flow (Cass et al., 2014; Renard et al., 2016).

Although considerable progress in understanding the role of neuromodulator systems in the synaptic plasticity of the HPC-mPFC pathway, there are still many gaps that need further elucidation. Regarding monoamines and eCBs, for example, little is known about *in vivo* LTD. All monoamines present two primary receptor families, which pose functional dichotomies that mediate facilitation or suppression of synaptic efficacy (D_1 vs. D_2, β vs. α_2, and $5HT_1$ vs. $5HT_2$). *In vitro* studies have shown the critical roles of DA and NA in PFC and HPC-mPFC LTD (Marzo et al., 2009, 2010; Goto et al., 2010; Banks et al., 2015). Similarly, eCB neuromodulation induces a robust LTD in mPFC slices (Auclair et al., 2000; Riedel and Davies, 2005). Therefore we hypothesize that monoamines and eCB are also critical for LTD in the intact brain. However, we may also reason that while CB_1 produces a ubiquitous LTD, monoamines may conflict between synaptic facilitation and suppression, which may manifest itself in an inverted U-shape dose dependence, such as in D_1 functions. In this sense, we highlight that we still miss basic knowledge of pharmacological modulation of HPC-mPFC LTP. Namely, we still do not know the roles of 5-HT2, many of the nAChR, nor any NA receptors in the mPFC and several components related to the eCB system, such as 2-AG and $TRPV_1$.

Also, one crucial question is how the *in vitro* findings can be transposed to *in vivo* preparations. Notably, a well-established *in vitro* form of LTD induced by activation of M_1 receptors lacks *in vivo* demonstration to our knowledge. Although the reviewed results are not directly comparable (i.e., different drug used and experimental design), it does not appear that the *in vivo* effects of muscarinic activation on synaptic efficacy follow the same magnitude and dynamics as the *in vitro* response. Indeed, despite the optimal spatial resolution and control of confounding factors, *in vitro* preparation lacks the intact circuitry and network response that can modulate the interaction in specific circuits. These inputs can include brainstem and forebrain neuromodulatory pathways that produce a physiological complex synergy that allows cognitive and emotional processing. In this respect, most studies investigating monoamine and cholinergic modulation of LFPs were performed in rodents during spontaneous behavioral states or anesthesia. It is reasonable to consider that the HPC-PFC network may present more distinctive patterns of activity and modulation under specific situations such as cognitive demands, motivational states during goal-directed behaviors, or adaptive vs. maladaptive stress coping strategies. These issues should be investigated in the future.

Future Directions

The concept that neuropsychiatric symptoms could emerge from alterations in information processing within neural networks rather than an imbalance of a specific neurotransmitter is now consolidated (Castrén, 2005). In this view, a promising approach is to investigate the effects of neuropsychiatric drugs on specific networks of the brain. Relevant clinical drugs with non-selective actions make it hard to interpret mechanisms, but investigating their effects in cognitive circuits could be exceptionally informative about the therapeutic effect and elucidate the underlying network functioning that can compensate for the cognitive symptoms. For instance, there is evidence that the different systems may interact to promote efficient modulation of synaptic function (Meunier et al., 2017). Indeed, all monoamines are known to interact between themselves (González-Burgos and Feria-Velasco, 2008; Xing et al., 2016) and with both eCB (Cohen et al., 2019) and cholinergic systems (Lester et al., 2010). However, there is still no knowledge on how these interactions may influence HPC-PFC synapses. In this review, we focused on the basic mechanisms of modulation. However, there is significant evidence that some manipulations only show effects when counteracting the impacts of stress or developmental deficits (Dupin et al., 2006; Goto and Grace, 2006), suggesting a more general role in regulating synaptic homeostasis rather than increasing or decreasing connectivity.

We found a profound difference in research interest on the modulations of the intermediate against the ventral hippocampal inputs to the PFC. While monoamines and cannabinoids were only studied in vHPC inputs, cholinergic modulation was mainly investigated in iHPC. Although evidence has accumulated for a functional distinction across the septotemporal axis of the HPC, we still do not know if the ventral and intermediate HPC inputs into the mPFC are differently modulated by the systems addressed here. Interestingly, most modulation transmitters are released into the PFC in a non-synaptic manner, suggesting a convergence of these modulations onto both inputs. However, the coincident phasic activation of brainstem efferent systems could modulate each input differently across time. For instance, vHPC stimulation markedly increases VTA activity and DA release (Lisman and Grace, 2005). However, iHPC tetanic stimulation was shown to decrease monoamine levels (Lopes Aguiar et al., 2008), suggesting distinct influences of the HPC inputs over the neuromodulatory systems.

One important aspect to recognize is that most studies adopted an artificial stimulation protocol to probe synaptic plasticity. Such robust protocols elicit synchronous spike activity from a large number of neurons in a prolonged and

high-frequency manner that unlikely replicate what happens in a physiological state (Bocchio et al., 2017). Thus, it is essential to understand the spontaneous network electrophysiological activities related to synaptic plasticity. Understanding the network effects of synaptic plasticity in the LFP measures while controlling for the artificial stimulation could be useful to understand the dynamics of HPC-mPFC interaction during behavior. It is postulated that oscillatory synchrony facilitates neural communication creating a temporal window in which one region could influence or coordinate the activity in another distant brain region (Fries, 2015). Hence, it is theorized that an increase in synaptic influence would also generate a stronger LFP coupling. Zheng and Zhang (2015) showed that LTP induction in the vHPC-mPFC pathway increased theta phase coupling rather than the power of either region. Also, stimulation protocols that resemble more physiological activity, such as TBS, induce theta-gamma cross-frequency coupling and interfere with memory consolidation (Radiske et al., 2020). Future studies need to investigate the relationship between synaptic plasticity and network coupling in freely moving animals and control for the effects of stimulation *per se*. Furthermore, the effects of other forms of synaptic plasticity in HPC-mPFC network coupling, such as LTD and PPF, remain to be investigated.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Friend or Foe? The Varied Faces of Homeostatic Synaptic Plasticity in Neurodegenerative Disease

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Homeostatic synaptic plasticity (HSP) regulates synaptic strength both pre- and postsynaptically to ensure stability and efficient information transfer in neural networks. A number of neurological diseases have been associated with deficits in HSP, particularly diseases characterised by episodic network instability such as migraine and epilepsy. Recently, it has become apparent that HSP also plays a role in many neurodegenerative diseases. In this mini review, we present an overview of the evidence linking HSP to each of the major neurodegenerative diseases, finding that HSP changes in each disease appear to belong to one of three broad functional categories: (1) deficits in HSP at degenerating synapses that contribute to pathogenesis or progression; (2) HSP induced in a heterosynaptic or cell non-autonomous manner to support the function of networks of which the degenerating synapses or cells are part; and (3) induction of HSP within the degenerating population of synapses to preserve function and to resist the impact of synapse loss. Understanding the varied manifestations of HSP in neurodegeneration will not only aid understanding mechanisms of disease but could also inspire much-needed novel approaches to therapy.

Keywords: neurodegeneration, synaptic plasticity, synaptic scaling, Alzheimer's, Parkinson's, Huntington's, amyotrophic lateral sclerosis

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INTRODUCTION

The ability of physiological systems to respond to and resist perturbations is vital to their continued normal and healthy functioning. In addition to the well-understood homeostatic systems that allow for the maintenance of key variables such as temperature or blood pressure within set bounds, it has become apparent that there are a variety of mechanisms responsible for maintaining the function of neuronal circuits and networks within an appropriate range. The best-studied of these mechanisms are those of synaptic scaling, otherwise known as homeostatic synaptic plasticity (HSP), which adjust synaptic strengths across either the whole neuron or a functionally relevant subregion (e.g., dendritic branch) in order to prevent excessive strengthening or weakening of synapses by the potential positive feedback loop created by Hebbian plasticity (Davis, 2013; Vitureira and Goda, 2013). HSP is therefore a critical check that is required to maintain both neuronal health and the fidelity of information transfer (Turrigiano, 2012). HSP utilizes a number of mechanisms that partly overlap with those mediating other forms of synaptic plasticity; while most of these are intrinsic to neurons, under some conditions glial-secreted factors such as TNFα or IL-33 may also be required (Wang et al., 2021).

In addition to HSP, a number of other homeostatic mechanisms can also be deployed to stabilise neuronal activity across individual cells and networks. These include regulation of synapse number (Kirov et al., 1999; Wierenga et al., 2006), regulation of intrinsic neuronal excitability (Marder and Goaillard, 2006; Turrigiano, 2011), metaplastic changes to the thresholds for induction of Hebbian plasticity in response to previous synaptic activity (Abraham, 2008) and shifting the balance of excitatory and inhibitory activity (E/I ratio) within networks (Maffei et al., 2004; Gonzalez-Islas and Wenner, 2006).

A number of neurological disease states have been associated with disruption of the mechanisms of HSP, including migraine (Welch, 2003), autism (Nelson and Valakh, 2015), epilepsy (Turrigiano, 2011) and Rett syndrome (Della Sala and Pizzorusso, 2014). HSP dysfunction has also been proposed as a significant factor in the development of schizophrenia (Dickman and Davis, 2009), and it appears to be particularly important in mood disorders occurring either alone or associated with other neurological conditions. Indeed, it is becoming clear that not only does modulation of HSP offer a potentially fruitful therapeutic approach to mood disorder treatment, but some of the most effective current treatments for these disorders actually work *via* this mechanism (Kavalali and Monteggia, 2020). Many of these conditions have in common a tendency towards episodic dysregulation of activity across neuronal networks, which might be expected if key homeostatic regulators of activity are compromised. For example, inherited mutations in the pore-forming subunit of the Ca^{2+} channel $Ca_V2.1$, a key presynaptic effector of homeostatic synaptic plasticity (Jeans et al., 2017), can be an inherited cause of either migraine or epilepsy, which result from the aberrant, episodic depression or enhancement of activity, respectively, across networks (Haan et al., 2008).

It is increasingly becoming evident that neurodegenerative diseases constitute part of this group of conditions associated with deficits in HSP. Indeed, it has been recognised for some years that neuronal network-level function is pathologically unstable in several neurodegenerative diseases, potentially accounting for the day-to-day shifts in performance status that characterise many of them (Palop et al., 2006). This mini review will focus on the emerging literature supporting a role for HSP in the major neurodegenerative diseases and in particular highlight an intriguing dichotomy that is becoming apparent. In certain settings, as with non-degenerative neurological diseases, the failure of mechanisms of HSP likely contributes to pathogenesis and progression. Yet in others, HSP has a very different significance as it can be recruited to resist disease progression and confer a degree of protection against the loss of neuronal and network function.

ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a substantial and increasing societal burden that is predicted to affect over 100 million people by 2050 (Prince et al., 2013). The disease is thought to be triggered by the pathological accumulation of soluble oligomers of amyloid β (Aβ; Mucke and Selkoe, 2012), which exert a variety of effects on different cell types (De Strooper and Karran, 2016). Alterations in synaptic transmission are among the earliest observed effects, and these include enhanced excitatory activity in cortico-hippocampal networks (Busche and Konnerth, 2016) and impaired Hebbian synaptic plasticity at glutamatergic synapses, specifically a marked attenuation of long-term potentiation (LTP) and facilitation of long term depression (LTD; Walsh et al., 2002; Hsieh et al., 2006; Shankar et al., 2008; Li et al., 2009, 2011). Both of these phenomena precede the appearance of pathology and are considered to be the key cellular substrates of early cognitive impairment in AD.

There is a substantial body of evidence in support of the idea that deficiencies in HSP might contribute to the initiation and/or progression of AD pathogenesis. At the most fundamental level, functional dysregulation of a variety of key protein effectors of various aspects of HSP has been associated with the development of AD. These proteins include β-secretase and presenilin 1, which are responsible for different steps in the cleavage pathway that regulates the production of Aβ from the amyloid precursor protein (APP). Changes in the activity of either due to AD-causative mutations in the protein itself (presenilin 1), or mutations in the sequence within APP that is recognised and cleaved by the protein (β-secretase), are associated with enhanced production of Aβ and consequently AD pathogenesis (Hutton and Hardy, 1997; Zhang et al., 2017). Presenilin 1 has also been shown to be required for Akt-dependent homeostatic upscaling of synaptic strength following chronic blockade of action potential firing with TTX (Pratt et al., 2011), while β-secretase is essential for homeostatic increases in synaptic strength in the visual cortex following dark exposure, as well as for normalization of synaptic transmission by subsequent exposure to light (Petrus and Lee, 2014). These observations might be at least partly explained by the role that Aβ itself appears to play in HSP since a recent study has demonstrated that soluble, secreted Aβ, but not the parent protein APP nor other APP cleavage products, mediates the homeostatic upscaling of synaptic strength following TTX treatment in cultured neurons (Galanis et al., 2021). While this study suggests that Aβ may act in this context *via* postsynaptic mechanisms, other work indicates that it can regulate presynaptic function in response to chronically altered synaptic activity (Abramov et al., 2009), and the locus or loci of action of Aβ in HSP, therefore, remains an open question.

Other HSP-associated proteins linked to AD include cyclin-dependent kinase 5 (CDK5), a pleiotropic effector acting at various intracellular locations that has been strongly implicated in AD pathogenesis. CDK5 becomes both inappropriately activated and delocalised following Aβ exposure (Patrick et al., 1999), phosphorylating a range of targets, including tau, that contribute to the synaptic decline and eventual neuronal loss (Shukla et al., 2012). CDK5 also homeostatically regulates synaptic strength following experimental neuronal silencing, acting *via* distinct presynaptic (Kim and Ryan, 2010) and postsynaptic (Seeburg et al., 2008) mechanisms. TNFα appears to play multiple roles in AD pathogenesis since it is present at elevated levels in AD brains, and inhibiting TNFα signalling in a mouse model of AD lowered Aβ production and rescued

cognitive deficits (Chang et al., 2017). TNFα of glial origin is also critical for the induction of HSP in response to chronic activity blockade (Stellwagen and Malenka, 2006). EphA4 is required for the synaptotoxic effects of oligomeric Aβ (Vargas et al., 2014), and controls excitatory synaptic strength during HSP by regulating AMPAR levels *via* CDK5 activity (Fu et al., 2007, 2011; Peng et al., 2013). Conversely, repressor element-1 silencing transcription factor (REST) appears to play a neuroprotective role in AD. REST induction in the cortex and hippocampus is a part of normal ageing, and the protein acts as a potent protector from oxidative stress and Aβ-associated toxicity. However, REST is lost in AD brains, and conditional deletion of REST in mouse brains leads to age-related neurodegeneration (Lu et al., 2014). During HSP, REST reduces the strength of excitatory synapses in response to chronic network hyperactivity by acting presynaptically to decrease the size of functional synaptic vesicle pools (Pecoraro-Bisogni et al., 2018). These examples are all mediators of classical HSP; however, an even greater variety of proteins is implicated in both AD pathogenesis and more broadly defined processes of cellular and network homeostasis, and these have been well summarised elsewhere (Jang and Chung, 2016; Styr and Slutsky, 2018).

There is, therefore, a large body of evidence to support the idea that deficits in various components of the HSP machinery are essential for AD pathogenesis, although the evidence currently falls short of definitively placing HSP in a central role. However, a recently proposed hypothesis raises exactly this possibility by invoking HSP, together with non-synaptic homeostatic mechanisms, as a functional link between the two most consistently observed early neural signatures of AD, enhanced excitatory activity in cortical and hippocampal networks and deficits in Hebbian synaptic plasticity (Styr and Slutsky, 2018).

A region-specific increase in tonic excitatory activity is one of the earliest abnormalities that can be observed in both AD patients (Mondadori et al., 2006; Filippini et al., 2009; Sperling et al., 2009; Bateman et al., 2012; Reiman et al., 2012) and animal models of the disease (Busche et al., 2008, 2012, 2015; Rudinskiy et al., 2012; Maier et al., 2014), usually preceding the emergence of overt pathology in AD patients by years (Busche and Konnerth, 2016). It appears to play a significant role in symptomatology and possibly pathogenesis, since reducing network hyperactivity pharmacologically has been shown to improve cognitive performance in patients with mild cognitive impairment (MCI), considered to be the prodromal stage of Alzheimer's (Bakker et al., 2012, 2015), and in AD model mice (Sanchez et al., 2012; Nygaard et al., 2015). In addition to simple hyperactivity, network hypersynchrony has been observed in AD animal models, resulting in epileptiform and seizure activity (Palop et al., 2007; Minkeviciene et al., 2009; Palop and Mucke, 2016). Consistent with this, AD patients are at elevated risk for epileptic seizures (Hauser et al., 1986; Romanelli et al., 1990), particularly those with autosomal dominant, early-onset familial AD (Palop and Mucke, 2016). The recently proposed "failure of firing homeostasis and plasticity" (FHP) hypothesis argues that such early changes are the result of a failure of homeostasis of network activity that arises when two necessary conditions have been met: firstly, some kind of perturbation of activity is imposed on susceptible brain networks, and secondly, that one or more of the core (meaning non-redundant) homeostatic mechanisms that would normally functionally compensate the perturbation are lost or compromised (Styr and Slutsky, 2018). As discussed above, the second of these criteria seems to be clearly met in AD, since several mechanisms of HSP, including a number that normally serves to downscale synaptic strength, are dysregulated. The perturbation could be any of a variety of environmental or age-related phenomena that in healthy individuals are readily compensated; in AD, drivers of the early network hyperactivity might include the build-up of Aβ, which is a positive regulator of neurotransmitter release (Abramov et al., 2009). According to the FHP hypothesis, the resultant chronically elevated network-level activity might drive maladaptive, principally postsynaptic changes *via* various mechanisms, including mechanisms of HSP that remain intact. Synapse weakening and elimination in AD might, therefore, simply represent a functional compensation that is insufficient to renormalize hyperactivity (Styr and Slutsky, 2018). These events would almost certainly impact Hebbian plasticity, both because of its mechanistic overlap with HSP, and because of the likely recruitment of metaplasticity processes, which set thresholds for Hebbian plasticity in line with the recent history of activity at the synapse (Abraham, 2008). In summary, therefore, a primary failure in mechanisms regulating HSP could lead to chronically enhanced network activity in the initial stages of the disease, which in turn could drive compensatory synapse weakening and eventual elimination, together with the plasticity deficits (impaired LTP and facilitation of LTD) that are a key substrate of cognitive decline in the early stages of AD (Mucke and Selkoe, 2012; Styr and Slutsky, 2018; **Figure 1**). Accordingly, the overall scheme of AD pathogenesis appears to be one in which loss of HSP function leads to deleterious consequences for affected synapses.

While this account of AD pathogenesis is highly consistent with existing experimental data obtained in a variety of model systems and with clinical observations, there is an alternative possible interpretation of the current evidence. It remains possible that postsynaptic weakening is a primary event, possibly the result of pathological signalling downstream of postsynaptic binding of pathogenic conformations of Aβ (Um et al., 2012; Kim et al., 2013), and this engages mechanisms of HSP to enhance presynaptic function and thereby synaptic activity in order to preserve synaptic strength (Taylor et al., 2020). Although this view seems much less favoured by the available evidence, it would again place HSP in an important role in pathogenesis, albeit as a maladaptive response to a pathological insult rather than as a primary, upstream event.

PARKINSON'S DISEASE

Like AD, Parkinson's disease (PD) is a progressive neurodegenerative disease that shows a strong association with age. The pathology of PD is principally characterised by the degeneration of the nigrostriatal tract composed of axonal projections from dopaminergic neurons located in the midbrain substantia nigra, and is a "dying-back" axonopathy in which

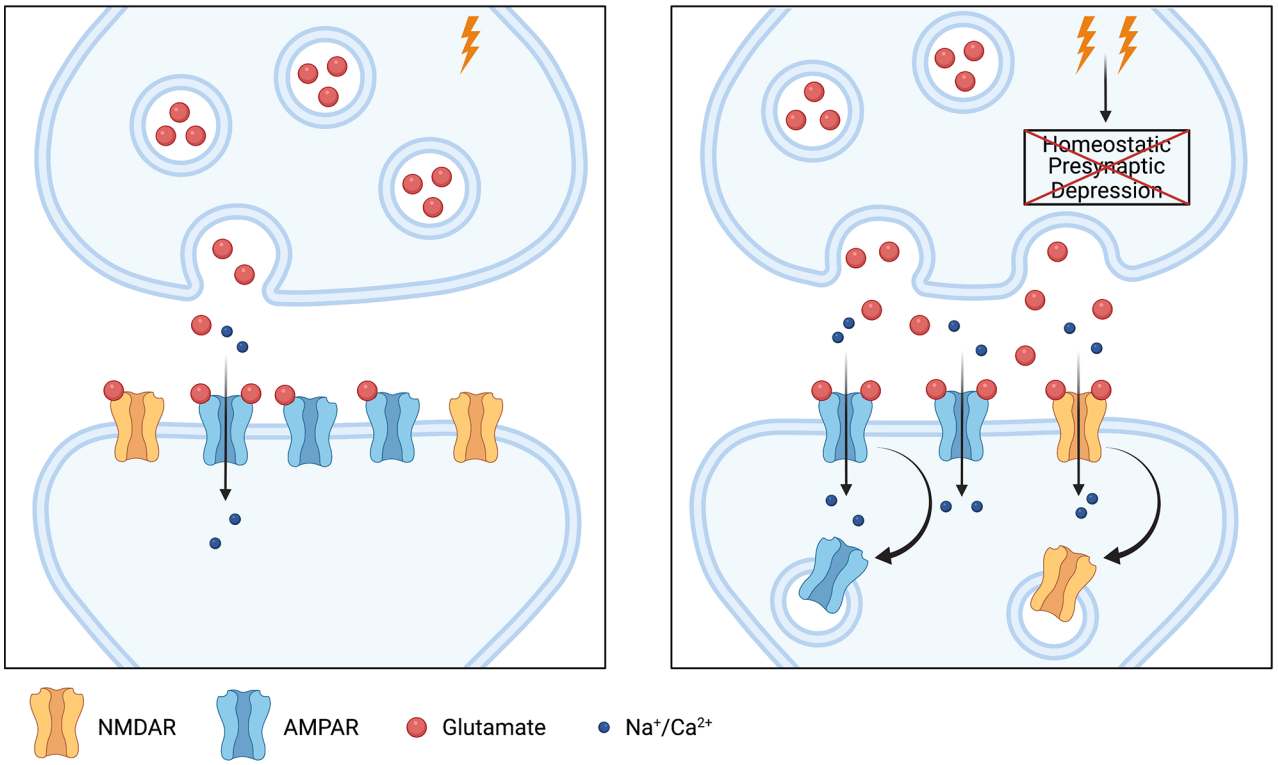

FIGURE 1 | A potential role for homeostatic synaptic plasticity (HSP) in Alzheimer's disease (AD) pathogenesis. The left panel shows a glutamatergic synapse under normal physiological conditions. The right panel shows a synapse during the early stages of AD. There is enhanced synaptic activity in affected networks, with increased synaptic glutamate release. This would normally be compensated by one or more mechanisms of HSP but if these fail, as many appear to in the context of AD, then elevated synaptic release persists and can drive a downregulation of postsynaptic strength *via* various mechanisms including postsynaptic mechanisms of HSP that remain intact, or potentially even recruitment of Hebbian plasticity (LTD). This internalization of glutamate receptors (AMPAR/NMDAR) could alter the ability of the synapse to support (further) Hebbian plasticity, with resultant effects on learning and cognition. Image created with Biorender.com. LTD, long term depression.

degeneration begins in the synaptic terminals and proceeds to the cell body (Burke and O'Malley, 2013). Clinically, PD presents with classical symptoms including bradykinesia, muscular rigidity and tremor; the first two of these are a direct result of dopaminergic neuron loss, while tremor is thought to relate to altered activity in neural circuits elsewhere in the brain (Helmich et al., 2012).

Somewhat analogous to Aβ in AD, the key trigger for PD pathogenesis is thought to be an accumulation of the protein α-synuclein (Stefanis, 2012). There is, however, a more limited body of evidence supporting the involvement of HSP in pathogenesis, at least in a cell-autonomous manner within the degenerating cell population. Physiologically, α-synuclein regulates assembly of the presynaptic SNARE protein complex that controls neurotransmitter release (Chandra et al., 2005; Burre et al., 2010), and it is thought that in the context of pathology, α-synuclein acts *via* both gain- and loss-of-function mechanisms to produce diverse effects including impairment of evoked neurotransmitter release in various PD model systems (Garcia-Reitbock et al., 2010; Nemani et al., 2010; Scott et al., 2010). The presynaptic localisation of α-synuclein and its interaction with synaptic vesicles, together with the observations that it is trafficked within neurons in an activity-dependent manner (Fortin et al., 2005), and that its expression is regulated during learning in songbirds (George et al., 1995), all argue for a physiological role in synaptic plasticity. However, this appears not to be classical HSP, but more likely the maintenance of different functional pools of synaptic vesicles that are important for healthy synaptic function in the very long term (Burre, 2015). While there is, therefore, currently no clear evidence of an effect, either loss- or gain-of-function, on HSP in PD, this remains a possibility in view of the importance of α-synuclein in synaptic regulation. Here, however, it is important to recognise that the mode of transmission at dopaminergic synapses is fundamentally different than that occurring at glutamatergic synapses of the sort affected in AD. The effects of dopamine at nigrostriatal synapses are mediated *via* volume transmission, in which the released neurotransmitter diffuses a relatively large distance to bind postsynaptic metabotropic receptors at multiple sites (Borroto-Escuela et al., 2018). This mode of synaptic transmission contrasts with the input specificity of glutamatergic synapses, which require independent regulation as well as co-regulation in functional groups of synapses across cells or networks. Indeed, it may be because of the need to balance

these sometimes competing demands that HSP plays such an important role at glutamatergic synapses (Vitureira and Goda, 2013) and, conversely, may be less significant at dopaminergic synapses.

While there is little evidence for a role for HSP at the degenerating nigrostriatal synapses, there is evidence that dopamine depletion triggers the induction of a variety of heterosynaptic and cell non-autonomous homeostatic mechanisms within the striatum in order to preserve the function of basal ganglia circuits of which the degenerating synapses are part. These adaptations are principally seen in various classes of spiny projection neurons and include structural changes, such as changes in the density of dendritic spines (Stephens et al., 2005), changes in cellular excitability (Fieblinger et al., 2014) and changes in the ability of corticostriatal synapses to support Hebbian plasticity (Thiele et al., 2014). There is also evidence for changes in basal strength of striatal synapses following dopamine depletion, which are likely to represent typical HSP (Villalba and Smith, 2018). In particular, there is a specific reduction in the strength of glutamatergic corticostriatal synapses on to D2 receptor-expressing spiny neurons of the indirect pathway (Suarez et al., 2016), the inhibitory arm of the system that represents the major regulator of the initiation and amplitude of voluntary movements. This change appears to require the local upregulation of TNFα, an established mediator of HSP (Lewitus et al., 2014), and serves to maintain the strength of output from spiny neurons that would otherwise be pathologically enhanced by the loss of heterosynaptic D2R-mediated inhibition accompanying the loss of dopaminergic afferents (**Figure 2A**). In addition, there is a loss of both synaptic strength and connectivity amongst the recurrent collateral synapses that mediate inhibitory synaptic activity amongst the spiny neurons themselves (Taverna et al., 2008), although the functional significance of this is less clear.

These observations, therefore, suggest another potential role for HSP in neurodegenerative disease, quite distinct from its involvement in driving degeneration through loss of function as in AD. In the context of PD, HSP can be recruited in a heterosynaptic or cell non-autonomous manner at various loci of control within basal ganglia circuits to resist the effects of synaptic/neuronal loss, specifically the loss of dopaminergic nigrostriatal projections, elsewhere.

HUNTINGTON'S DISEASE

Huntington's disease (HD) is an inherited autosomal dominant neurodegenerative disorder caused by an elongation of the CAG repeat region of the gene encoding the huntingtin (Htt) protein (MacDonald et al., 1993). The disease usually manifests in middle age and is characterised initially by loss of motor coordination, frequently accompanied by evidence of cognitive impairment and psychiatric changes. These early symptoms develop into a hyperkinetic movement disorder and, usually, an overt dementia. The rate of decline and severity of symptoms is correlated with the length of the pathogenic CAG repeat. Like PD, HD is a condition that affects the basal ganglia, and the motor symptoms are a direct result of degeneration of a specific class of striatal spiny projection neurons, an effect that is thought to be mediated *via* a toxic gain-of-function of the mutant Htt (Ross and Tabrizi, 2011).

In common with other neurodegenerative diseases HD does, therefore, exhibit a degree of selective neuronal vulnerability (Fu et al., 2018). However, a variety of neuronal populations may be involved, which is perhaps expected since HD is a genetic disease, and all cells will express the toxic mutant Htt protein. Although the degeneration of striatal spiny neurons is perhaps the best-recognized feature of HD pathology, cortical neurons are also affected relatively early in the course of the disease, accounting for the high incidence of cognitive and psychiatric symptoms in HD (Ross and Tabrizi, 2011). Cortical neurons are much more accessible and tractable for study than striatal neurons and have yielded the strongest evidence to suggest dysregulation of HSP in HD. In one study, cultured cortical neurons from HD model mice were shown to lack the ability to induce HSP in response to network silencing, a phenotype which could be rescued by enhancing BDNF signalling (Smith-Dijak et al., 2019). *In vivo*, loss of HSP is a likely explanation for the failure of dendritic spines in the barrel cortex to adapt to the loss of sensory input as expected following whisker trimming (Murmu et al., 2015). There is also some evidence from human HD patients, with data from transcranial magnetic stimulation of the motor cortex revealing various abnormalities including impairments in LTP and LTD-like plasticity at cortical synapses that might partly reflect defective homeostatic scaling processes (Calabresi et al., 2016).

In addition, there is some indirect evidence for a role for HSP in Huntington's disease (HD), although this is less compelling. A study of the Htt interactome and of perturbation genes relevant for HD pathology endpoints showed enrichment of both datasets for HSP-related genes, suggesting that interactions of mutant Htt with HSP-related proteins may contribute to pathogenesis or progression in HD (Wang et al., 2017). Furthermore, the microRNA miR-485, expression of which is dysregulated in HD, plays a key regulatory role in HSP, again raising the possibility of some overlap between these processes (Cohen et al., 2011). Overall, therefore, the picture emerging is one of loss and/or dysregulation of function in pathways mediating HSP, although the pathogenic significance of this is not yet clear, as these effects remain relatively little explored. Given the importance of HSP in maintaining healthy neuronal and network function, it seems likely that these deficits will play a role in pathogenesis or progression of HD, as well as possibly in the generation of motor symptoms, and HD would in this case belong to the broad functional class of HSP involvement as AD, where changes in HSP are deleterious. It does, however, remain possible, if unlikely, that the dysregulation of HSP is merely a bystander phenomenon that contributes little to pathogenesis, and further work will be required to resolve these issues.

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease principally affecting upper and lower motor neurons and presenting as progressive motor

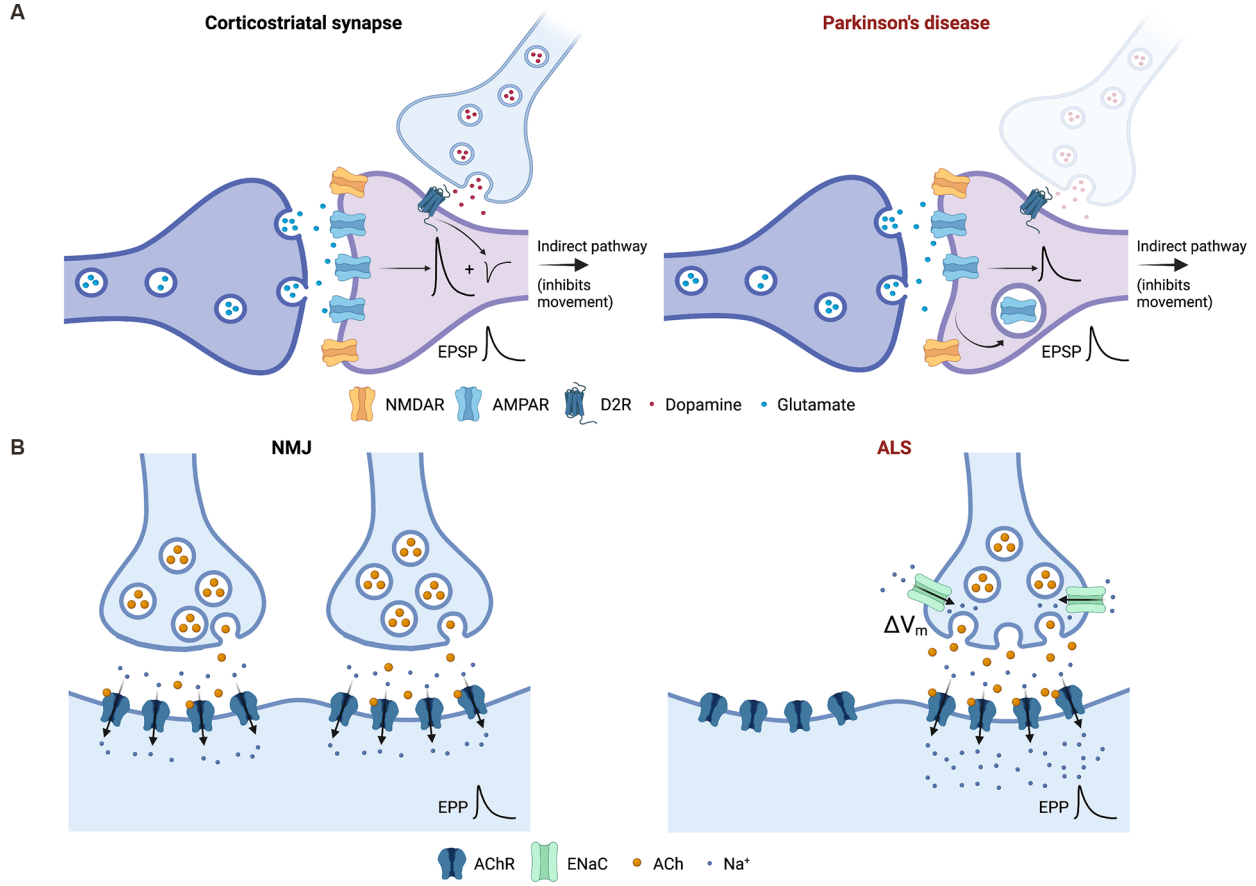

FIGURE 2 | Induction of homeostatic synaptic plasticity (HSP) can support the functioning of neuronal networks and/or populations of synapses in the context of synaptic degeneration. **(A)** Heterosynaptic and cell non-autonomous induction of HSP supports the function of basal ganglia circuits following degeneration of dopaminergic synapses in Parkinson's disease (PD). One example of this is shown involving synapses of the indirect pathway, which serves to negatively regulate voluntary movement. In models of PD, the strength of glutamatergic corticostriatal synapses onto medium spiny neurons is scaled down *via* mechanisms of HSP to compensate for the loss of D2 receptor-dependent inhibition (conceptually represented in the left panel as an inhibitory potential) of the same neuron by the degenerating nigrostriatal dopaminergic projections (right panel). Thus, heterosynaptic induction of HSP ensures that the overall strength of excitatory postsynaptic potentials (EPSPs) measured at the medium spiny neuron soma is preserved. **(B)** Induction of HSP at the degenerating neuromuscular junction (NMJ) supports motor function and opposes disease progression in amyotrophic lateral sclerosis (ALS) pathogenesis. The left panel shows the NMJ under physiological conditions. The right panel represents early stage ALS, in which innervation of the muscle and motor function is compromised by the loss of motor neuron synapses. This results in the induction of presynaptic HSP in remaining synaptic terminals *via* the membrane insertion of epithelial sodium channels (ENaC) that constitutively depolarise the presynaptic membrane (ΔV_m) and augment neurotransmitter release. This serves to: (1) preserve the strength of degenerating synapses; and/or (2) maintain normal or near-normal levels of postsynaptic depolarisation and end plate potentials (EPP) despite the loss of synaptic terminals, as shown here. Image created with Biorender.com.

deficits that develop over weeks or months. In later stages, there may also be cortical involvement with cognitive symptoms (van Es et al., 2017). Like other neurodegenerative diseases, the aetiology is not well understood but appears to be a complex mix of genetics and environmental factors. The greatest insights into pathogenesis thus far have come from the identification of a number of genes associated with familial variants of ALS, which group functionally into three main categories: RNA biology, protein turnover and axonal transport, suggesting that deficits in these processes play a causal role in pathogenesis (Renton et al., 2014). The discovery of causative genes has also assisted greatly with the production of suitable animal models of ALS for further mechanistic studies.

Because the most prominent feature is usually lower motor neuron degeneration with resultant muscular weakness, ALS is frequently thought of as a disease of the neuromuscular junction (NMJ; van Es et al., 2017). There is evidence in support of potential adaptive or homeostatic changes at NMJs in various animal models of ALS, and some in particular demonstrate enhanced spontaneous neurotransmission (Dzieciolowska et al., 2017; Bose et al., 2019), although it is not clear whether this is functionally significant or beneficial.

One intriguing feature of ALS, which is by no means unique to this disease, is that a significant loss of synapses can occur in affected regions, principally the NMJ, prior to the onset of symptoms (Moloney et al., 2014). This observation suggests that endogenous mechanisms may, to some degree, be

recruited to preserve function in the face of reduced synaptic number, although until recently the existence and identity of such mechanisms had never been confirmed. In 2020, a study initiated at the *Drosophila* NMJ showed that classical presynaptic HSP is induced at degenerating NMJs and that it functionally opposes the effects of motor neuron degeneration and loss (Orr et al., 2020). Presynaptic HSP is known to depend on the presynaptically localised epithelial Na^+ channel, ENaC (Younger et al., 2013), which was the basis for most of the manipulations used in this study. The authors went on to demonstrate that presynaptic HSP is induced similarly in a mouse model of motor neuron degeneration (Orr et al., 2020), supporting its relevance in mammalian systems. These exciting results establish a new paradigm for HSP in neurodegenerative disease whereby it is induced within the degenerating population of synapses themselves to functionally oppose the effects of disease progression (**Figure 2B**). It remains unclear whether this process is initiated within individual terminals during the process of degeneration in order to augment their own declining function, or whether it may be initiated by orphan postsynaptic elements in regions of synapse loss to enhance release from remaining terminals nearby. Alternatively, both of these mechanisms may operate, and further work will be required to resolve the various possibilities. In addition to the functional rescue, Orr et al. demonstrated that the induction of HSP at NMJs actually arrests degeneration. They hypothesize that this might be because the loss of synaptic transmission could lead to a loss of trophic support (growth and survival factors) normally released by the innervated muscle (Orr et al., 2020).

CONCLUSIONS

The body of evidence in support of a role for HSP in a variety of neurodegenerative diseases is growing steadily. What, then, is the nature of this role? We have presented here an overview of the currently available evidence, which suggests that the significance of HSP may be distinctly different in each of the major diseases associated with neurodegeneration.

In this context, HSP appears able to function in three broad ways, notwithstanding that there may be more yet to be recognised. The first is a direct involvement in pathogenesis, usually when the failure of one or more mechanisms of HSP in degenerating synapses leads to consequences that drive the development of disease. A substantial body of evidence suggests that this is a critical mechanism underlying Alzheimer's disease, and it also seems likely to be relevant to Huntington's disease, although here there is currently much less evidence to support the conclusion. Secondly, HSP may be induced in a heterosynaptic or cell non-autonomous manner in connected neurons to preserve the function of networks of which the degenerating synapses are part, as may happen in Parkinson's disease. Thirdly and finally, HSP may be recruited within degenerating populations of synapses themselves to oppose functional decline and even mitigate synapse loss, as in amyotrophic lateral sclerosis.

Recognition of the diverse manifestations of HSP in the disease context will aid the development of new therapeutic approaches, which are acutely needed. These may aim to restore or replace a dysfunctional protein or pathway, or to further augment a beneficial homeostatic response. It may also be possible to initiate homeostatic responses in cells in which these may be dormant or not otherwise induced. Such an approach has recently been validated in a *Drosophila* model of *C9orf72*-associated motor neuron loss in which presynaptic HSP is normally inactive at degenerating NMJs, but can be experimentally induced to restore synaptic strength (Perry et al., 2017).

AUTHOR CONTRIBUTIONS

HT and AJ conceived the review and wrote and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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Phosphorylation of Spastin Promotes the Surface Delivery and Synaptic Function of AMPA Receptors

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Synaptic plasticity is essential for cognitive functions such as learning and memory. One of the mechanisms involved in synaptic plasticity is the dynamic delivery of AMPA receptors (AMPARs) in and out of synapses. Mutations of *SPAST*, which encodes SPASTIN, a microtubule-severing protein, are considered the most common cause of hereditary spastic paraparesis (HSP). In some cases, patients with HSP also manifest cognitive impairment. In addition, mice with Spastin depletion exhibit working and associative memory deficits and reduced AMPAR levels. However, the exact effect and molecular mechanism of Spastin on AMPARs trafficking has remained unclear. Here, we report that Spastin interacts with AMPAR, and phosphorylation of Spastin enhances its interaction with AMPAR subunit GluA2. Further study shows that phosphorylation of Spastin can increase AMPAR GluA2 surface expression and the amplitude and frequency of miniature excitatory synaptic currents (mEPSC) in cultured hippocampal neurons. Moreover, phosphorylation of Spastin at Ser210 is crucial for GluA2 surface expression. Phosphorylation of Spastin K353A, which obliterates microtubule-severing activity, also promotes AMPAR GluA2 subunit trafficking to the surface and increases the amplitude and frequency of mEPSCs in cultured neurons. Taken together, our data demonstrate that Spastin phosphorylation promotes the surface delivery of the AMPAR GluA2 subunit independent of microtubule dynamics.

Keywords: spastin, phosphorylation, AMPA receptor, synaptic plasticity, microtubule

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INTRODUCTION

AMPA-type glutamate receptors (AMPARs) are heterotetrameric assemblies of four highly homologous subunits, GluA1–4, that are highly enriched at synapses (Herguedas et al., 2016; Diering and Huganir, 2018). In the central nervous system, postsynaptic AMPARs mediate the majority of fast excitatory transmission. Interestingly, AMPARs are not static components at synapses, rather, they are continuously being delivered and removed in and out of the synapses (Moretto and Passafaro, 2018). The dynamic trafficking of AMPARs into and out of the synaptic membrane is crucial for synaptic plasticity, which is thought to be one of the key cellular mechanisms underlying cognitive functions such as learning and memory (Anggono and Huganir, 2012; Henley and Wilkinson, 2013).

Generally speaking, an increased number of synaptic AMPARs leads to long-term potentiation (LTP), which promotes learning and memory, whereas the removal of surface AMPARs results in long-term depression (LTD; Anggono and Huganir, 2012; Henley and Wilkinson, 2013). This dynamic behavior of AMPAR involves a complex protein-protein interaction network, from receptor biosynthesis to their transport along dendrites and finally insertion and removal from the postsynaptic membrane (Greger and Esteban, 2007; Kneussel and Hausrat, 2016). Thus, elucidating how proteins regulate the trafficking of AMPARs is critical for our understanding of synaptic plasticity and human cognitive behavior.

Hereditary spastic paraplegia (HSP) is a heterogeneous group of genetic neurodegenerative disorders, characterized by distinct lower limb spasticity and weakness (Novarino et al., 2014; Walusinski, 2020). To date, there are about 100 loci/88 spastic paraplegia genes (SPG) involved in the pathogenesis of HSP (Elsayed et al., 2021). More than 40% of HSP cases originate from mutations in the *SPG4* gene, which encodes SPASTIN, a microtubule-severing protein (Kara et al., 2016; Erfanian Omidvar et al., 2021). Spastin is widely expressed in the spinal cord and brain (Solowska et al., 2008, 2010). In the central nervous system, it is mainly distributed in regions such as the cerebral cortex, cerebellum, hippocampus, amygdala, substantia nigra, and striatum (Solowska et al., 2008). The classic function of Spastin is to sever long microtubules into a number of short segments which mainly relies on the microtubule-binding domain (MTBD) and AAA ATPase catalytic domain (Blackstone et al., 2011). The MTBD is responsible for binding tubulin in an ATP-independent manner and the AAA domain forms a circular hexamer with a central pore. It is proposed that the C-terminal tail of tubulin is pulled into this hexamer, generating a mechanical force that breaks the microtubules (Salinas et al., 2005; Roll-Mecak and Vale, 2008). As HSP is a motor neuron disease caused by a progressive degeneration of the motor axons of the corticospinal tract (Salinas et al., 2008), numerous studies have focused on the causative mechanism of motor axon injuries of the corticospinal tract by Spastin. These studies have shown that the severing ability of Spastin has a crucial role in axon growth and axonal transport (Yu et al., 2008; Kasher et al., 2009; Stone et al., 2012; Fassier et al., 2013). Thus, impairment of axonal growth and transport caused by insufficient microtubule cleavage is considered to be one of the reasons for the pyramidal syndrome described in SPG4-linked HSP (Solowska and Baas, 2015).

Originally, SPG4-linked HSP had been considered as a pure form (in which only a pyramidal syndrome is found), however, later studies reported that patients with SPG4-HSP also exhibit cognitive impairment (Orlacchio et al., 2004; Murphy et al., 2009; Chelban et al., 2017; Akaba et al., 2021; Erfanian Omidvar et al., 2021; Giordani et al., 2021). In addition, mice with Spastin depletion exhibit working and associative memory deficits and reduced AMPA receptor levels (Lopes et al., 2020). One of the mechanisms explaining the influence of synaptic plasticity on cognitive behavior is AMPAR trafficking (Forrest et al., 2018); thus, further exploration of how Spastin regulates AMPAR trafficking is warranted. Our study of cultured hippocampal neurons showed that in addition to its influence on axon outgrowth, Spastin also promotes dendrite development (Ji et al., 2018). It interacts with collapsin response mediator proteins (CRMPs) to promote dendrite outgrowth and branch formation (Ji et al., 2018; Li et al., 2021). Moreover, phosphorylation of Spastin was found to play a key role during this process (Li et al., 2021). Phosphorylation, as the most common and important posttranslational modification of proteins, has critical and well-known functions in diverse cellular processes. Although a recent study has shown that Spastin depletion reduces AMPA receptor (AMPAR) levels (Lopes et al., 2020), the role of Spastin and its phosphorylation on AMPA receptor trafficking is not fully understood.

In this study, we reported that Spastin interacts with all four subunits of AMPA receptors. Then, using phosphorylation site mutations, we found that phosphorylation of Spastin enhances its interaction with AMPAR subunit GluA2. Further investigation showed that overexpression of phosphorylated Spastin mutation increases the surface expression of AMPAR levels. Meanwhile, the synaptic function was also increased. Moreover, our results identified Ser210 as the key phosphorylation site of Spastin involved in AMPAR trafficking. Finally, by phosphorylation of Spastin K353A, which obliterates microtubule-severing activity, we clarified that microtubule motility was not involved in Spastin phosphorylation-mediated AMPAR trafficking. Taken together, our data provide new and important insights into the role of Spastin in AMPAR trafficking and advances our understanding of the synaptic plasticity and cognitive dysfunction in HSP.

MATERIALS AND METHODS

Animals

The experiments were undertaken with 1-month-old and 1-day-old specific pathogen-free Sprague Dawley (SD) rats purchased from the Experimental Animal Center of Sun Yat-sen University. We conducted the animal experiments in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals produced by the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at Jinan University, China. All efforts were taken to minimize the suffering and the number of animals used.

Construction of Plasmids

Green fluorescent protein (GFP)-Spastin, mCherry-Spastin, and Glutathione-S-transferase (GST)-Spastin constructs were described previously (Cha et al., 2016; Ji et al., 2018). Point mutations S210A, S233A, T271A, S562A (mutation of serine and threonine to alanine to mimic dephosphorylated Spastin), S210D, S233D, T271D, S562D (mutation of serine and threonine to aspartic acid to mimic phosphorylated Spastin), K353A (mutation of lysine to alanine), and R464C (mutation of arginine to cysteine) were generated by using the Quickchange Kit (Agilent, Santa Clara, CA), and the positive clone was confirmed by sequencing.

Hippocampal Neuron Culture and Transfection

Standard hippocampal neuron culture and transfection were performed as described in our previous study (Zhang et al., 2012). Briefly, hippocampi were extracted from 1-day-old SD rats. After hippocampi were cut into pieces, they were digested using 0.125% trypsin. Finally, rat hippocampal neurons were plated onto a poly-D-lysine coated glass coverslip at a density of 1×10^4 cells/cm^2. When cells were cultured for 13 days *in vitro* (DIV 13), different constructs were transfected into neurons using the calcium-phosphate method. All experiments were performed after transfection for 48 h.

COS1 and HEK293T Cell Culture and Transfection

COS1 cells and HEK293T cells were cultured in a 5% CO_2 incubator at 37°C. Transfection of the constructs was performed in 24-well plates or 10-cm dishes with Lipofectamine 2000 (Invitrogen, Waltham, MA). After transfection for 48 h, COS1 cells were fixed for performing fluorescence immunostaining. HEK293T cells were harvested for performing immunoprecipitation.

GST Pull-Down Assay

The plasmids for GST-Spastin and its mutants were transformed into the BL21 strain of *E. coli* (Invitrogen). The GST-fusion proteins expression was performed as described previously (Ji et al., 2018). Approximately, 400 μg brain protein from 1-month-old SD rats was incubated with 5 μg GST-fusion protein under gentle rotation at 4°C overnight. Then the binding proteins were eluted and analyzed using western blotting.

Co-immunoprecipitation Assay

HEK293T cells were co-transfected with Flag-GluA2 and GFP, GFP-Spastin, and its phosphomimetic and dephosphomimetic mutants. The co-IP assay was performed after transfection for 48 h, as per a previously described method (Cheng et al., 2022). HEK293T cells were lysed with a cold immunoprecipitation (IP) lysis buffer (Beyotime, Shanghai, China; 25 mM Tris-Cl pH 7.4, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 0.5% NP40) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland) for 30 min. The lysates were harvested and centrifuged at 12,000 rpm at 4°C for 20 min. Cell extracts were determined using the bicinchoninic acid assay (BCA) and incubated with anti-GFP agarose beads (KT Health, Shenzhen, China) at 4°C for 3 h. Following this, the beads were collected and washed twice with IP lysis buffer and once with IP wash buffer with 0.05% NP-40 in PBS. The immune complexes were collected and eluted six times with IP wash buffer. The samples were analyzed by Western blotting using anti-Flag and anti-GFP antibodies.

Fluorescence Immunostaining

Immunofluorescence staining was performed after cells were transfected for 48 h, as per a previously described method (Zhang et al., 2012; Cheng et al., 2022). For tubulin immunofluorescence staining, COS1 cells were permeabilized by 0.1% (v/v) Triton X-100 dissolved in tris-buffered saline. The primary antibody targeting tubulin (Abcam, Cambridge, United Kingdom) was used at dilution 1:500 and the secondary antibody Alexa Fluor 555 (Life Technologies, Carlsbad, CA) was diluted at 1:1,000. For surface GluA2 immunofluorescence staining, hippocampal neurons and HEK293T cells expressing GluA2 subunits were not permeabilized. The primary antibodies targeting GluA2 (Millipore, Burlington, MA) were used at 1:200 dilutions. The secondary antibodies Alexa Fluor 647 and Alexa Fluor 555 (Life Technologies, Carlsbad, CA) were diluted to 1:800. After staining, images were randomly captured in a blinded manner under a Carl Zeiss LSM 700 confocal microscope (Zeiss, Oberkochen, Germany) with a 63× oil microscope. Fluorescence-integrated density measurements were made using ImageJ software (version ImageJ 2.x; NIH). Briefly, three dendritic regions of ∼25 μm for each neuron were randomly selected and their average was obtained. Surface GluA2 was calculated by dividing the intensity corresponding to the transfected cells by the values corresponding to the non-transfected cells. Twenty neurons were counted from three independent experiments.

Electrophysiology

After culturing hippocampal neurons for 13 DIV, they were transfected with GFP, GFP-Spastin, and other constructs. After transfection for 48 h, electrophysiology assays were performed as per a previously described method (Zhang et al., 2020). Miniature excitatory synaptic currents (mEPSCs) were obtained using whole-cell patch-clamp recordings at 20°C–22°C. During recordings, cultured hippocampal neurons were bathed in an extracellular solution (in mM): 128 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 20 HEPES, 15 glucose, 1 tetrodotoxin, and 100 μM picrotoxin. The intracellular solution contained the following (in mM): 147 KCl, 5 Na_2-phosphocreatine, 2 EGTA, 10 HEPES, 2 MgATP and 0.3 Na_2GTP. Recordings were performed in voltage clamp mode, at a holding potential of −70 mV, using a Multiclamp 700 B amplifier (Molecular Devices, San Jose, CA) and Clampex 10.5 software (Axon Instruments, Union City, CA). Series resistance below 30 MΩ was monitored during recordings. Signals were sampled at 10 kHz, filtered at 1 kHz and MiniAnalysis software was used for analyzing signals (Synaptosoft, Inc., Fort Lee, NJ).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA). All data are presented as the mean ± SEM. One-way ANOVA followed by Tukey's *post-hoc* tests was used to determine differences among multiple groups. P-value < 0.05 was considered statistically significant.

RESULTS

Phosphorylation of Spastin Increased Its Interaction With AMPAR GluA2 Subunit

To investigate the relationships between Spastin and AMPAR, we first constructed GST-Spastin plasmid and purified the

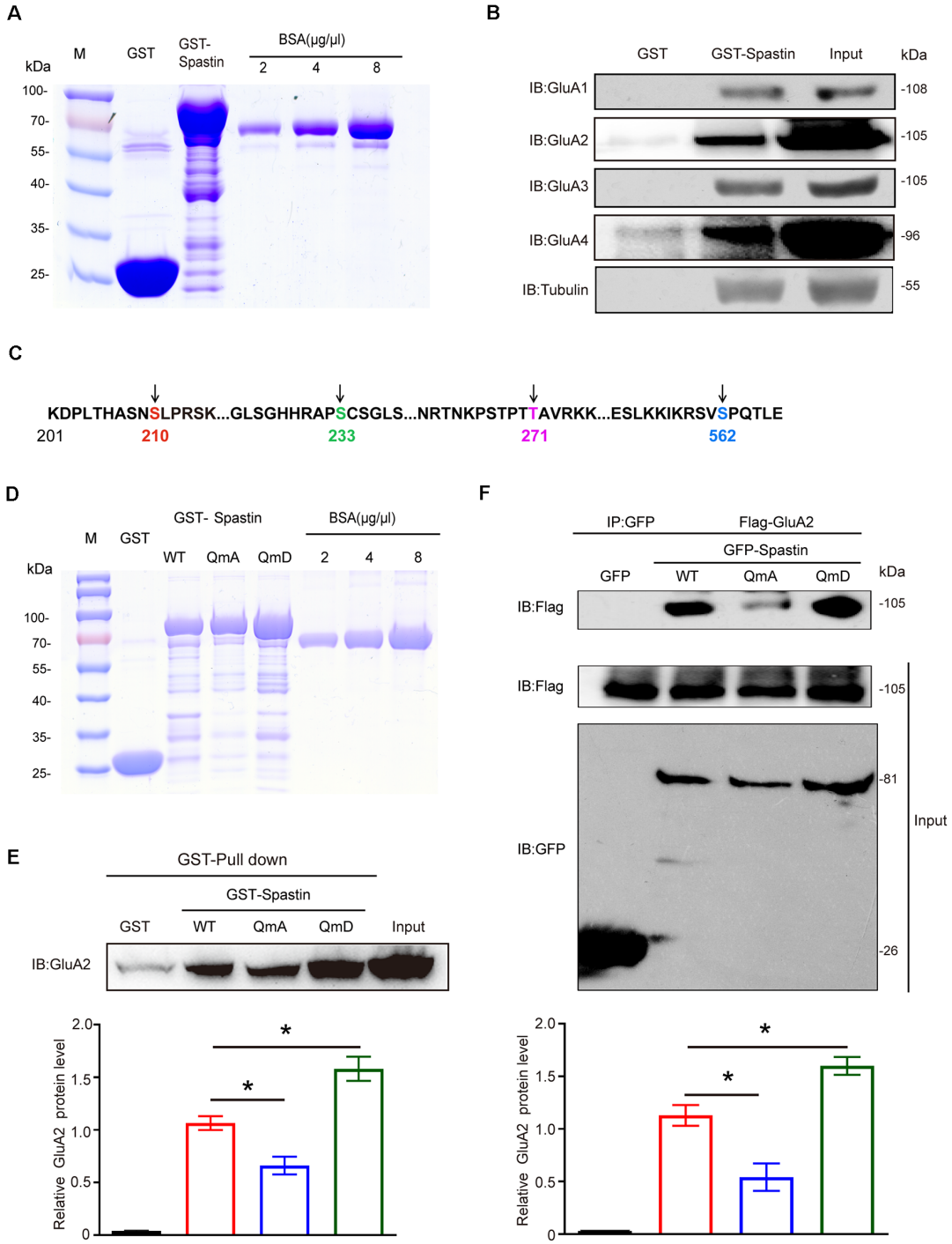

FIGURE 1 | Spastin interacted with AMPARs and phosphorylation of Spastin increased its binding ability with GluA2. **(A)** Purified glutathione-S-transferase (GST), GST-Spastin proteins. **(B)** Pulldown assay was performed using GST-tagged Spastin or GST incubated with 1-month-old SD rat brain lysates. Immunoblotting of input and bound proteins was performed using antibodies against GluA1-A4 and tubulin. **(C)** Schematic of the four phosphorylation sites within the rat Spastin sequence. Numbers represent amino acid residues within the Spastin sequence. **(D)** Purified proteins of GST, GST-Spastin, and its phosphomimetic and dephosphomimetic mutants. **(E)** Pulldown assay was performed using GST, GST-tagged Spastin, and its mutants incubated with 1-month-old SD rat brain lysates. Immunoblotting of input and bound proteins was performed using antibodies against GluA2 (top); quantification of the relative binding of GluA2 to GST, GST-Spastin, and its mutants (bottom), $n = 3$ independent experiments, $^*p < 0.05$ compared to GST-Spastin WT group. **(F)** HEK293T cells co-transfected with Flag-GluA2 and GFP, GFP-Spastin, and its mutants were lysed for co-IP assay. Immunoblotting of input and bound proteins was performed using antibodies against GFP and Flag respectively (top). Quantification of the relative binding of GluA2 to GFP, GFP-Spastin, and its mutants (bottom), $n = 3$ independent experiments, $^*p < 0.05$ compared to GFP-Spastin WT group.

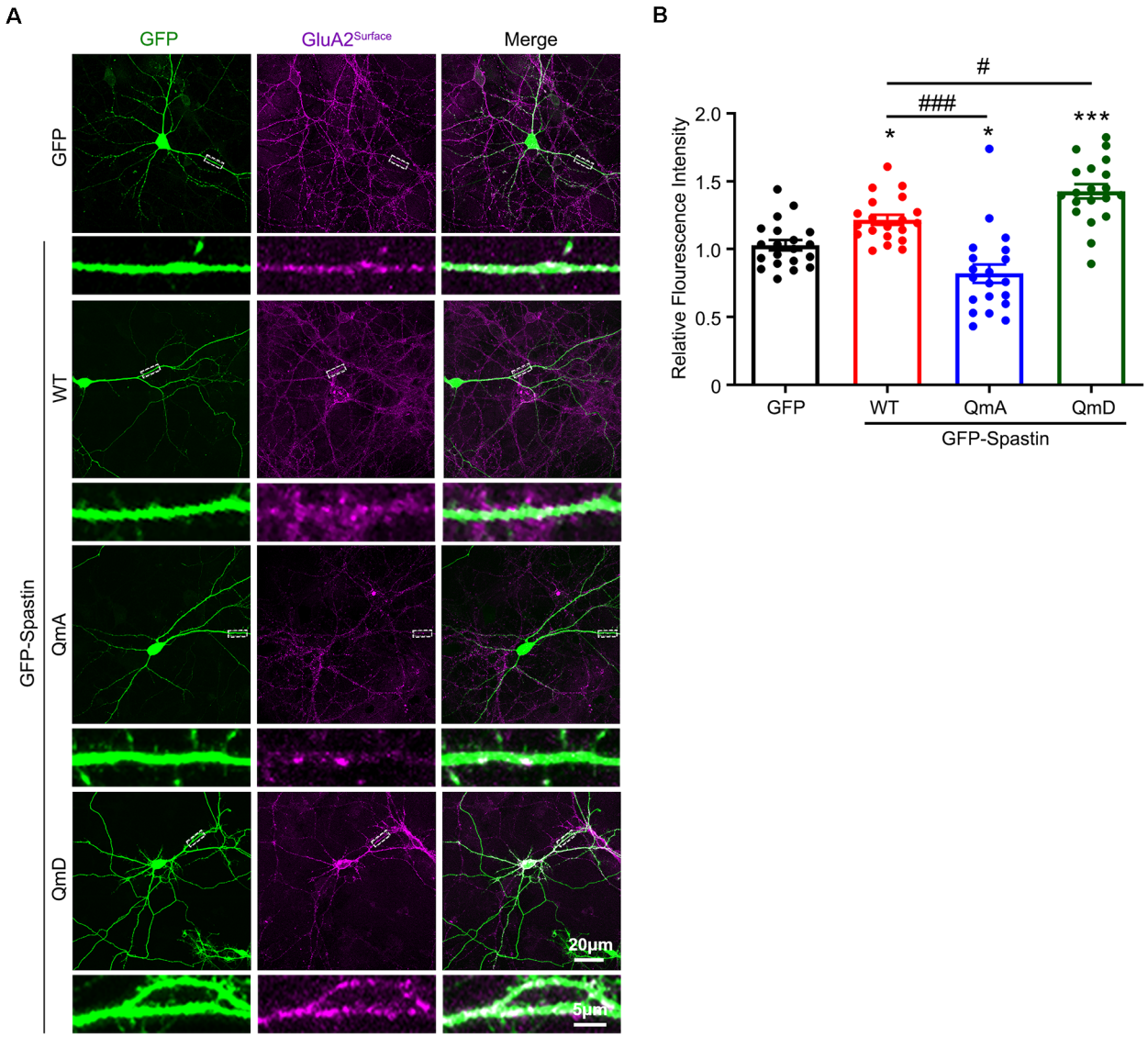

FIGURE 2 | Phosphorylation of Spastin promoted GluA2 surface expression. **(A)** Confocal micrographs showing surface GluA2 in DIV 15 hippocampal neurons overexpressing GFP, GFP-Spastin WT, QmA, and QmD. Scale bar, 20 μm. The rectangle details were enlarged. In the magnified dendrite, the scale bar corresponds to, 5 μm. **(B)** Quantification of relative fluorescence intensity in neurons overexpressing GFP, GFP-Spastin WT, QmA, and QmD. In each group, $n = 20$ cells from three independent experiments, $^{*}P < 0.05$, $^{***}P < 0.001$, as compared to the GFP group, $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, as compared to the GFP-Spastin WT group.

protein (**Figure 1A**). Then GST and GST-Spastin fusion protein was purified and incubated with the brain lysates of 1-month-old rats. GST pulldown analysis showed that Spastin interacted with all the four subunits (GluA1-GluA4) of AMPA receptors (**Figure 1B**). Simultaneously, to ensure the accuracy of the experimental system, we also performed a pulldown assay to examine the relationship between Spastin and tubulin as a positive control (**Figure 1B**). Since our previous study has shown that phosphorylation of Spastin can change its binding ability to interacted proteins, we speculated that phosphorylation of Spastin may also influence Spastin–AMPAR interaction. As a vast majority of AMPARs are GluA1/2 and GluA2/3 heteromers and most of the synaptic AMPARs within the brain contain GluA2 subunits (Bats et al., 2013), we selected GluA2 to evaluate whether Spastin phosphorylation affects the binding ability between Spastin and AMPAR. Large-scale mass spectrometry analyses have revealed four different potential phosphorylation sites in Spastin. These sites are S210, S233, T271, and S562 in rats (**Figure 1C**), corresponding to human S245, S268, T303, and S597, and are evolutionarily conserved in rats, humans, and mice[1]. With the exception of S268, which is reported to be phosphorylated by HIPK2, the upstream kinase at other sites is still unknown (Pisciottani et al., 2019). Therefore, in

[1]https://www.phosphosite.org

our study, we chose a genetic approach wherein we mutated all the phosphorylation sites (Ser210, Ser233, Thr271, and Ser562) of Spastin and constructed a phosphomimetic mutant (Spastin QmD) and dephosphomimetic mutant (Spastin QmA) of Spastin. In Spastin QmD, Ser210, Ser233, Thr271, and Ser562 were mutated to aspartic acid, whereas, in Spastin QmA, Ser210, Ser233, Thr271, and Ser562 were mutated to alanine. After the two mutants were successfully constructed, fusion proteins of GST, GST-Spastin WT, QmA, and QmD were purified (**Figure 1D**) for GST pulldown assays. As shown in **Figure 1E**, the binding ability of phosphomimetic mutant Spastin QmD with GluA2 was enhanced, while the binding ability of dephosphomimetic mutant Spastin QmA with GluA2 was impaired, when compared with the WT group. This phosphorylation-dependent interaction between Spastin and GluA2 was further confirmed by a Co-IP assay. As shown in **Figure 1F**, the interaction between Spastin QmA with GluA2 decreased significantly, whereas the opposite was seen for the binding ability of Spastin QmD to GluA2. Together, these results suggested that phosphorylation of Spastin increased its interaction with AMPAR GluA2.

Phosphorylation of Spastin Increased the Surface Expression of AMPAR GluA2 and Synaptic Function

To further clarify the effects of the interaction between Spastin and GluA2 on AMPAR levels, we monitored surface AMPAR levels *via* immunofluorescence staining of nonpermeabilized neurons with anti-GluA2 antibodies. As shown in **Figure 2**, neurons overexpression of Spastin exhibited an increasement of surface GluA2 fluorescence intensity compared with neurons overexpressing GFP. Additionally, neurons overexpressing Spastin QmA showed a decreased level of surface GluA2 fluorescence intensity, whether compared with neurons overexpressing GFP or Spastin WT. However, neurons overexpressing Spastin QmD exhibited an increased level in surface GluA2 fluorescence intensity when compared with neurons overexpressing GFP and Spastin WT. These data illustrated that Spastin phosphorylation increased the surface expression of AMPAR GluA2.

Does the increased surface expression of AMPAR reflect in synaptic function? To determine whether the synaptic functional level of neurons overexpressing Spastin and its phosphorylation were changed, whole-cell patch clamp recordings were performed to measure mEPSCs levels in target neurons. As shown in **Figure 3**, neurons overexpressing Spastin WT exhibited an increased level in both amplitude and frequency of mEPSCs than the neurons overexpression GFP, indicating an increased number of functional synapses. Furthermore, compared with neurons overexpressing Spastin WT, neurons overexpressing Spastin QmD exhibited an increased level in amplitude and frequency of mEPSC, while neurons overexpressing Spastin QmA showed a decreased level in amplitude and frequency of mEPSC. All these data demonstrated that phosphorylation of Spastin increased the surface expression of AMPAR GluA2 and synaptic function.

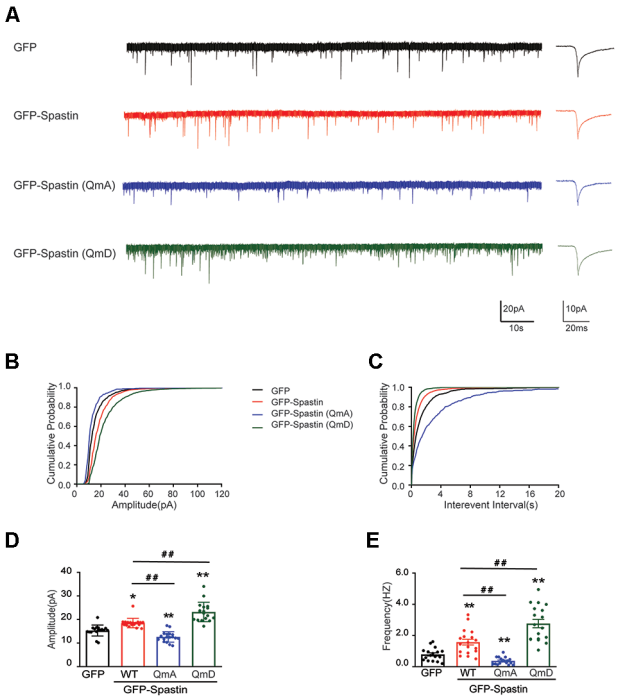

FIGURE 3 | Phosphorylation of Spastin increased the amplitude and frequency of mEPSCs. **(A)** Representative recordings of mEPSCs from neurons overexpressing GFP, GFP-Spastin WT, QmA, and QmD. Representative traces of individual mEPSC are on the right. **(B,C)** Cumulative distributions of mEPSC amplitudes and the inter-mEPSC event intervals in neurons overexpressing GFP, GFP-Spastin WT, QmA, and QmD. **(D,E)** Quantification of mEPSCs amplitude and frequency in neurons overexpressing GFP, GFP-Spastin WT, QmA, and QmD. In GFP group $n = 14$ cells; in the WT, QmA and QmD group, $n = 18$ cells respectively; from three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, as compared to the GFP group, $^{\#\#}P < 0.01$, as compared to the GFP-Spastin WT group.

The Ser210 Site Phosphorylation of Spastin Contributed to the Surface Delivery of AMPAR

After clarifying the relationship between Spastin phosphorylation and AMPAR transport, we asked which of the four phosphorylation sites played an important role during this process. To answer this question, we first generated a series of mutants with Ser210, Ser233, Thr271, and Ser562 replaced with alanine (S210A, S233A, T271A, and S562A) or aspartic acid (S210D, S233D, T271D, and S562D) to mimic dephosphorylated or phosphorylated Spastin, respectively. Next, we examined the level of GluA2 surface expression in HEK293T cells stably expressing the GluA2 subunit of AMPAR. HEK293T cells stably expressing the GluA subunits make it easier for us to observe the effects of AMPAR binding proteins on AMPAR trafficking (Wei et al., 2016; Li et al., 2018; Cheng et al., 2022). We found that cells transfected with Spastin S210D mutants, similarly to cells transfected

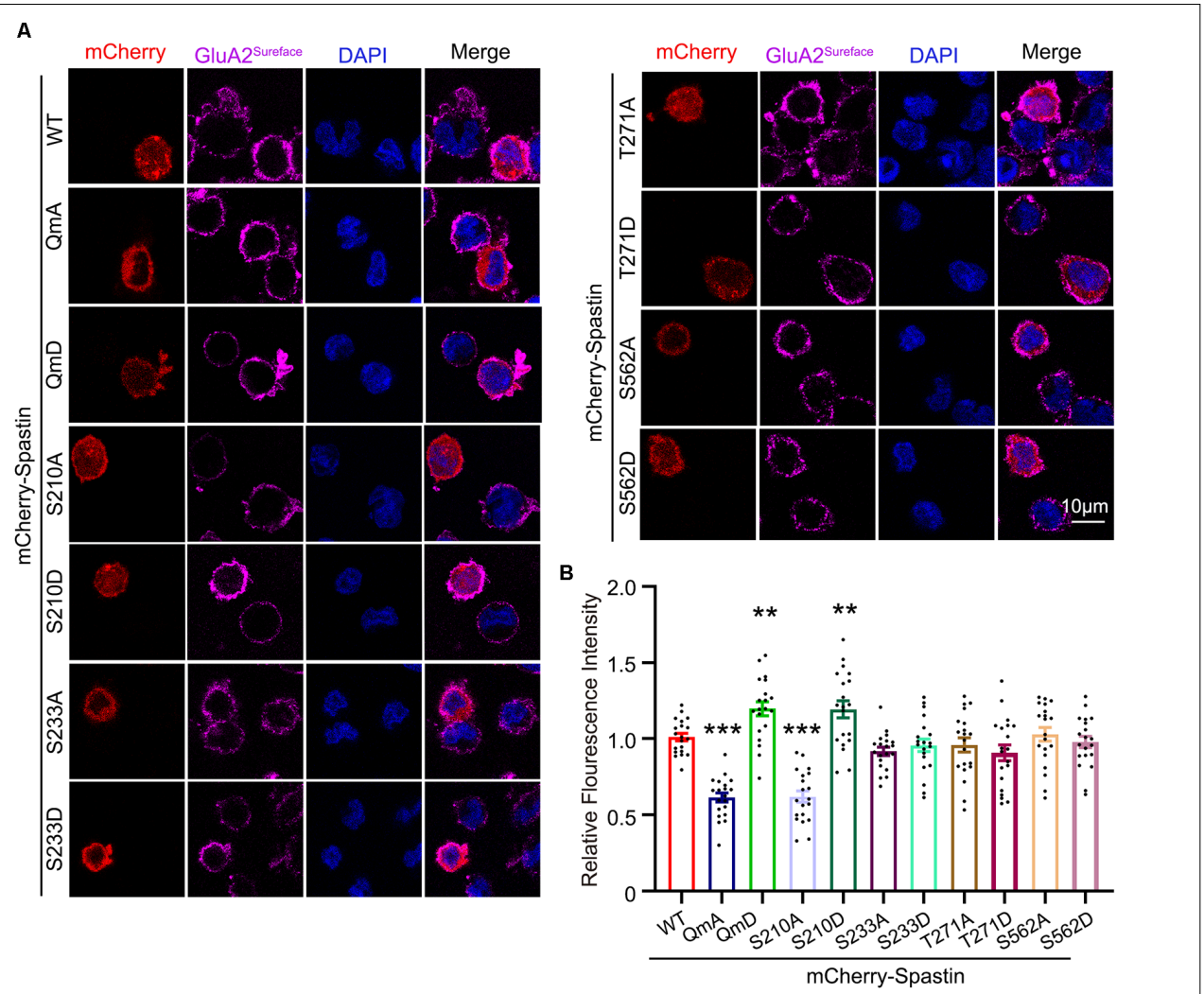

FIGURE 4 | Phosphorylation of Spastin at Ser210 promoted GluA2 surface expression in HEK293T cells expressing GluA2 subunit. **(A)** Confocal micrographs showing surface GluA2 in HEK293T cells stably expressing the GluA2 subunit of AMPA receptor expressing mCherry-Spastin WT and its phosphomimetic and dephosphomimetic mutants, respectively. Scale bar, 10 μm. **(B)** Quantification of relative fluorescence intensity in HEK293 cells overexpressing mCherry-Spastin WT and its phosphomimetic and dephosphomimetic mutants, respectively. In each group, $n = 20$ cells from three independent experiments, $^{**}P < 0.01$, $^{***}P < 0.001$, as compared to the mCherry-Spastin WT group.

with Spastin QmD mutants, displayed an increased surface expression of GluA2, whereas cells transfected with Spastin S210A mutants showed a reduced surface expression of GluA2 (**Figure 4**). To further explore the effect of Spastin S210 phosphorylation on excitatory synapses, whole-cell patch-clamp recordings were performed to analyze mEPSC of neurons transfected with Spastin WT, Spastin S210A, and Spastin S210D mutants. As shown in **Figure 5**, neurons overexpressing Spastin S210A showed a reduction in both amplitude and frequency of mEPSC, while neurons overexpressing Spastin S210D exhibited an increased level in amplitude and frequency of mEPSC, compared with neurons overexpressing GFP control. Moreover, the same phenomenon also appeared when compared with neurons overexpressing Spastin WT. Taken together, these results indicated that phosphorylation of Spastin at Ser210 alone may increase the surface expression of AMPAR GluA2.

Phosphorylation of Spastin Promoted AMPAR Glua2 Surface Expression Independently of Microtubule Dynamics

The classical function of Spastin is to cut microtubules and we recently reported that phosphorylation of Spastin decreased the severing function of microtubules (Li et al., 2021). To determine whether the increased level of surface GluA2 is associated with their binding to Spastin or with the microtubule-severing efficiency of Spastin, we first generated two mutants of Spastin without the ability to sever microtubules: Spastin K353A (mutation of lysine to alanine) and Spastin R464C

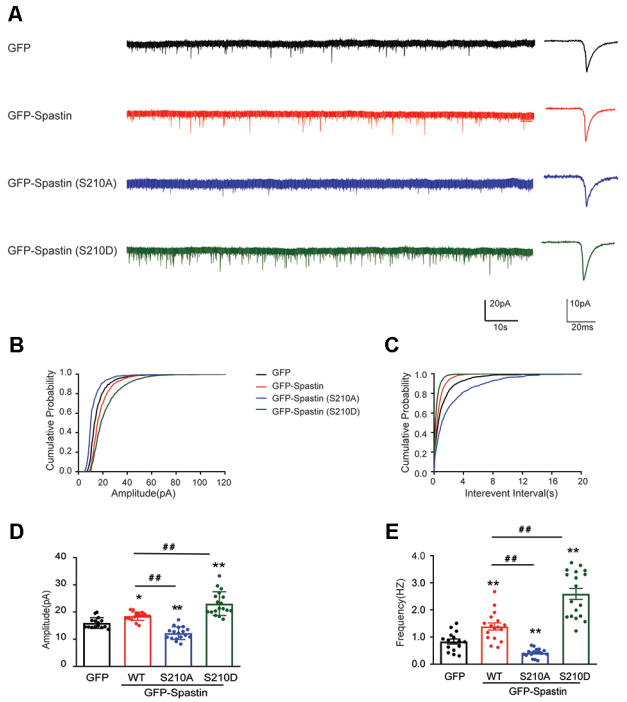

FIGURE 5 | Phosphorylation of Spastin at Ser210 increased the amplitude and frequency of mEPSCs. **(A)** Representative recordings of mEPSCs from neurons overexpressing GFP, GFP-Spastin WT, S210A, and S210D. Representative traces of individual mEPSC are on the right. **(B,C)** Cumulative distributions of mEPSC amplitudes and the inter-mEPSC event intervals in neurons overexpressing GFP, GFP-Spastin WT, S210A, and S210D. **(D,E)** Quantification of mEPSCs amplitude and frequency in neurons overexpressing GFP, GFP-Spastin WT, S210A, and S210D. In GFP group $n = 15$ cells; in the WT and S210A group, $n = 17$ cells respectively; in QmD group, $n = 18$ cells; from three independent experiments, $^{*}P < 0.05$, $^{**}P < 0.01$, as compared to the GFP group, $^{\#\#}P < 0.01$, as compared to the GFP-Spastin WT group.

(mutation of arginine to cysteine) according to a previous study (Evans et al., 2005). Then, these two mutants were transfected into COS1 cells to verify their microtubule-severing function. Microtubule immunofluorescence staining showed that the microtubules in the COS1 cells transfected with the Spastin WT were severed into microtubule segments or fragments when compared with the cells transfected with GFP control, while cells overexpressing Spastin K353A or Spastin R464C had intact microtubules retained (**Figure 6A**). The quantitative analysis showed that the relative microtubules' fluorescence intensity decreased significantly in cells overexpressing Spastin WT when compared with those in control cells overexpressing GFP. However, there was no difference in microtubule fluorescence intensity, among cells overexpressing K353A and R464C and control cells overexpressing GFP (**Figure 6B**). These data indicated that K353A and R464C obliterated the ability to sever microtubules, which is consistent with previous research (Evans et al., 2005).

In addition, we also evaluated if the interaction between GluA2 and Spastin would be affected by the two mutants using Co-IP assays. We found that Spastin WT, Spastin K353A, and Spastin R464C coimmunoprecipitated almost an equal amount of GluA2 (**Figure 6C**). These data suggested that mutation of Spastin at K353 and R464 did not affect the interaction between Spastin and GluA2.

After clarifying that Spastin K353A and R464C can lose the microtubule-severing function without changing the binding ability between Spastin and GluA2, Spastin phosphomimetic and dephosphomimetic mutants with impaired microtubule-severing activity (K353A/S210A and K353A/S210D) were generated. Then, immunofluorescence staining and electrophysiology assessments were performed to explore the effect of Spastin S210 phosphorylation on the trafficking of AMPAR after Spastin K353A, Spastin K353A/S210A, and Spastin K353A/S210D were transfected into the hippocampal neurons. As shown in **Figure 7**, neurons overexpressing Spastin K353A/S210A showed a decreased surface GluA2 fluorescence intensity compared with neurons overexpressing Spastin K353A. However, neurons overexpressing Spastin K353A/S210D exhibited increased surface GluA2 fluorescence intensity when compared with neurons overexpressing Spastin K353A. Simultaneously, electrophysiological findings revealed that neurons overexpressing Spastin K353A/S210A exhibited a reduction in both amplitude and frequency of mEPSC compared with neurons overexpressing Spastin K353A. In contrast, neurons overexpressing Spastin K353A/S210D exhibited an increased level in amplitude and frequency of mEPSC when compared with neurons overexpressing Spastin K353A (**Figure 8**). These data indicated that phosphorylation of Spastin at Ser210 promoted AMPAR GluA2 surface expression independent of microtubule dynamics.

DISCUSSION

The present study demonstrated that Spastin interacted with AMPAR and phosphorylation of Spastin enhanced its interaction with AMPAR subunit GluA2. Further immunostaining and electrophysiology experiments showed that phosphorylation of Spastin increased the surface expression of AMPAR GluA2 subunits and synaptic function. Additionally, our study further clarified that it was the Ser210 site phosphorylation of Spastin that contributed to the surface delivery of AMPAR and this process was not dependent on microtubule dynamics.

First, our results provide evidence that Spastin acts as a regulatory protein on postsynaptic AMPA receptors. In our previous study, we reported that Spastin promotes dendrite outgrowth (Ji et al., 2018). In this research, the results showed that overexpression of Spastin increased surface expression of AMPA receptors (**Figure 2**). Meanwhile, the synaptic function had also been strengthened as reflected by the amplitude and frequency of mEPSC (**Figure 3**). Increased amplitude and frequency of mEPSC indicate the presence of an increased number of AMPARs at the synapse. Interestingly, phosphorylation of Spastin further increased AMPAR surface expression and synaptic function, whereas dephosphorylated Spastin had the opposite effect. Here, Spastin showed the ability

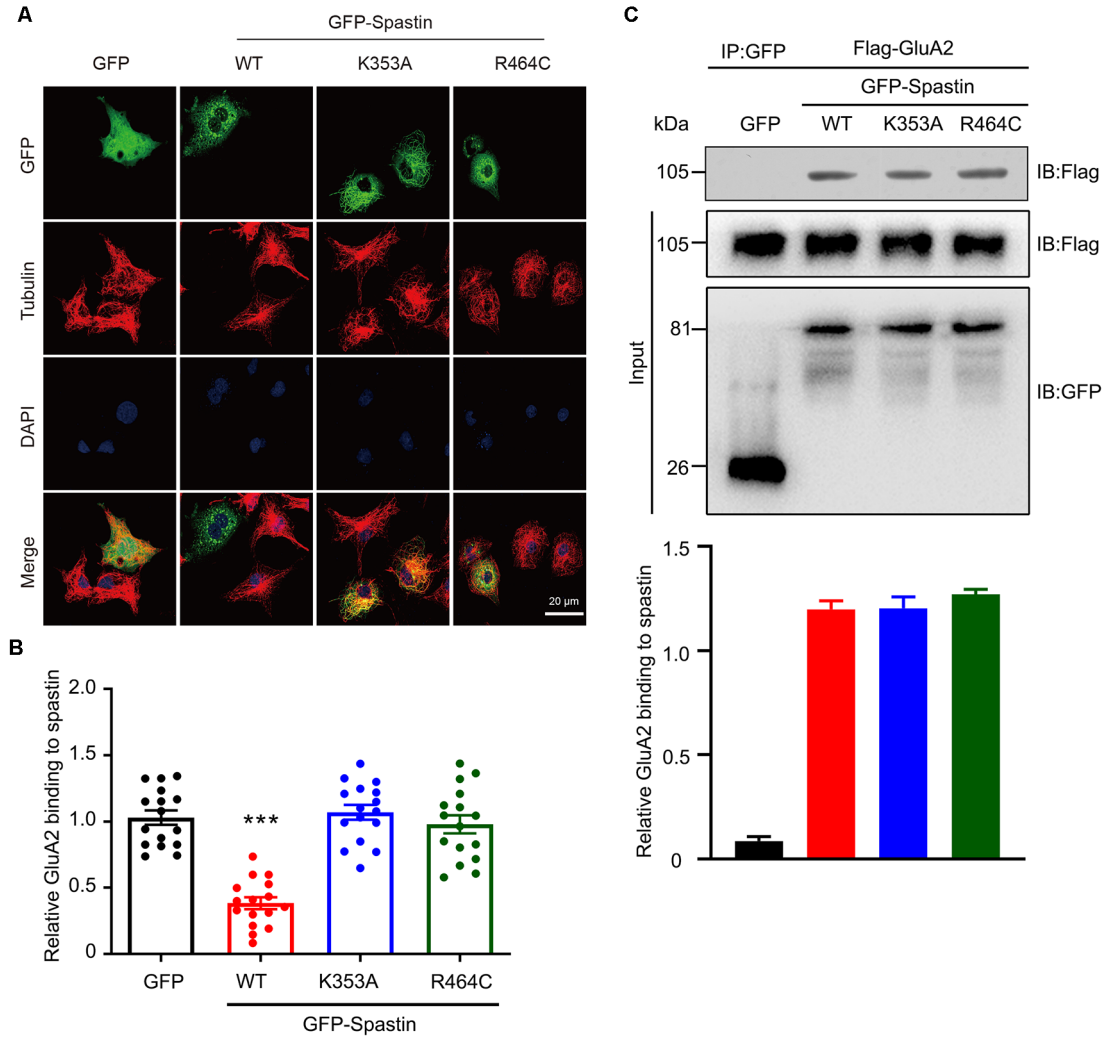

FIGURE 6 | Mutation of Spastin at K353 and R464 inhibited microtubule-severing activity of Spastin but did not affect its binding to GluA2. **(A)** Confocal micrographs showing microtubules (red) in COS1 cells overexpressing GFP, GFP-Spastin WT, K353A, and R464C. Scale bar, 20 μm. **(B)** Quantification of relative fluorescence intensity of microtubules in cells overexpressing GFP, GFP-Spastin, and its mutants. In each group, $n = 16$ cells from three independent experiments, $^{***}P < 0.001$ compared to the GFP group. **(C)** HEK293T cells co-transfected with Flag-GluA2 and GFP, GFP-Spastin, GFP-Spastin K353A, and GFP-Spastin R464C were lysed for Co-IP assay. Immunoblotting of input and bound proteins was performed using antibodies against GFP and Flag, respectively (top). Quantification of the relative binding of GluA2 to GFP, GFP-Spastin, GFP-Spastin K353A, and GFP-Spastin R464C (bottom), $n = 3$ independent experiments.

to transfer AMPARs to the membrane. This was especially true in the case of phosphorylated Spastin.

Second, one of the most striking results obtained from our research is the phosphorylation-dependent interaction between Spastin and AMPAR GluA2 subunit. Studies have shown that AMPAR transport involves a complex protein-protein interaction network. In our study, Spastin showed a degree of association with all the four subunits of AMPARs (**Figure 1B**). Although Spastin binding to AMPA receptors did not exhibit subunit specificity, it suggested that Spastin was an AMPAR binding protein. Due to the predominance of GluA2-containing AMPARs in hippocampal neurons (Bats et al., 2013), we focused on investigating the binding ability between Spastin and GluA2. Pulldown and Co-IP assays provided direct evidence that Spastin interacted with GluA2 and that their interaction was regulated by Spastin phosphorylation (**Figures 1E,F**). Protein phosphorylation, the most studied post-translational modification, is employed by cells to transiently alter protein properties such as their localization and conformation, as well as their interactions with other proteins (Sharma et al., 2014). Our recently published article also reported a phosphorylation-dependent binding of Spastin with tubulin. Phosphorylated Spastin weakens its binding to microtubules, thereby its microtubule-severing ability is attenuated (Li et al., 2021). On the contrary, here, phosphorylated Spastin showed increased binding ability with GluA2. This means that phosphorylated Spastin is dissociated from the microtubules to assist receptor transport.

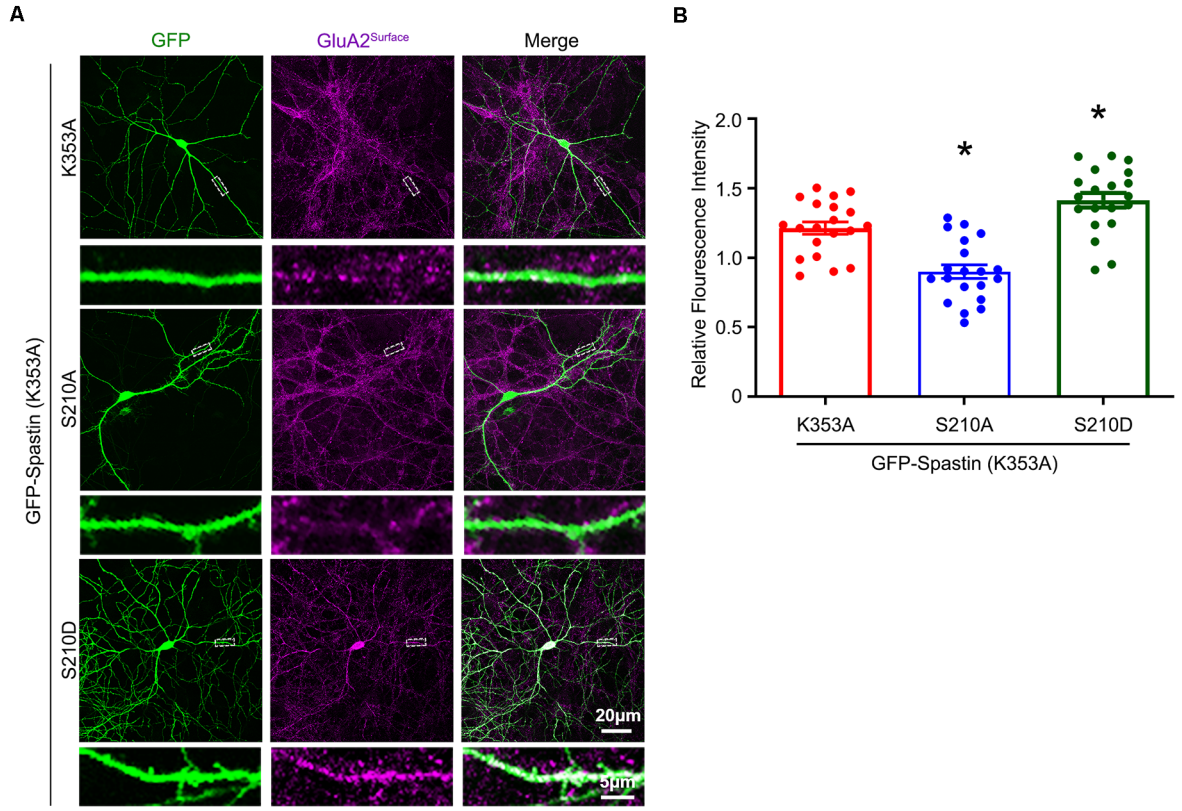

FIGURE 7 | Phosphorylation of Spastin at Ser210 with impaired microtubule-severing activity promoted GluA2 surface expression. **(A)** Confocal micrographs showing surface GluA2 in DIV 15 hippocampal neurons overexpressing GFP-Spastin K353A, GFP-Spastin K353A/S210A, and GFP-Spastin K353A/S210D. Scale bar, 20 μm. The rectangle details were enlarged. In the magnified dendrite, the scale bar corresponds to 5 μm. **(B)** Quantification of relative fluorescence intensity in neurons overexpressing GFP-Spastin K353A, GFP-Spastin K353A/S210A, and GFP-Spastin K353A/S210D. In each group, $n = 20$ cells from three independent experiments, $^*P < 0.05$, as compared to the GFP-Spastin K353A group.

The number of synaptic AMPARs is regulated through endocytosis, exocytosis, and endosomal sorting, which results in the recycling of AMPARs back to the plasma membrane or degradation in the lysosome (van der Sluijs and Hoogenraad, 2011; Parkinson and Hanley, 2018). Here, we confirmed that phosphorylated Spastin promotes receptor expression on the membrane by increasing its binding ability to GluA2, however, the specific step underlying the AMPARs Spastin trafficking pathway remains unclear. Previous studies have reported that Spastin interacts with IST1 and CHMP1B, the ESCRT-III-associated proteins, to control endosome recycling (Campsteijn et al., 2016; Connell et al., 2020). The process of AMPA receptors trafficking is dependent on endosomal recycling (van der Sluijs and Hoogenraad, 2011). It is therefore hypothesized that Spastin may serve as an intermediate protein to interact with AMPAR subunits in hippocampal neurons, thereby affecting the entry and exit of AMPAR subunits to the membrane, and positively regulating the synaptic function. In addition, Spastin QmA decreased synaptic function and AMPAR surface expression even more than GFP, which was not anticipated. We speculate that the site mutation may have a greater impact on the conformation of non-phosphorylated spastin. On one hand, dephosphorylation greatly reduces the recruitment of spastin to AMPA receptors, and on the other hand, it may also affect the binding of proteins related to endosome recycling. However, these speculations require further investigation.

Third, our results identified the key phosphorylation site of Spastin involved in AMPAR trafficking. There are four different potential phosphorylation sites in Spastin. In our study, we started by mutating all four Spastin phosphorylation sites to construct phosphomimetic (Spastin QmD) and dephosphomimetic mutants (Spastin QmA) of Spastin. After clarifying the role of Spastin phosphorylation in AMPAR transportation, we generated a series of mutants, with Ser210, Ser233, Thr271, and Ser562 replaced with alanine (S210A, S233A, T271A, and S562A) or aspartic acid (S210D, S233D, T271D, and S562D), respectively, to mimic dephosphorylated or phosphorylated Spastin and determine which site played a key role in this process. We found that like the "quadruple phosphomutants," the "single phosphomutants" at S210 of Spastin could promote the GluA2 surface expression and the amplitude and frequency of mEPSCs. Other phosphorylation sites of Spastin did not

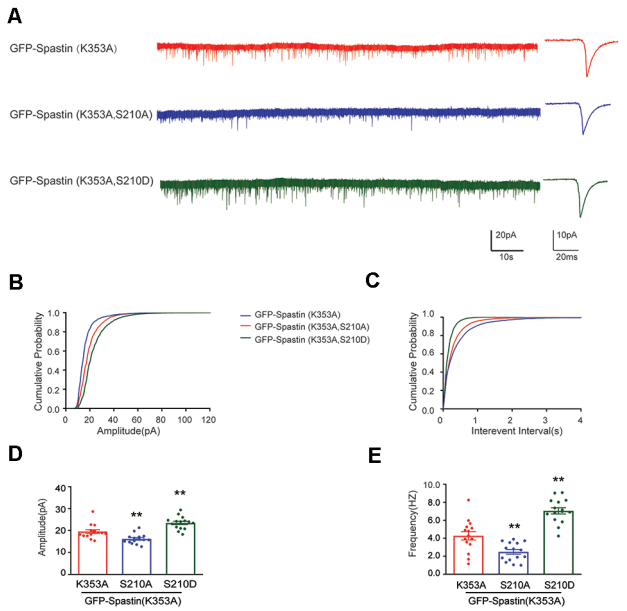

FIGURE 8 | Phosphorylation Spastin at Ser210 with microtubule-severing activity impaired increased the amplitude and frequency of mEPSCs. **(A)** Representative recordings of mEPSCs from neurons overexpressing GFP-Spastin K353A, GFP-Spastin K353A/S210A and GFP-Spastin K353A/S210D. Representative traces of individual mEPSC are on the right. **(B,C)** Cumulative distributions of mEPSC amplitudes and the inter-mEPSC event intervals in neurons overexpressing GFP-Spastin K353A, GFP-Spastin K353A/S210A, and GFP-Spastin K353A/S210D. **(D,E)** Quantification of mEPSCs amplitude and frequency in neurons overexpressing GFP-Spastin K353A, GFP-Spastin K353A/S210A, and GFP-Spastin K353A/S210D. In each group, $n = 15$ cells from three independent experiments, $^{**}P < 0.01$, as compared to the GFP-Spastin K353A group.

show any effect. Thus, S210 phosphorylation appears to be crucial for Spastin in AMPAR trafficking. Simultaneously, S210 phosphorylation also has been reported to play a role in neurite outgrowth and branching (Li et al., 2021). In addition, HIPK2 phosphorylates Spastin at S268 contributes to midbody localization for successful abscission in cytokinesis (Pisciottani et al., 2019). This suggests that Spastin regulates different biological functions through phosphorylation at different sites. However, with the exception of S268, which is reported to be phosphorylated by HIPK2, the upstream kinase at other sites is still unknown. Thus, investigating the role and the upstream kinase of other phosphorylation sites will be an interesting topic for future research.

Finally, our study clarified whether microtubule motility was involved in Spastin phosphorylation-mediated AMPAR trafficking. Previous studies have revealed that loss of Spastin can cause abnormalities in the stability of the neuronal microtubule cytoskeleton and lead to synaptic growth and neurotransmission defects (Trotta et al., 2004; Ji et al., 2018). Through its MTBD domain, Spastin binds to the microtubule, then the fence-like structure of microtubules is disrupted by its AAA ATPase domain. Thus, long microtubules are severed into small microtubule fragments by Spastin (Garnham and Roll-Mecak, 2012). Moreover, in our previous study, we reported that phosphorylation of Spastin decreased the microtubule-severing ability (Li et al., 2021). Therefore, we needed to clarify whether the surface expression of GluA2 increased by Spastin phosphorylation is related to the dynamics of microtubules. In our present study, we generated two Spastin mutants, which obliterated the microtubule-severing function. Subsequently, we confirmed that their microtubule-severing function was indeed impaired and that their interaction with GluA2 was not changed by immunofluorescence staining of microtubules and by Co-IP assay, respectively (**Figure 6**). Therefore, Spastin phosphomimetic and dephosphomimetic mutants with impaired microtubule-severing activity (K353A/S210A and K353A/S210D) were produced as a tool for studying the effect of Spastin S210 phosphorylation on AMPAR trafficking. In the case that Spastin obliterated its microtubule cleavage activity, phosphorylation of Spastin at S210 could still promote the GluA2 surface expression and improve the frequency and amplitude of mEPSCs (**Figures 7**, **8**). Thus, a pivotal role of Spastin S210 phosphorylation in the trafficking of AMPARs was confirmed, and this process was independent of microtubule dynamics. Therefore, it is plausible to conclude that the binding ability between Spastin and GluA2 is critical for AMPAR delivery to neuronal membranes.

Mutations in *SPAST* are the most common cause of HSP (Kara et al., 2016; Erfanian Omidvar et al., 2021). Most research on Spastin has focused on its relationship with movement disorders of HSP. Although increasing research has shown that SPG4-linked HSP is associated with cognitive dysfunction (Orlacchio et al., 2004; Murphy et al., 2009; Chelban et al., 2017; Akaba et al., 2021; Erfanian Omidvar et al., 2021; Giordani et al., 2021), the studies on its mechanism remain few. Brain abnormalities in regions including the cerebral cortex, corpus callosum, hippocampus, and thalamus were observed in patients with SPG4-HSP (Orlacchio et al., 2004; Murphy et al., 2009; Servelhere et al., 2021). In addition, mice with spastin depletion exhibited working and associative memory deficits and reduced function of hippocampal synapses (Lopes et al., 2020). These studies suggest that the function of spastin is closely related to synaptic plasticity and cognitive function. Thus, we chose to use cultured hippocampal neurons to study the relationship between spastin and AMPA receptors. Our study primarily, demonstrated that phosphorylation of Spastin promotes the surface delivery and synaptic function of AMPA receptors; however, whether this will cause changes in synaptic plasticity and cognitive functions needs to be further confirmed *in vivo*. In addition, since the upstream kinase of Spastin S210 phosphorylation is unknown, we relied on overexpression of various constructs in primary hippocampal neurons. Pharmacological manipulations will shed light on this physiological process once the upstream kinases are identified. In addition, our previous studies have confirmed spastin phosphorylation at site S210 in rat brain tissue, although it is unclear whether the Spastin S210 phosphorylation level is reduced in the brain tissue of patients with SPG4-HSP. Nevertheless, Spastin phosphorylation is beneficial for enhancing AMPA receptor function, which may be a potential target for

improving cognitive dysfunction in patients with SPG4-HSP in the future.

In summary, this study reveals a novel role of Spastin on AMPAR trafficking. It provides the first evidence that Spastin interacts with AMPAR and demonstrated that their phosphorylation-dependent interactions rather than microtubule dynamics are required for GluA2 surface delivery. Finally, we also verified that it is Ser210 site phosphorylation of Spastin that contributed to GluA2 AMPAR trafficking. Our study provides important evidence on the role of Spastin phosphorylation in AMPAR trafficking, which will advance our understanding of cognitive dysfunction in HSP.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Jinan University.

AUTHOR CONTRIBUTIONS

JifZ and GG conceived and designed the study. JifZ wrote the article. LC, HW, and SC performed most of the experiments and analyzed the data. JL, JiaZ, and JW helped with pulldown and Co-IP experiments. All authors contributed to the article and approved the submitted version.

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Multiple Mechanistically Distinct Timescales of Neocortical Plasticity Occur During Habituation

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Recognizing familiar but innocuous stimuli and suppressing behavioral response to those stimuli are critical steps in dedicating cognitive resources to significant elements of the environment. Recent work in the visual system has uncovered key neocortical mechanisms of this familiarity that emerges over days. Specifically, exposure to phase-reversing gratings of a specific orientation causes long-lasting stimulus-selective response potentiation (SRP) in layer 4 of mouse primary visual cortex (V1) as the animal's behavioral responses are reduced through habituation. This plasticity and concomitant learning require the NMDA receptor and the activity of parvalbumin-expressing (PV+) inhibitory neurons. Changes over the course of seconds and minutes have been less well studied in this paradigm, so we have here characterized cortical plasticity occurring over seconds and minutes, as well as days, to identify separable forms of plasticity accompanying familiarity. In addition, we show evidence of interactions between plasticity over these different timescales and reveal key mechanistic differences. Layer 4 visual-evoked potentials (VEPs) are potentiated over days, and they are depressed over minutes, even though both forms of plasticity coincide with significant reductions in behavioral response. Adaptation, classically described as a progressive reduction in synaptic or neural activity, also occurs over the course of seconds, but appears mechanistically separable over a second as compared to tens of seconds. Interestingly, these short-term forms of adaptation are modulated by long-term familiarity, such that they occur for novel but not highly familiar stimuli. Genetic knock-down of NMDA receptors within V1 prevents all forms of plasticity while, importantly, the modulation of short-term adaptation by long-term familiarity is gated by PV+ interneurons. Our findings demonstrate that different timescales of adaptation/habituation have divergent but overlapping mechanisms, providing new insight into how the brain is modified by experience to encode familiarity.

Keywords: primary visual cortex, learning, adaptation, habituation, inhibition, novelty, stimulus-selective response potentiation, NMDA receptors

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SIGNIFICANCE STATEMENT

Habituation is a foundational cognitive process that reduces the requirement for neural resources to be allocated to innocuous stimuli, thereby freeing up attention and energy to detect and explore salience. Memories of innocuous familiar stimuli must be formed so that they can be selectively ignored while novel stimuli, which have the potential for significance, are detected. Within the visual system, we have previously shown that increases in neural activity in cerebral cortex occur

during habituation that emerges over days, but many forms of habituation must occur over shorter timescales to allow allocation of resources to appropriate stimuli within a single session. Here we characterize cortical plasticity and habituation over seconds, minutes, and days within the same subjects, revealing short-term plasticity that diminishes neural activity, an opposing effect to the better characterized long-term plasticity. In addition, we have revealed overlapping but distinct molecular and cellular mechanisms mediating these different timescales of plasticity. Elucidating the mechanisms that underlie habituation will inform us how the brain can learn to recognize familiar stimuli and thereby detect novelty. This work also provides unique insight into core processes of learning that are affected in the disordered brain, where habituation and novelty detection are commonly dysfunctional.

INTRODUCTION

Learning and memory enable organisms to adapt to altered pressures in the environment to produce appropriate responses to stimulus and context over a variety of timescales (McGaugh, 2000). Substantial gaps remain in our understanding of the neural underpinnings of these processes, in part due to difficulties in observing and intervening in underlying plasticity as learning and memory occur (Neves et al., 2008). Habituation is one relatively robust, easy to observe and apparently simple form of learning, in which organisms acquire familiarity with innocuous stimuli and selectively reduce behavioral responses to those stimuli over seconds, minutes, and days (Cooke and Ramaswami, 2020). Habituation forms a foundation for further learning by enabling energy and attention to be devoted to stimuli of already established salience, or novel stimuli that may have future significance (Rankin et al., 2009; Schmid et al., 2014) and disruptions in this process likely contribute to a range of psychiatric and neurological disorders (Ramaswami, 2014; McDiarmid et al., 2017). This form of learning has commonly been ascribed to a neural process known as adaptation, which reduces feedforward synaptic activity in response to repeated non-associative stimulation (Groves and Thompson, 1970), especially over shorter timescales (Chung et al., 2002). However, a competing theory, known as the comparator model (Sokolov, 1963), suggests the formation of long-lasting memory of familiar stimuli through Hebbian synaptic potentiation, which in turn suppresses behavioral output by recruiting inhibitory systems. It remains possible that both models apply but over different timescales (Cooke and Ramaswami, 2020). In this study, we have assessed plasticity in primary visual cortex (V1) of mice in response to repeated presentations of oriented, phase reversing visual stimuli to assess whether different directions of plasticity can be observed across different timescales.

It is now well established that the magnitude of visual-evoked potentials (VEPs) recorded in layer 4 of mouse binocular V1 increases dramatically over days of repeated stimulation through an orientation-specific form of plasticity known as stimulus-selective response potentiation (SRP) (Frenkel et al., 2006; Cooke and Bear, 2010). This form of plasticity is also manifest as an increase in the peak firing rate of V1 neurons (Aton et al., 2014; Cooke et al., 2015) and many of the known molecular mechanisms are consistent with the involvement of Hebbian synaptic potentiation, notably including a requirement for the NMDA receptor during induction and AMPA receptor insertion during expression (Frenkel et al., 2006; Cooke and Bear, 2010). Importantly, mice produce behavioral responses to the onset of these visual stimuli that exhibit significant orientation-selective habituation over days (Cooke et al., 2015; Kaplan et al., 2016; Fong et al., 2020; Finnie et al., 2021), and this process also requires the presence of NMDA receptors in V1. In addition, a cortical cell-type that exerts exquisite inhibitory control over excitatory cell activity, the parvalbumin-expressing (PV+) inhibitory interneurons (Atallah et al., 2012), are critical for differential cortical and behavioral responses to familiar and novel stimuli after SRP and accompanying habituation (Kaplan et al., 2016). Thus, SRP comprises a robust and relatively well understood form of plasticity that occurs concomitantly with and shares mechanism with long-term memory.

One fascinating feature of SRP is that it does not manifest within a ∼30-min recording session but starts to emerge the following day (Frenkel et al., 2006) and recent work has demonstrated that SRP is dependent on consolidation processes that occur during sleep (Aton et al., 2014; Durkin et al., 2017). Activity in the primary visual relay nucleus of the thalamus, the dorsal lateral geniculate nucleus (dLGN), does increase over the course of 30 min prior to the emergence of SRP in the cortex (Durkin et al., 2017), but there has so far been no description of what happens over this time-course in V1. Although we have previously described evidence for a faster adaptation that is apparent when comparing the beginning of a 200-phase reversal block with the end (Kim et al., 2020), we have not described the time-course of this adaptation during this 100-s block. In neither case is there any understanding of the underlying mechanism. In the current study, we show that cortical plasticity accompanying behavioral habituation occurs across seconds, minutes, and days of repeated stimulus experience. Notably, these forms of plasticity diverge in direction and mechanism, and there is evidence of an interaction in which long-term familiarity suppresses adaptation. In striking opposition to our observations of SRP during long-term habituation (Cooke et al., 2015), layer 4 response magnitude decreases over seconds and minutes in V1. Loss of expression of NMDA receptors from neurons in V1 impairs plasticity and adaptation across all timescales. However, inactivation of PV+ neurons has a more nuanced effect, revealing the existence of two separable forms of fast adaptation within a stimulus block. Moreover, we show that the interaction between long-lasting familiarity and adaptation requires the activity of PV+ neurons. Thus, a range of mechanistically separable forms of plasticity can be assayed across different timescales in the same learning mouse.

MATERIALS AND METHODS

Animals

All procedures were carried out in accordance with the guidelines of the National Institutes of Health

and protocols approved by the Committee on Animal Care at the Massachusetts Institute of Technology. **Figures 1**, **2** are composed of data from male C57B6/J mice (Charles River laboratory international, Wilmington, MA). NMDA knock-down experiments (**Figures 3**, **4**) make use of GRIN$^{fl/fl}$ mice (B6.129S4-*Grin1*^{tm2Stl}/J—Jackson laboratory). PV+ interneuron inactivation (**Figure 5**) uses PV-Cre mice (B6;129P2-*Pvalb*$^{TM1(cre)Arbr}$/J—Jackson laboratory). All animals had food and water available *ad libitum* and were maintained on a 12-h light-dark cycle.

Viral Transfection

In the NMDAR knock-down and PV+ inactivation experiments viral vectors were administered *via* stereotaxic injections into the mice. For the NMDA knock-down, GRIN$^{fl/fl}$ mice (B6.129S4-*Grin1*^{tm2Stl}/J—Jackson laboratory) underwent surgery at ∼ 1 month. AAV8-hSyn-GFP-Cre (knockdown; UNC viral core) or AAV8-hSyn-GFP (control; UNC viral core; generated by Dr. Bryan Roth's laboratory) were injected in quantities of 13.5 nl 10 times at depths 600, 450, 300, and 150 μm bellow surface. Each injection was separated by 15 s and after repositioning 5 min was allowed. For the PV+ inactivation experiment, AAV9-hSyn-DIO-HA-hM4D(Gi)-IRES-mCitrine virus (UNC viral core—generated by B. Roth's laboratory) was injected into PV-Cre or WT-littermates in quantities of 81 nl at depths 600, 450, and 300 μm below surface, including a 5-min delay after repositioning. Viral transfections were performed in both hemispheres and were immediately followed by V1 electrode implantation, outlined below. Following surgery, mice were allowed 3 weeks for full viral expression.

V1 Electrode Implantation

Mice were anesthetized with an intraperitoneal (i.p) injection of 50 mg/kg ketamine and 10 mg/kg xylazine for surgery. 1% lidocaine hydrochloride anesthetic was injected locally under the scalp and 0.1 mg/kg Buprenex was delivered sub-cutaneously for analgesia. Iodine and 70% ethanol were used to clean the scalp. The skull was cleaned, dried, and scored using a blade. A steel headpost was fixed over the frontal suture using super glue (ethyl cyanoacrylate). Burr holes were drilled 3.1 mm lateral to lambda (to target binocular V1). Tungsten recording electrodes (FHC, Bowdoinham, ME, United States) were implanted 450 μm below surface in both hemispheres. Silver wire reference electrodes were placed in prefrontal cortex bi-laterally.

Visual Stimuli

Visual stimuli were generated using software developed by Jeff Gavornik.[1] The display was 20 cm in front of the mouse, and mean luminance was 27 cd/m^2. Sinusoidal phase reversing gratings were presented full field, reversing at 2 Hz. In most experiments, blocks consisted of 200 phase reversals, each block was presented 5 times interleaved with 30 s of gray screen. Gamma-correction was performed to maintain constant luminance between gratings and gray screen. The 5 blocks were repeated until day 6. On the final day, day 7, the familiar orientation (X°) was pseudo-randomly interleaved (such that no more than 2 blocks of the same orientation were shown in sequence) with a novel orientation (X+90°). Orientations were never within 25° of horizontal. In the PV+ inactivation experiment (**Figure 5**) 10 blocks were shown. On day 7 familiar (X°) and novel (X°-60°) stimuli were shown. Then CNO was administered at 5 mg/kg *via* intraperitoneal (i.p) injection. After a 15-min wait, the familiar stimulus (X°) was presented with a new novel stimulus (X°+60°).

[1]https://github.com/jeffgavornik/VEPStimulusSuite

In vivo Data Acquisition and Analysis

Mice recovered from electrode implantation then underwent 2 days of habituation, followed by the 7-day protocol outlined above. All data was acquired using the Plexon data acquisition system (Plexon Inc., Dallas, TX, United States). Local field potentials (LFP) were collected from V1 in both hemispheres, and piezoelectrical signal was reduced in amplitude and digitized into a third recording channel. Animals were head fixed at the opening of a metal cylinder tube and positioned on a piezoelectric transducer placed under the front paws but touching the metal cylinder. This piezoelectric signal therefore consists mainly of front paw movement but hind paw/whole body movements also contribute to the signal due to vibrations *via* the metal tube. All digital channels were recorded at 1 kHz sampling and run through a 500 Hz low-pass filter. Data was extracted into Matlab using custom software. For the analysis over days, 450 ms traces following stimulus onset were averaged over 1,000 phase reversals (5 blocks × 200 phase reversals). For the across block analysis, traces were averaged over 200 phase reversals. For the within-block analysis (1v2, 1v200), each individual phase reversal was averaged over 5 blocks. VEP magnitude was taken as the minimum microvolt value from 1 to 100 ms following onset subtracted from the maximum microvolt value taken from 75 to 250 ms following onset.

Statistics

All data is expressed as mean ± SEM and number of animals is represented by n. All statistical analysis is non-parametric due to small n numbers negating true testing of normality. For comparisons between two groups or time points, a paired Wilcoxon signed rank test is used, for adaptation ratio analysis a one-sample Wilcoxon signed rank test is used with a μ of 1. Repeated measures Friedman test is used for analysis across multiple time points within one group. Where multiple tests have been performed, all *p*-values are adjusted using false discovery rate (FDR) correction.

Data Collection and Use

Data was originally collected by Sam Cooke in Mark Bear's lab (MIT). Raw data used in **Figures 1**, **2** was previously published by Kim et al. (2020). Raw data used in **Figures 3**, **4** was previously published by Cooke et al. (2015), and **Figure 5** was published by Kaplan et al. (2016). Extended data analysis was performed

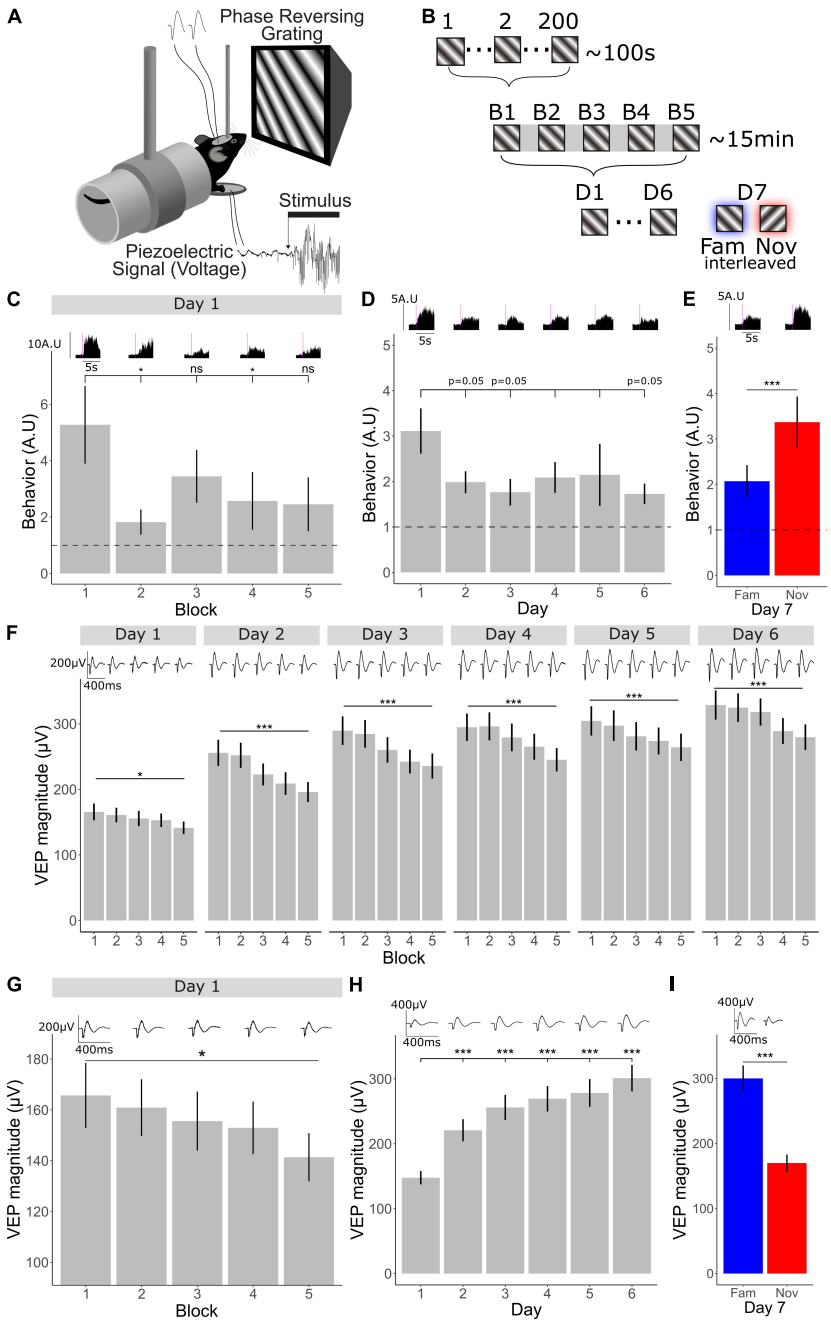

FIGURE 1 | V1 plasticity accompanying long- and short-term habituation occurs in opposing directions. **(A)** Schematic of recording set-up. Mice viewed phase reversing gratings while layer 4 local-field potentials were recorded through implanted tungsten electrodes and movement was recorded through a piezo-electrical device. **(B)** 1 through 200 individual phase reversals were shown lasting approximately 100 s (1 block). Five blocks were shown lasting approximately 15 min within one session. One session of 5 blocks was shown for 6 days. On the 7th day, the familiar orientation (previously viewed) and a novel orientation were shown pseudo-randomly interleaved. **(C)** Comparison of behavior across blocks ($n = 30$). Friedman test $\chi^2(4) = 13.8$, $p = 0.008$. *Post-hoc* analysis of individual comparisons of blocks 1–2: $p = 0.02$, blocks 1–3: $p = 0.7$, blocks 1–4: $p = 0.04$, blocks 1–5: $p = 0.5$. FDR correction for multiple comparisons. **(D)** Behavioral change over days 1–6 ($n = 30$). Freidman test $\chi^2(5) = 6.55$, $p = 0.3$. *Post-hoc* analysis of individual comparisons of days 1–2: $p = 0.05$, days 1–3: $p = 0.05$, days 1–4: $p = 0.2$, days 1–5: $p = 0.09$, days 1–6: $p = 0.05$. FDR correction for multiple comparisons. **(E)** Behavioral response to familiar and novel ($n = 30$). Wilcoxon signed-rank test fam vs. nov: $p < 0.001$. **(F)** VEP magnitude from block 1 to 5 over 6 days ($n = 33$). Comparison across blocks, Friedman test, day 1: $\chi^2(4) = 12.8$, $p = 0.01$, day 2: $\chi^2(4) = 69.8$, $p < 0.001$, day 3: $\chi^2(4) = 55.1$, $p < 0.001$, day 4: $\chi^2(4) = 43.8$, $p < 0.001$, day 5: $\chi^2(4) = 32.5$, $p < 0.001$, day 6: $\chi^2(4) = 38.6$, $p < 0.001$. FDR correction for multiple comparisons. **(G)** VEP magnitude from block 1 to 5 on day 1 (n = 33). Friedman test across blocks on day 1; $p = 0.01$. **(H)** VEP magnitude potentiation over days 1–6 ($n = 33$). Freidman test $\chi^2(5) = 95.9$, $p < 0.001$. *Post-hoc* analysis of individual comparisons of days 1, 2, 3, 4, 5, day 6: all $p < 0.001$, FDR correction for multiple comparisons. **(I)** VEP magnitude response to familiar and novel ($n = 33$). Wilcoxon signed-rank test fam vs. nov: $p < 0.001$. Asterisks throughout denote significance (*$p < 0.05$, ***$p < 0.001$) while ns denotes non-significant. Where $p = 0.05$, this is explicitly stated.

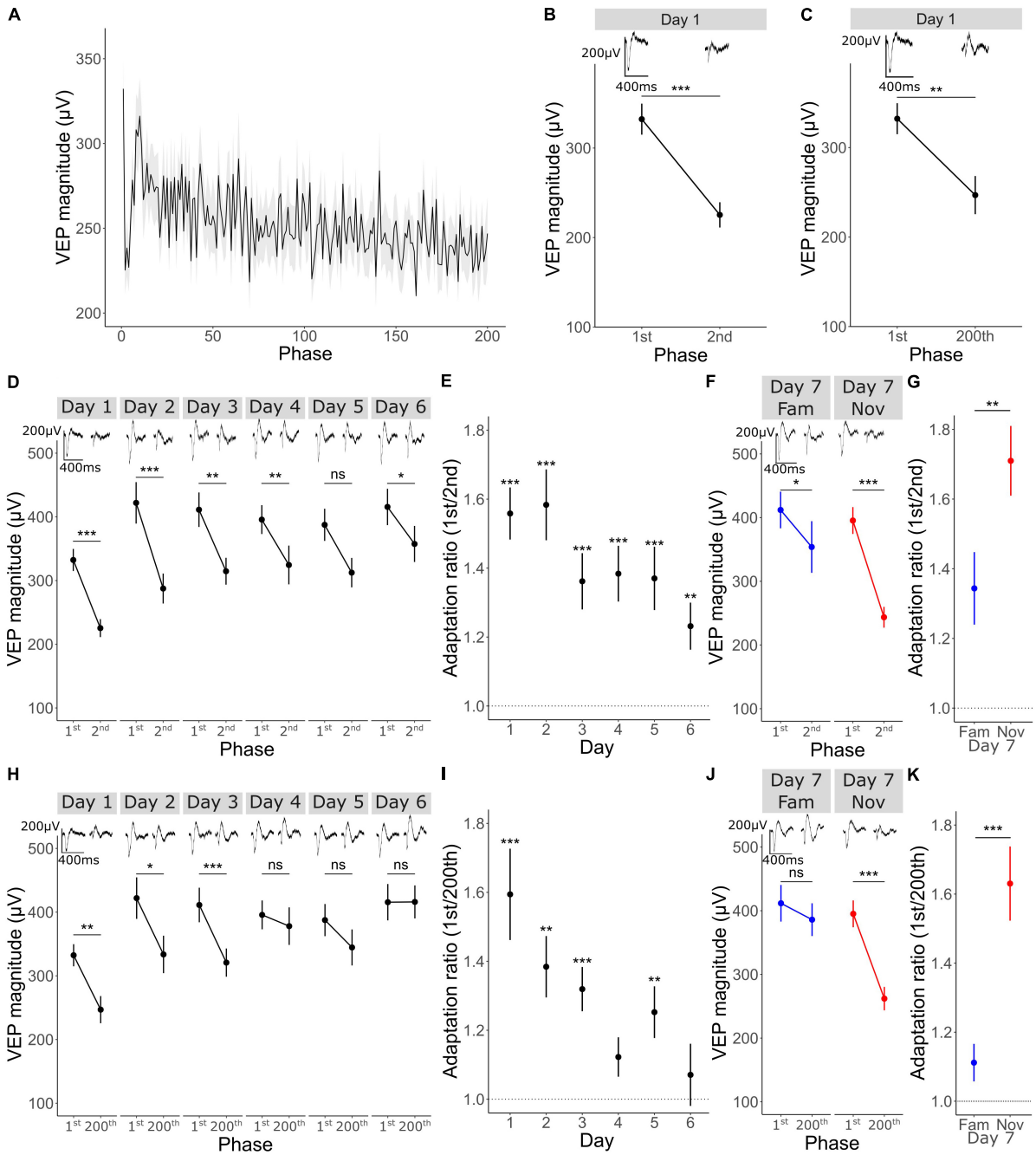

FIGURE 2 | Short-term adaptation occurs within a stimulus block and is modulated by familiarity. **(A)** Mean ± SEM VEP magnitude for phase reversal 1–200 ($n = 33$). **(B)** VEP magnitude in response to the first phase reversal and the 2nd, Wilcoxon signed rank 1st vs. 2nd: $p < 0.001$ ($n = 33$). **(C)** VEP magnitude in response to the first phase reversal and the 200th, Wilcoxon signed rank 1st vs. 200th: $p = 0.001$ ($n = 33$). **(D)** VEP potential magnitude in response to the 1st vs. 2nd phase reversal over 6 days ($n = 33$). Wilcoxon signed rank 1st vs. 2nd day 1: $p < 0.001$, day 2: $p < 0.001$, day 3: $p = 0.002$, day 4: $p = 0.008$, day 5: $p = 0.05$, day 6: $p = 0.04$. FDR correction for multiple comparisons. **(E)** Adaptation ratio (1st/2nd) over 6 days. Wilcoxon signed-rank test on AR ($\mu = 1$) day 1: $p < 0.001$, day 2: $p < 0.001$, day 3: $p < 0.001$, day 4: $p < 0.001$, day 5: $p < 0.001$, day 6: $p = 0.002$. FDR correction for multiple comparisons. **(F)** VEP potential magnitude in response to the 1st vs. 2nd phase reversal on day 7 ($n = 33$). Wilcoxon signed rank 1st vs. 2nd day 7 fam: $p = 0.02$, day 7 nov: $p < 0.001$. FDR correction for multiple comparisons. **(G)** Adaptation ratio (1st/2nd) on day 7. Wilcoxon signed-rank test fam vs. nov: $p = 0.009$. **(H)** VEP potential magnitude in response to the 1st vs. 200th phase reversal over 6 days ($n = 33$). Wilcoxon signed rank 1st vs. 200th day 1: $p = 0.008$, day 2: $p = 0.04$, day 3: $p < 0.001$, day 4: $p = 1$, day 5: $p = 0.4$, day 6: $p = 1$. **(I)** Adaptation ratio (1st/200th) over 6 days. Wilcoxon signed-rank test on AR ($\mu = 1$) day 1: $p < 0.001$, day 2: $p = 0.001$, day 3: $p < 0.001$, day 4: $p = 0.1$, day 5: $p = 0.006$, day 6: $p = 1$. FDR correction for multiple comparisons. **(J)** VEP potential magnitude in response to the 1st vs. 2nd phase reversal on day 7 ($n = 33$). Wilcoxon signed rank 1st vs. 200th day 7 fam: $p = 1$, day 7 nov: $p < 0.001$. **(K)** Adaptation ratio (1st/200th) on day 7. Wilcoxon signed-rank test fam vs. nov: $p < 0.001$. Asterisks throughout denote significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) while ns denotes non-significant.

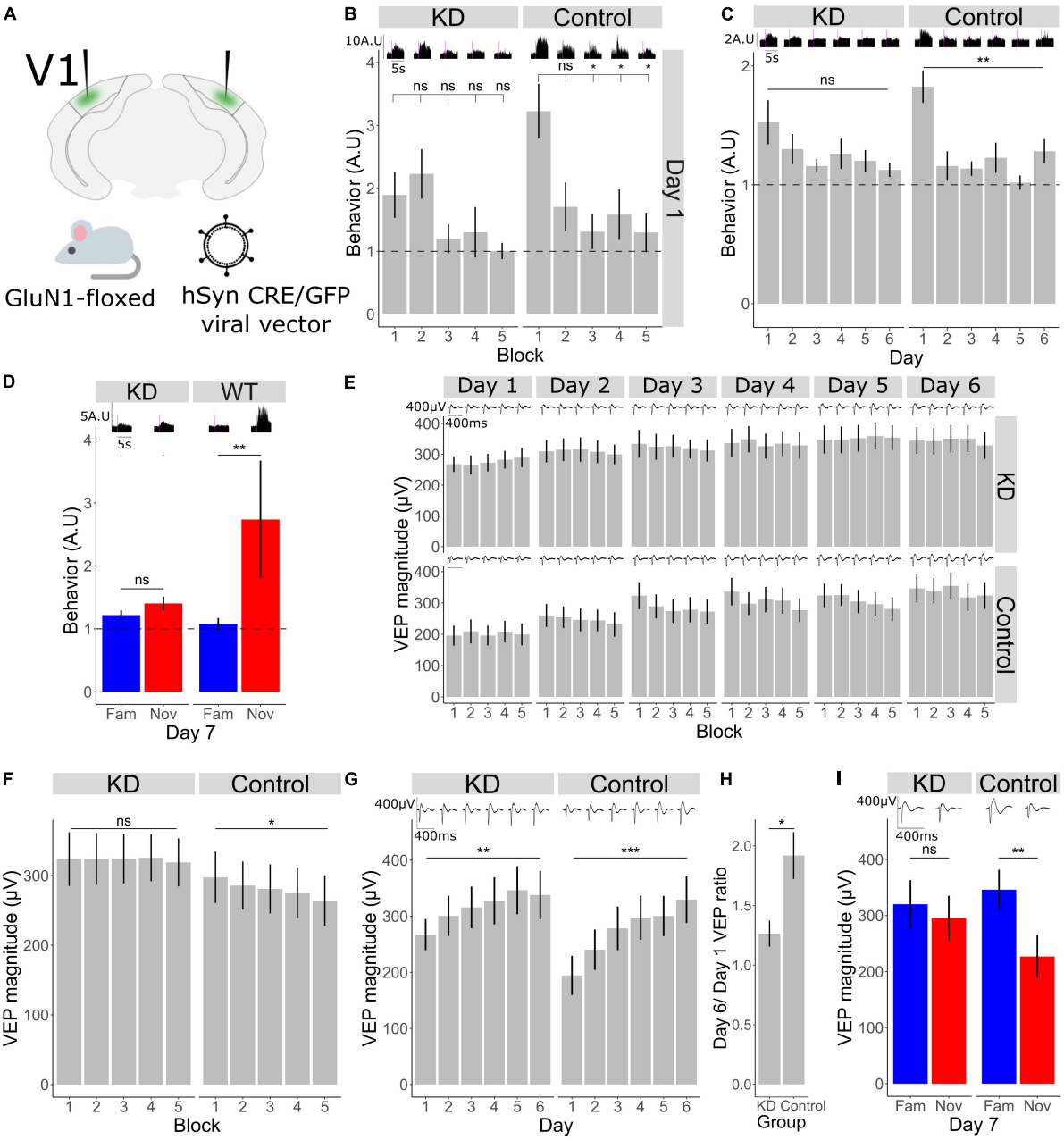

FIGURE 3 | Bidirectional plasticity occurring in V1 during short- and long-term habituation require NMDA receptors in V1. **(A)** Schematic of the experimental set-up in which a Cre recombinase was locally expressed bilaterally in binocular V1 using an AAV viral vector to knockdown the mandatory GluN1 subunit of the NMDA receptor in GluN1-floxed mice. **(B)** Comparison of behavior across blocks for KD group (n = 11). Friedman test $\chi^2(4) = 4.7$, $p = 0.3$. *Post-hoc* analysis of individual comparisons of blocks 1–2: $p = 0.8$, blocks 1–3: $p = 0.5$, blocks 1–4: $p = 0.3$, blocks 1–5: $p = 0.1$. Comparison of behavior across blocks for WT group (n = 11). Friedman test $\chi^2(4) = 10.8$, $p = 0.03$. *Post-hoc* analysis of individual comparisons of blocks 1–2: $p = 0.1$, blocks 1–3: $p = 0.02$, blocks 1–4: $p = 0.02$, blocks 1–5: $p = 0.02$. FDR correction for multiple comparisons. **(C)** Behavioral change over days 1–6 in KD group (n = 11), Friedman test $\chi^2(5) = 5.9$, $p = 0.3$. In WT group (n = 11), Freidman test $\chi^2(5) = 21.6$, $p = 0.001$. FDR correction for multiple comparisons. **(D)** Behavioral response to familiar and novel. Wilcoxon signed-rank test fam vs. nov in KD group: $p = 0.2$, in WT group: $p = 0.009$. FDR correction for multiple comparisons. **(E)** VEP magnitude change from block 1 to block 5 for day 1 to day 6 (n = 11 for each group). **(F)** VEP potential magnitude averaged over day 1–6. Comparison over blocks for KD group, Friedman test $\chi^2(4) = 0.7$, p = 0.9 (n = 11). Comparison over blocks for WT group, Friedman test $\chi^2(4) = 12.1$, $p = 0.03$ (n = 11). FDR correction for multiple comparisons. **(G)** VEP magnitude across days 1–6 in knock-down (KD) and wild-type (WT groups). Friedman test for KD group: $\chi^2(5) = 15.4$, $p = 0.008$ (n = 11). Friedman test for WT group: $\chi^2(5) = 36.5$, $p < 0.001$ (n = 11). FDR correction for multiple comparisons. **(H)** Ratio of day 6 VEP magnitude to day 1 VEP magnitude in KD and control group. Wilcoxon signed rank between groups: $p = 0.04$. **(I)** VEP magnitude response to familiar and novel, Wilcoxon signed-rank test fam vs. nov for KD group: $p = 0.2$, for WT group: $p = 0.003$ (n = 11). FDR correction for multiple comparisons. Asterisks throughout denote significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) while ns denotes non-significant.

on this data which elucidated further phenotypes which are discussed below.

RESULTS

Habituation Can Be Observed Within and Across Days in the Same Animal

Visual stimuli were presented over multiple timescales to awake head-fixed mice within a longitudinal experimental design. This approach allowed for investigation into the change in neocortical activity across these different timescales as visual-evoked behavior was concomitantly monitored. Awake mice were head-fixed and viewed full field, oriented, 0.05 cycles/degree, 100% contrast, phase-reversing, sinusoidal grating stimuli while concurrently recording layer 4 local fields potentials (LFPs) with chronically implanted tungsten microelectrodes and behavior using a piezoelectric sensor (**Figure 1A**). After a 5-min period of gray screen (equivalent luminance to the grating stimuli to follow) to settle the animal into head-fixation, a stimulus of one fixed orientation (X°) was presented at a temporal frequency of 2 Hz for 200 phase reversals, resulting in ∼100 s of continuous stimulus presentation (we describe this as a stimulus block throughout). This block was repeated 5 times with 30-s-long gray screen intervals separating them. Overall, this session lasted approximately 15 min (5 min of gray followed by ∼10 min of stimulus blocks and intervening gray). These sessions, each containing 5 separated blocks, were then repeated once each over 6 days. On the 7th day, 5 blocks of the original orientation (X°) were presented pseudo-randomly interleaved with a novel orientation (X+90°), such that no more than 2 blocks of one orientation were presented in sequence (**Figure 1B**). This experimental design allowed for analysis of habituation and cortical plasticity across days and within a day.

We found that behavioral habituation occurred both within a day and across days. After the onset of a block of visual stimuli, animals produce a pronounced behavioral response, which we measured using a piezoelectric device and previously termed a vidget (Cooke et al., 2015). Using the vidget, we were able to observe behavioral habituation within a single recording session on day 1 (n = 30), when the X° stimulus was novel. The vidget magnitude dropped considerably by the second block and remained low (**Figure 1C**; Friedman test: p = 0.008, Wilcoxon signed-rank on B1–B2: p = 0.02, B1–B3: p = 0.7, B1–B4: p = 0.04, B1–B5: p = 0.5; FDR correction for multiple comparisons), indicating the occurrence of short-term habituation on day 1. When averaged over all 5 blocks, the overall magnitude of vidgets was greater on day 1 than on the following days (**Figure 1D**; Friedman test: p = 0.3; Wilcoxon signed-rank on days 1–2: p = 0.05, days 1–3: p = 0.05, days 1–4: p = 0.2, days 1–5: p = 0.09, days 1–6: p = 0.05; FDR correction for multiple comparisons), indicating the occurrence of long-term habituation. During presentation of blocks of a novel stimulus (X+90°), interleaved with the familiar X° stimulus on the final day, vidgets were increased in magnitude for the novel compared to the familiar stimulus (**Figure 1E**; Wilcoxon signed-rank test: p<0.001), just as we have described previously (Cooke et al., 2015; Kaplan et al., 2016; Fong et al., 2020).

V1 Plasticity Accompanying Long- and Short-Term Habituation Occurs in Opposing Directions

Phase-locked LFP responses from layer 4 were averaged together to assess changes in visual-evoked potential (VEP) magnitude within a day and across days (n = 33). We found that the changes in VEP magnitude occurred in differing directions dependent upon the timescale. A very clear decrement in VEP magnitude was apparent over the course of 5 blocks of stimulus presentation (∼10 min) within day 1 (**Figures 1F,G**; Friedman test across blocks on day 1; p = 0.01), following the trend of behavioral habituation. This effect became more pronounced after the first day of stimulus presentation (**Figure 1F**; Friedman test: day 1; p = 0.01, day 2–6 p < 0.001; FDR multiple comparisons corrected). In contrast, across days there was significant potentiation of VEP magnitude (**Figure 1H**; Friedman test: p < 0.001) and this potentiation was orientation specific, because VEP magnitude was reduced to baseline in response to the novel orientation (**Figure 1I**; Wilcoxon signed-rank test: p < 0.001). Thus, SRP is also present in these animals, just as described previously (Frenkel et al., 2006; Cooke and Bear, 2010). Importantly, a response decrement accompanies short-term habituation, while response potentiation accompanies long-term habituation in the same animals.

Short-Term Adaptation Occurs Within a Stimulus Block

Next, we wanted to determine whether even shorter timescales of plasticity could be identified within the same experiments, this time focusing on plasticity across a single stimulus block. We averaged VEP magnitude for each of the 200 phase reversals within a block across all 5 blocks on day 1 and across animals (n = 33). Over the course of 200 phase reversals (∼100 s) we observed a reduction in the VEP magnitude (**Figure 2A**). Most notably, there was an immediate reduction from phase 1 to phase 2 (**Figures 2A,B**; Wilcoxon signed-rank on phase 1–2: p < 0.001), followed by a striking rebound over the next few phase reversals. A steadier reduction in VEP magnitude was observed across all 200 phase reversals, culminating in a significant difference between phase reversal 1 and phase reversal 200 (**Figures 2A,C**; Wilcoxon signed-rank on phase 1–200: p = 0.001). Thus, clear evidence is apparent of adaptation within a stimulus block, indicating at least one, and perhaps two additional potential timescales of plasticity to be investigated.

Short-Term Adaptation Is Modulated by Stimulus Familiarity

Short-term adaptation occurred from both the first to the second and the first to the last phase reversal in a stimulus block when a stimulus was relatively novel on day 1, but did that plasticity persist for highly familiar stimuli? By assessing averaged within-block adaptation over the course of 6 days of long-term observation, we found that adaptation from the first to the second

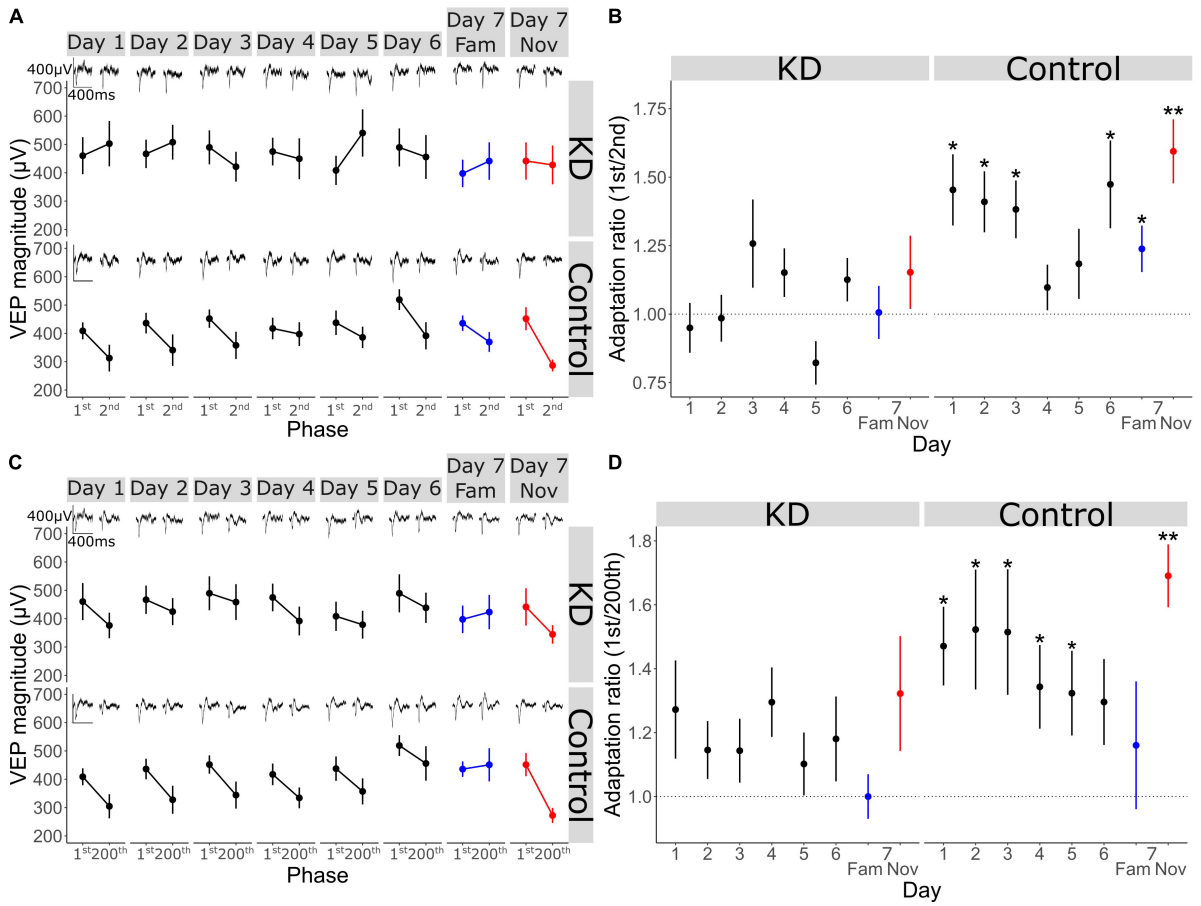

FIGURE 4 | V1 adaptation requires NMDA receptors in V1 across short timescales. **(A)** VEP magnitude in response to the 1st and 2nd phase reversal in GluN1 KD and WT group across all days (n = 11) **(B)** adaptation ratio (1st/2nd) across days. Wilcoxon signed-rank test on AR (μ = 1) in KD group on day 1: p = 0.6, day 2: p = 0.9, day 3: p = 0.5, day 4: p = 0.4, day 5: p = 0.4, day 6: p = 0.4, day 7 fam: p = 1, day 7 nov: p = 0.6. Wilcoxon signed-rank test on AR (μ = 1) in WT group on day 1: p = 0.02, day 2: p = 0.01, day 3: p = 0.02, day 4: p = 0.2, day 5: p = 0.3, day 6: p = 0.03, day 7 fam: p = 0.03, day 7 nov: p = 0.008. FDR correction for multiple comparisons. **(C)** VEP magnitude in response to the 1st and 200th phase reversal in KD and WT group across all days. **(D)** Adaptation ratio (1st/200th) across days. Wilcoxon signed-rank test on AR (μ = 1) in KD group on day 1: p = 0.3, day 2: p = 0.3, day 3: p = 0.3, day 4: p = 0.3, day 5: p = 0.5, day 6: p = 0.5, day 7 fam: p = 0.5, day 7 nov: p = 0.4. Wilcoxon signed-rank test on AR (μ = 1) in WT group on day 1: p = 0.02, day 2: p = 0.02, day 3: p = 0.02, day 4: p = 0.02, day 5: p = 0.05, day 6: p = 0.08, day 7 fam: p = 0.8, day 7 nov: p = 0.008. FDR correction for multiple comparisons. Asterisks throughout denote significance ($^*p < 0.05$, $^{**}p < 0.01$).

phase reversal was gradually reduced over days (**Figure 2D**; Wilcoxon signed-rank test on phase 1 vs. 2 on day 1: $p < 0.001$, day 2: $p < 0.001$, day 3: $p = 0.002$, day 4: $p = 0.008$, day 5: $p = 0.05$, day 6: $p = 0.04$; FDR correction for multiple comparisons). Although this adaptation from the first to the second phase reversal lessened as the stimulus became familiar over days, significant adaptation remained and the adaptation ratio (AR) (1st/2nd) was always significantly above 1 (**Figure 2E**; one sample Wilcoxon signed-rank test on AR (μ = 1) on day 1: $p < 0.001$, day 2: $p < 0.001$, day 3: $p < 0.001$, day 4: $p < 0.001$, day 5: $p < 0.001$, day 6: $p = 0.002$; FDR correction for multiple comparisons). On day 7, there was greater adaptation for the novel stimulus than for the familiar orientation in pseudo-randomly interleaved blocks (**Figure 2F**; Wilcoxon signed-rank test on phase 1 vs. 2 on day 7 fam: $p = 0.02$, day 7 nov: $p < 0.001$; FDR correction for multiple comparisons) and the AR (1st/2nd) for the familiar stimulus was significantly reduced compared to that in response to the novel stimulus (**Figure 2G**; Wilcoxon signed rank day 7 fam AR vs. day 7 nov AR: $p = 0.009$) suggesting modulation of adaptation from the 1st to 2nd phase reversal by long-term familiarity.

A more pronounced modulation of adaptation by long-term familiarity was observed for adaptation from the first to the last phase reversal. Adaptation from phase reversal 1 to 200 was no longer significant by day 4 and thereafter (**Figure 2H**; Wilcoxon signed-rank phase 1 vs. 200 on day 1: $p = 0.008$, day 2: $p = 0.04$, day 3: $p < 0.001$, day 4: $p = 1$, day 5: $p = 0.4$, day 6: $p = 1$; FDR correction for multiple comparisons). In this case, the adaptation ratio (1st/200th) became statistically indistinguishable from 1 by day 4 for the familiar orientation [**Figure 2I**; one sample Wilcoxon signed-rank test on AR (μ = 1) on day 1: $p < 0.001$, day 2: $p = 0.001$, day 3: $p < 0.001$, day 4: $p = 0.1$, day 5: $p = 0.006$, day 6: $p = 1$; FDR correction for multiple comparisons]. The adaptation

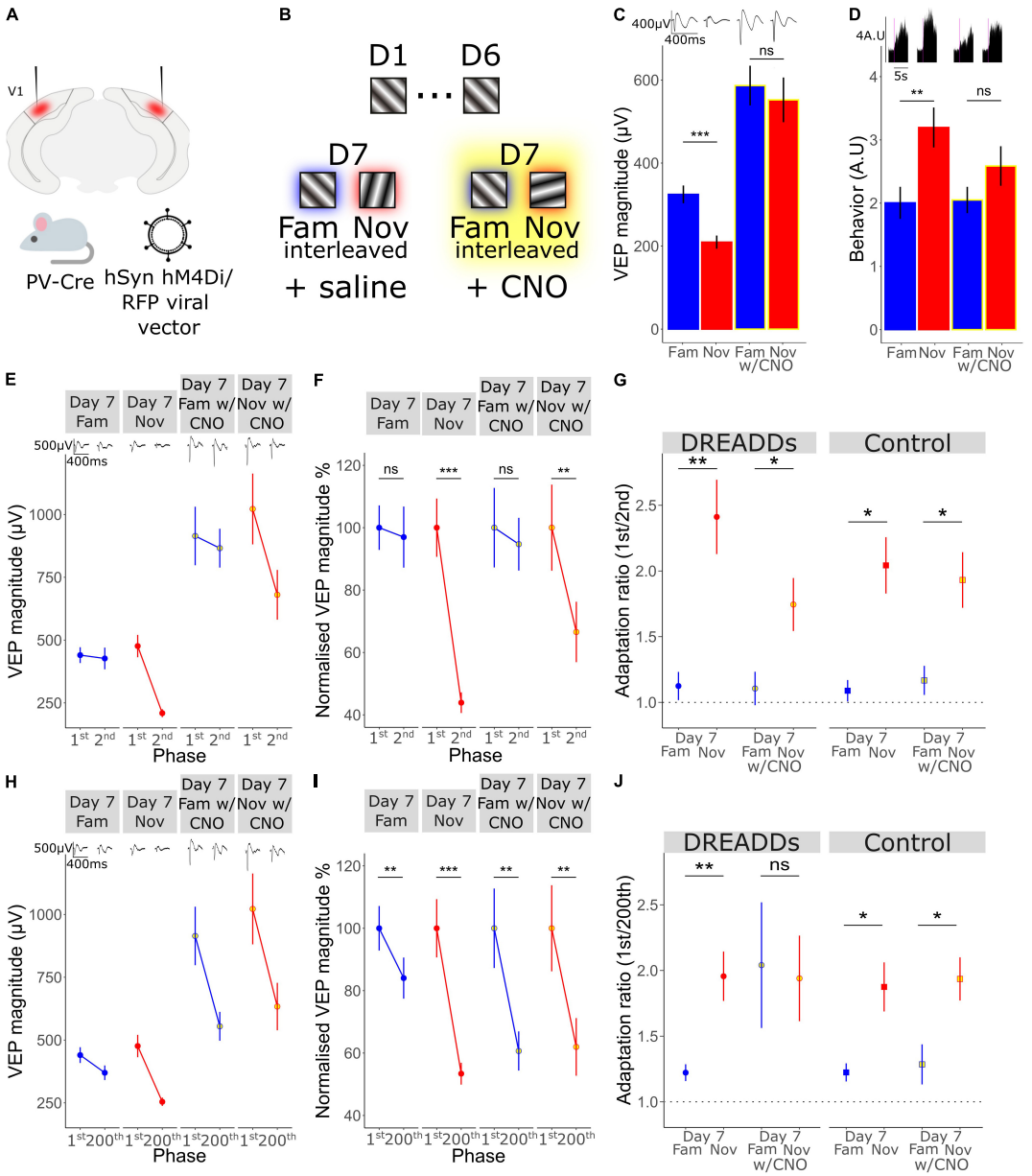

FIGURE 5 | A key role for the activity of Parvalbumin-expressing inhibitory interneurons in long-term familiarity exposes a mechanistic difference between timescales of adaptation. **(A)** Schematic of the experimental set-up in which hM4Di was selectively expressed in parvalbumin-expressing (PV) inhibitory neurons of V1 using an AAV viral vector in PV-Cre mice. **(B)** Schematic of visual presentation protocol in which all mice underwent a standard 6-day SRP protocol before testing response to familiar and novel stimuli during systemic saline injection or CNO application, which were administered prior to presentation of familiar and novel stimuli. **(C)** VEP magnitude in response to familiar and novel stimuli with and without CNO-induced PV+ neuronal inactivation. Wilcoxon signed rank day 7 fam vs. nov: $p < 0.001$. Wilcoxon signed rank day 7 fam vs. nov with CNO: $p = 0.09$. **(D)** Behavioral change in response to familiar and novel stimuli with and without CNO. Wilcoxon signed rank day 7 fam vs. nov: $p = 0.02$. Wilcoxon signed rank day 7 fam vs. nov with CNO: $p = 0.2$. **(E)** VEP magnitude in response to the 1st and the 2nd phase reversal for familiar and novel stimuli with and without CNO. **(F)** VEP magnitude to the 1st and the 2nd phase reversal normalized to the 1st phase reversal in response to familiar and novel stimuli with and without CNO. Wilcoxon signed rank phase 1 vs. 2 on day 7 fam ($n = 14$): $p = 0.5$, day 7 nov: $p < 0.001$, day 7 fam w/CNO: $p = 0.9$, day 7 nov w/CNO: $p = 0.004$. **(G)** Adaptation ratio (1st/2nd) in response to familiar and novel stimuli with and without CNO. Wilcoxon signed rank ($n = 14$): day 7 fam AR vs. day 7 nov AR in DREAADs group: $p = 0.007$; day 7 fam w/CNO AR vs. day 7 nov AR w/CNO in DREAADs group: $p = 0.02$. Wilcoxon signed rank ($n = 7$): day 7 fam AR vs. day 7 nov AR in WT group: $p = 0.03$; day 7 fam w/CNO AR vs. day 7 nov AR w/CNO in WT group: $p = 0.02$. FDR correction for multiple comparisons. **(H)** VEP magnitude in response to the 1st and the 200th phase reversal in response to familiar and novel stimuli with and without CNO. **(I)** VEP magnitude to the 1st and the 200th phase reversal normalized to the 1st phase reversal in response to familiar and novel stimuli with and without CNO. Wilcoxon signed rank phase 1 vs. 200 on day 7 fam: $p = 0.005$, day 7 nov ($n = 14$): $p < 0.001$, day 7 fam w/CNO: $p = 0.005$, day 7 nov w/CNO: $p = 0.003$. **(J)** Adaptation ratio (1st/200th) in response to familiar and novel stimuli with and without CNO. Wilcoxon signed rank day 7 fam AR vs. day 7 nov AR in DREAADs group ($n = 14$): $p = 0.007$; day 7 fam w/CNO AR vs. day 7 nov AR w/CNO in DREAADs group: $p = 0.8$. Wilcoxon signed rank ($n = 7$); day 7 fam AR vs. day 7 nov AR in WT group: $p = 0.03$; day 7 fam w/CNO AR vs. day 7 nov AR w/CNO in WT group: $p = 0.04$. FDR correction for multiple comparisons. Asterisks throughout denote significance ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) while ns denotes non-significant.

from reversal 1 to 200 only returned when a novel orientation was presented on the final day (**Figure 2J**; Wilcoxon signed-rank phase 1 vs. 200 on day 7 fam: $p = 1$, day 7 nov: $p < 0.001$; FDR correction for multiple comparisons). The AR (1st/200th) for the familiar stimulus was significantly different to that in response to the novel stimulus (**Figure 2K**; Wilcoxon signed rank day 7 fam AR vs. day 7 nov AR: $p < 0.001$) showing that adaptation from the 1st to 200th phase reversal is strongly modulated by long-term familiarity.

Both Short-Term and Long-Term Habituation Require NMDA Receptors in V1

Given the critical role of NMDA receptors (NMDAR) in a wide range of plasticity, and a known requirement in SRP and long-term habituation (Frenkel et al., 2006; Cooke et al., 2015), we sought to investigate habituation and accompanying plasticity over shorter timescales after local NMDAR knock-down in V1. Knock-down of NMDAR was achieved by expressing CRE recombinase *via* AAV viral vector injection bilaterally into V1 in a GluN1-floxed (GRIN1 fl/fl) mouse line (**Figure 3A**), thus knocking down expression of this mandatory subunit for NMDAR only within V1 ($n = 11$ mice). In the control condition, GRIN1 fl/fl littermates were injected with a comparable vector, sharing serotype, promoter and fluorophore, that lacked CRE recombinase ($n = 11$). As we have shown (**Figure 1**), behavioral habituation occurs both across days and within a day from block 1 to block 5. We found that loss of NMDARs from V1 affects both timescales. Behavioral activity usually drops from the first block to the second and remains low (**Figure 1**), and we found that to also be true in the WT littermate control mice (**Figure 3B**; Friedman test for block 1–5: $p = 0.003$, Wilcoxon signed-rank test in WT group B1–B2: $p = 0.1$, B1–B3: $p = 0.02$, B1–B4: $p = 0.02$, B1–B5: $p = 0.02$; FDR correction for multiple comparisons). However, knock-down of NMDARs in V1 prevents the reduction in behavior across blocks (**Figure 3B**; Friedman test for block 1–5: $p = 0.3$, Wilcoxon signed-rank in KD group B1–B2: $p = 0.8$, B1–B3: $p = 0.5$, B1–B4: $p = 0.2$, B1–B5: $p = 0.1$; FDR correction for multiple comparisons). As we reported previously (Cooke et al., 2015), behavioral habituation from day 1 to day 6 is absent in the KD group (**Figure 3C**; Friedman test in KD group: $p = 0.3$, in WT group: $p = 0.001$; FDR correction for multiple comparisons). On day 7 there was no difference in the behavioral response between the novel and familiar stimulus in the KD group, whereas in the WT group behavioral activity was higher in response to the novel stimulus (**Figure 3D**; Wilcoxon signed-rank fam vs. nov in KD: $p = 0.2$, in WT: $p = 0.009$).

Bidirectional Plasticity Occurring in V1 During Short- and Long-Term Habituation Require NMDA Receptors in V1

Within the same dataset, we now assessed the within-day VEP magnitude reduction that accompanies within-day habituation. The reduction in VEP magnitude across 5 blocks was modest in this dataset and was less apparent in these subjects than in the subjects described in **Figure 1** (**Figure 3E**). Nevertheless, by averaging the block-to-block VEP magnitudes observed during short-term habituation across days, a significant within-day VEP suppression was observed in the GRIN fl/fl littermate control animals (**Figure 3F**; $n = 11$; Friedman test in control group: $p = 0.03$; FDR correction for multiple comparisons). In contrast, this significant VEP decrement was not observed in the NMDAR KD mice (**Figure 3F**; $n = 11$; Friedman test in KD group: $p = 0.9$, FDR correction for multiple comparisons), indicating that the within-day reduction in VEP magnitude accompanying short-term habituation requires NMDAR, just as with the habituation itself. As previously reported (Cooke et al., 2015), VEP magnitude potentiation from day 1 to 6, or SRP, is reduced in the knock-down (KD) group compared to control (**Figure 3G**; $n = 11$; Friedman test in KD group: $p = 0.008$, WT group: $p < 0.001$; FDR correction for multiple comparisons). Comparing the ratio of day 6–1 in the control and KD group shows a significant reduction in this plasticity over days after NMDAR KD (**Figure 3H**; Wilcoxon signed rank between control and KD day 6/day 1 ratio: $p = 0.04$). On day 7, there was no difference in VEP magnitude between the familiar and novel orientation in the KD group, whereas the VEP magnitude to the novel stimulus in the control group was significantly different (**Figure 3I**; $n = 11$; Wilcoxon signed-rank fam vs. nov in KD: $p = 0.2$, control: $p = 0.003$; FDR correction for multiple comparisons).

V1 Adaptation Requires NMDA Receptors in V1 Across Short and Longer Timescales

As we have shown above, short-term adaptation within our paradigm ordinarily occurs from both the 1st to the 2nd phase reversal and the 1st to the 200th phase reversal but disappears as the stimulus becomes familiar (**Figure 2**). Within the GRIN1 fl/fl dataset, this adaptation was similarly present in the GRIN1 fl/fl controls on day 1 and the subsequent 2 days, eventually becoming non-significant by day 4 and thereafter for highly familiar stimuli [**Figures 4A,B**; one sample Wilcoxon signed-rank test on AR (1st/2nd) ($\mu = 1$) control group on day 1: $p = 0.02$, day 2: $p = 0.01$, day 3: $p = 0.02$, day 4: $p = 0.2$, day 5: $p = 0.3$, day 6: $p = 0.03$; FDR correction for multiple comparisons]. However, after knock-down of NMDAR in V1, adaptation from the 1st to the 2nd phase reversal was absent on day 1 and all subsequent days [**Figures 4A,B**; one sample Wilcoxon signed-rank test on AR (1st/2nd) ($\mu = 1$) KD group on day 1: $p = 0.6$, day 2: $p = 0.9$, day 3: $p = 0.5$, day 4: $p = 0.4$, day 5: $p = 0.4$, day 6: $p = 0.4$]. When blocks of stimuli for familiar and novel orientations were presented pseudo-randomly interleaved on day 7, this 1st/2nd reversal adaptation was reduced for familiar but not novel stimuli in the control mice [**Figures 4A,B**; one sample Wilcoxon signed-rank test on AR (1st/2nd) ($\mu = 1$) on day 7 fam: $p = 0.03$, day 7 nov: $p = 0.008$; FDR correction for multiple comparisons], but not present for either stimulus in the NMDAR KD mice [**Figures 4A,B**; one sample Wilcoxon signed-rank test on AR (1st/2nd) ($\mu = 1$) KD group on day 7 fam: $p = 1$, day 7 nov: $p = 0.6$]. The same phenotype was present when investigating adaptation from the 1st to the 200th phase reversal. Loss of

NMDARs prevented any short-term adaptation expression across all days and stimulus type [**Figures 4C,D**; one sample Wilcoxon signed-rank test on AR (1st/200th) ($\mu = 1$) KD group on day 1: $p = 0.3$, day 2: $p = 0.3$, day 3: $p = 0.3$, day 4: $p = 0.3$, day 5: $p = 0.5$, day 6: $p = 0.5$, day 7 fam: $p = 0.5$, day 7 nov: $p = 0.4$; FDR correction for multiple comparisons], while it remained present in the control mice over the first 5 days of stimulus presentation, and re-emerged to a novel stimulus on day 7 [**Figures 4C,D**; one sample Wilcoxon signed-rank test on AR (1st/200th) ($\mu = 1$) control group on day 1: $p = 0.02$, day 2: $p = 0.02$, day 3: $p = 0.02$, day 4: $p = 0.02$, day 5: $p = 0.04$, day 6: $p = 0.08$, day 7 fam: $p = 0.8$, day 7 nov: $p = 0.008$; FDR correction for multiple comparisons]. Thus, short-term adaptation of VEP magnitude in V1 requires the presence of functional NMDAR.

A Key Role for the Activity of Parvalbumin-Expressing Interneurons in Long-Term Familiarity Exposes a Mechanistic Difference Between Timescales of Adaptation

Previously, we have shown that parvalbumin-expressing (PV+) inhibitory neurons in V1 are critical for the expression of long-term familiarity. We inactivated these neurons using a cell type-specific chemo-genetic approach in which the hM4Di DREADDS receptor was expressed in PV+ neurons of V1, disrupting SRP expression (Kaplan et al., 2016). Therefore, we decided to assess whether these PV+ neurons in V1 are required for the modulation of adaptation by long-term familiarity that we have described in the current study (**Figure 2**). Bilateral injection of an AAV viral vector into V1 of a PV-Cre mouse to express hM4Di in these cells (**Figure 5A**) enabled subsequent inactivation of V1 PV+ interneurons after SRP and long-term habituation had been established over 6 days. Specifically, on day 7, familiar (X°) and novel (X+60°) orientations were pseudo-randomly interleaved in a standard design to test for selective SRP/habituation to the familiar orientation. After this, mice were systemically injected (i.p.) with clozapine-n-oxide (CNO), which binds to hM4Di to inactivate expressing neurons, before re-testing response to blocks of the familiar and a new novel stimulus (X-60°) to assess modulation of adaptation by long-term familiarity (**Figure 5B**). Prior to inactivation of PV+ neurons, VEP magnitude was significantly potentiated in response to the familiar stimulus and therefore significantly greater in magnitude than response to the novel stimulus (**Figure 5C**; Wilcoxon signed-rank day 7 fam vs. nov: $p < 0.001$; FDR correction for multiple comparisons). However, as we have reported previously (Kaplan et al., 2016), after inactivation of PV+ interneurons, there was no significant difference in VEP magnitude in response to familiar and novel stimuli (**Figure 5C**; Wilcoxon signed-rank day 7 fam vs. nov w/CNO: $p = 0.09$; FDR correction for multiple comparisons). It is important to note that after inactivation of PV+ interneurons, the general VEP magnitude was higher due to the loss of inhibition in the cortex. The inactivation of V1 PV+ inhibitory neurons also impaired behaviorally manifest novelty detection as the behavioral response to a novel stimulus was significantly greater than the response to the familiar stimulus before inactivation of PV+ neurons (**Figure 5D**; Wilcoxon signed-rank day 7 fam vs. nov: $p = 0.02$; FDR correction for multiple comparisons), but was suppressed after inactivation of these neurons and no longer different during PV+ inactivation (**Figure 5D**; Wilcoxon signed-rank day 7 fam vs. nov w/CNO: $p = 0.2$; FDR correction for multiple comparisons).

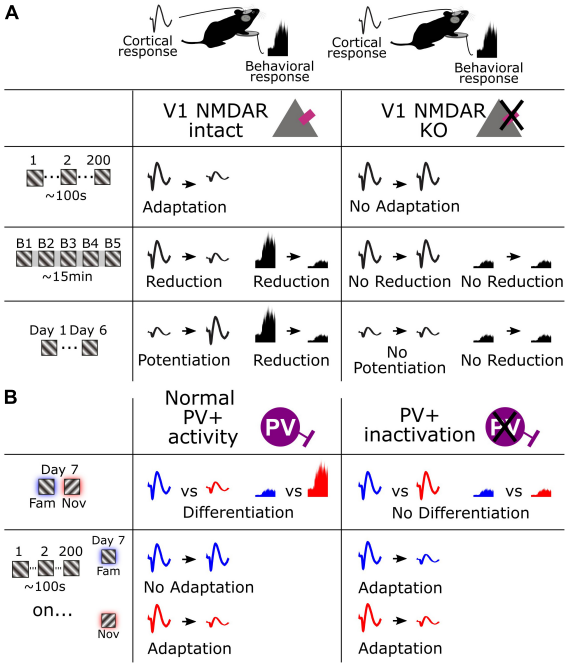

FIGURE 6 | Schematic summarizing fundamental cortical and behavioral changes across multiple timescales. **(A)** Cortical and behavioral changes over seconds, minutes, and days (left), and the result of V1 NMDAR KO on these changes. **(B)** Cortical and behavioral changes in response to a familiar and novel stimulus and the associated adaptation (left), and the result of PV+ interneuron inactivation on these changes.

As we have shown in the current study, short-term adaptation from the first to the second phase reversal progressively reduces as the stimulus becomes familiar and is selectively suppressed on day 7 to highly familiar stimuli, but not novel stimuli (**Figure 2**). Here we show that, although VEP magnitude generally increases, inactivation of PV+ interneurons had no effect on the modulation of 1st/2nd phase reversal short-term adaptation (**Figures 5E–G**). Strong adaptation from the first to the second phase reversal was absent when the stimulus was familiar and present when the stimulus was novel, regardless of whether PV+ neurons were inactivated. This observation is most clear when we normalize to the magnitude of the first phase reversal in order to remove the confound of increased overall response after PV+ inactivation [**Figure 5F**; normalized to the first phase reversal; Wilcoxon signed rank phase 1 vs. 2 on day 7 fam: $p = 0.5$, day 7 nov: $p < 0.001$, day 7 fam w/CNO: $p = 0.9$, day 7 nov w/CNO: $p = 0.004$ ($n = 14$)]. The adaptation ratio (1st/2nd) was significantly different between the familiar and the novel stimulus both before and after PV+ neuronal inactivation [**Figure 5G**; Wilcoxon signed rank on day 7 fam AR vs. day 7 nov AR: $p = 0.007$, Wilcoxon signed rank on day 7 fam w/CNO AR vs.

day 7 nov w/CNO AR: $p = 0.02$ ($n = 14$)]. Thus, inactivation of PV+ interneurons does not affect the short-term adaptation from the 1st to the 2nd phase reversal, nor its suppression by long-term familiarity.

Strikingly, the adaptation from the first to the last phase reversal of a stimulus block follows a different pattern. While adaptation is suppressed by familiarity on day 7 but present for the novel stimulus before PV+ neuronal inactivation (**Figures 5H–J**), it is strongly apparent for both familiar and novel stimuli during PV+ neuronal inactivation [**Figure 5I**; normalized to the first phase reversal; Wilcoxon signed rank phase 1 vs. 200 on day 7 fam: $p = 0.005$, day 7 nov: $p < 0.001$, day 7 fam w/CNO: $p = 0.005$, day 7 nov w/CNO: $p = 0.003$ ($n = 14$)]. The adaptation ratio (1st/200th) is significantly different for familiar and novel stimuli before PV+ inactivation [**Figure 5J**; Wilcoxon signed rank on day 7 fam AR vs. day 7 nov AR: $p = 0.007$ ($n = 14$)]. After application of CNO the AR is equivalent for both the familiar and novel stimuli (**Figure 5J**; Wilcoxon signed rank on day 7 fam w/CNO AR vs. day 7 nov w/CNO AR: $p = 0.8$). Therefore, the modulation of the short-term adaptation from the 1st/200th phase reversal by familiarity is not present after inactivation of PV+ interneurons, which differs from the effect on adaptation from the 1st/2nd phase reversal, indicating two mechanistically distinct processes.

DISCUSSION

In the current study we have identified multiple timescales of visual response adaptation that occur during habituation in mice. We have expanded on our previous characterization of stimulus-selective response potentiation (SRP), a form of long-term cortical response potentiation that occurs concomitantly with long-term habituation, to reveal that the reverse effect of response decrement coincides with short-term habituation. Moreover, we have identified shorter-term forms of adaptation that occur over seconds. We also reveal that the NMDA receptor serves as a key molecular mechanism shared by all these forms of plasticity (**Figure 6A**). In addition, we show that these various forms of plasticity are not isolated phenomena, because short-term adaptation and SRP over days clearly interact, such that adaptation no longer occurs for highly familiar stimuli. We also demonstrate that this suppression of adaptation across hundreds of stimuli by long-term familiarity is gated by the activity of PV+ inhibitory interneurons in V1 because inactivating these neurons causes short-term adaptation to re-emerge to highly familiar stimuli (**Figure 6B**). Finally, we make the important observation that the fastest form of adaptation that we have measured, occurring within a second of stimulus presentation, remains suppressed for familiar stimuli even after inactivation of PV+ interneurons, indicating that there may be at least two mechanistically separable timescales of adaptation present within our paradigm. Thus, we have revealed a multitude of forms of cortical plasticity that can be assessed in passively viewing mice to gain a deeper understanding of the processes of habituation.

The longest-term form of plasticity we have described here is already well characterized: potentiation of the VEP in layer 4 over days is described as SRP due to its high degree of stimulus-selectivity (Frenkel et al., 2006; Cooke and Bear, 2010) and it occurs concurrently with long-term behavioral habituation (Cooke et al., 2015; Kaplan et al., 2016; Fong et al., 2020; Finnie et al., 2021), just as we further confirm here. Despite the clear reliance of SRP and accompanying habituation on V1 NMDA receptors, selective knock-down of NMDARs in excitatory neurons of layer 4, the locus where SRP is manifest, does not impair SRP or accompanying habituation (Fong et al., 2020). This observation indicates that the potentiation is an echo of plasticity occurring elsewhere in V1, or in a different cell type within layer 4. Therefore, the direct strengthening of synapses at thalamocortical inputs to layer 4 now seems an unlikely explanation for SRP. Although local field potentials are thought to primarily report synaptic activity rather than action potentials (Katzner et al., 2009; Buzsáki et al., 2012), potentiation of VEP magnitude may reflect a loss of shunting inhibition that allows an increased synaptic response to thalamic input, rather than a potentiation of the synaptic input itself. We have previously shown that parvalbumin-expressing (PV+) inhibitory interneurons, which provide this powerful shunting inhibition, show reduced activity over days as the stimulus becomes familiar during SRP (Hayden et al., 2021). In addition, cell-specific interventional approaches reveal that a normal range of activity in PV+ neurons is required for differential response to familiar or novel stimuli after SRP, either cortically or behaviorally (Kaplan et al., 2016). Thus, it seems likely that SRP reflects a loss of PV+ inhibition. How this contributes to a decrement in behavior, as is observed in the concomitant long-term habituation, remains unclear (Montgomery et al., 2021). One possible arrangement is that increased cortical output recruits another form of inhibition to suppress behavioral output. This arrangement would accord with the comparator model of habituation, in which long-lasting memory is formed in the cortex through elevated synaptic activity that enables recognition of familiarity and suppresses output through feedforward inhibition, as suggested by Sokolov (1963) and others (Konorski, 1967; Wagner, 1981). To confirm that SRP conforms to this model will require measurement of V1 output from the deeper layers of neocortex, with the prediction that this activity is suppressed by superficial layers as they exhibit potentiation. It will also be critical to identify the inhibitory intermediary that leads to this cortical output. One strong candidate for this inhibitory suppression has recently emerged (Pluta et al., 2019).

The behavioral response decrement over the course of minutes, reflecting habituation over an intermediate time-scale, has been investigated by others (Sanderson and Bannerman, 2011). The reduction in VEP magnitude that coincides with this within session habituation has not formally been described by us previously. Our observations of a decrement in VEP magnitude are notable because of the striking contrast with SRP, which coincides with a similar reduction in behavior in the same animals, but in that case over days (**Figure 1**). Visual cortical activity decreases during repetitive presentation of natural movies (Deitch et al., 2021), suggesting that this reduced activity can occur in response to multiple different types of visual stimuli, and the well-documented phenomenon

of mis-match negativity, in which novel oddball stimuli evoke increased magnitudes of event-related potentials (ERP) relative to repetitions of increasingly familiar stimuli, occurs across similar timescales (Näätänen et al., 2007; Garrido et al., 2009). In a similar paradigm to ours, thalamic activity has been observed to increase over ∼30 min (Durkin and Aton, 2019), and it remains possible that the plasticity they have observed is, through some unidentified inversion, the origin of cortical decrement and behavioral habituation. However, the reliance of both VEP decrement and concomitant habituation on NMDARs within V1 strongly suggests that this is not the case (**Figure 3**). Dual recordings of thalamic and cortical neurons may be required to resolve the origins of these effects, and targeted interventions in the thalamus may also prove informative. Investigation of changes over the course of minutes in response to both a familiar and novel grating (currently not possible due to the interleaving of these stimuli) would elucidate if this reduction of cortical activity is indiscriminate to the type of visual stimulus being shown or is also orientation specific, indicating cortical plasticity that is potentially very similar to the familiarity effect observed leading up to mismatch negativity. Recent work has shown that mismatch negativity depends upon activity of the somatostatin-expressing (SST+) inhibitory interneurons (Hamm and Yuste, 2016), suggesting that modification of SST+ inhibition may account for our observations. This class of interneurons primarily target dendrites of excitatory cells and PV+ interneurons (Cottam et al., 2013; Pfeffer et al., 2013; Xu et al., 2013; Rikhye et al., 2021) and they have been shown to be strongly influenced by stimulus familiarity (Kato et al., 2015; Makino and Komiyama, 2015; Hayden et al., 2021). Inhibition on the dendrites of excitatory neurons, where the majority of synaptic contacts are made, may contribute to reduced synaptic activity during habituation (Natan et al., 2015), or these cells may influence the activity of PV+ neurons to mediate the reduction in V1 response, as they are known to do in layer 4 (Xu et al., 2013). It would be informative to measure the activity of these inhibitory neurons in layer 4 of V1 across this timescale and more informative still to monitor inhibitory responses in principle excitatory neurons during this within-session habituation. Given the dependency of the phenomenon that we have described on NMDARs, one intriguing hypothesis is that excitatory synapses onto SST+ neurons are potentiated during repeated stimulus presentation. Knocking down the NMDAR expression within these cells would test this hypothesis. It also remains possible that other types of inhibition are increasingly engaged to produce habituation, as has recently been hypothesized (Ramaswami, 2014). In line with the NMDAR dependence of the reduced behavioral responses, again, this process may involve synaptic depression of excitatory synapses within V1. Much further work is required to investigate the underlying mechanisms of this intermediate form of behavioral and cortical response adaptation.

Over even shorter timescales of seconds, the VEP adaptation that we observe here within continuous blocks of stimulation is a commonly reported phenomenon (Chung et al., 2002; Beierlein et al., 2003; von der Behrens et al., 2009; Cruikshank et al., 2010). The most parsimonious explanation for response decrement is that it reflects a depression of excitatory synapses within the canonical excitatory pathway of V1 through a process of adaptive filtration, which is perhaps the dominant theory of habituation (Horn, 1967; Groves and Thompson, 1970). This depression could potentially occur through Hebbian depression mechanisms (Lee et al., 1998) at excitatory synapses within the cortex (Chen et al., 2015), or the thalamus (Li et al., 2003), or through short-term effects on synaptic release (Moulder and Mennerick, 2006). That the origin of response depression is cortical is supported by its reliance on V1 NMDARs. Specifically, we show that both the adaptation from the 1st to the 2nd phase reversal (0.5 s), and the adaptation from the 1st to 200th phase reversal (100 s) is impaired by a loss of NMDAR expression in V1 (**Figure 3**). This somewhat surprising finding implicates the occurrence of a Hebbian form of plasticity that is at least induced post-synaptically at short timescales (Bliss and Collingridge, 1993). Additionally, we have made the intriguing additional observation that a loss of activity in PV+ neurons after chemo-genetic inactivation re-instates short-term adaptation even to highly familiar stimuli (**Figure 4**). The immediate conclusion from this observation is that short-term adaptation does not rely in any way on inhibition mediated by PV+ neuronal activity, in striking contrast to long-term familiarity. The reinstated short-term adaptation may therefore arise from the cortex responding to a familiar stimulus as if it were novel. Alternatively, it remains possible that the loss of adaptation with long-term familiarity arises from a gradual reduction in PV+ mediated inhibition through the course of a stimulus block that perfectly matches excitatory synaptic depression. Inactivation of PV+ neurons would remove this gradual effect and expose the depression occurring at those excitatory inputs. Using calcium imaging, we have previously observed the gradual loss of PV+ neuronal engagement across phase reversals for familiar but not novel stimuli, so this remains a plausible arrangement (Hayden et al., 2021). Interestingly, using a similar method in excitatory neurons we have also previously reported a perplexing mismatch with the electrophysiological measurements of SRP: when measuring VEP magnitude or peak unit firing rate, a pronounced potentiation is observed (Cooke et al., 2015), while a reduction of signal is observed with calcium imaging (Kim et al., 2020). In the current study we have added to that conundrum, as we reveal short-term adaptation across seconds that is limited to novel stimuli (**Figure 2**), while we previously revealed a similar effect with calcium imaging but limited to familiar stimuli (Kim et al., 2020). The only likely explanation for these curiously mismatched observations is that our electrophysiological methods have detected a fast phasic effect which is potentiated by familiarity over days and diminished to novel stimuli over seconds, while the calcium sensors detect a more sustained diminishment of calcium flux as a result of familiarity over either time-course. Further experiments comparing phasic and drifting gratings or using intracellular electrophysiology may be informative in this regard. It will also be interesting to use calcium imaging to assess the intermediate timescale that we have reported here which occurs from block to block over minutes within a session (**Figure 1**), to determine if the mismatch between the two methods persists even across this timescale. Our prior study indicates that for this timescale,

at least, findings with electrophysiology and calcium imaging will align (Kim et al., 2020).

The storage and retrieval of familiarity plays a major role in reserving energy and attention for only those stimuli that are most pertinent to a task or context and is therefore critical for survival and wellbeing. Understanding how these apparently simple forms of learning and memory are implemented is a greater challenge than expected and there appear to be multiple solutions to the same problem, some of which engage feedforward plasticity, others which engage inhibitory systems and more complicated circuitry. These various mechanisms may all play out within one structure but across different timescales. In this study, we have revealed the measurement of multiple mechanistically distinct forms of plasticity occurring in the same animals across seconds, minutes, and days of repeated stimulus presentation, providing great potential to gain a deep understanding of a foundational set of learning and memory processes. We have monitored these changes using LFP recordings, suggesting that much of the observed phenomenology is likely to translate to non-invasive electroencephalogram (EEG) recordings, providing future potential for translation into human subjects, where forms of plasticity such as mismatch negativity have already been described (Näätänen et al., 2007).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee on Animal Care at the Massachusetts Institute of Technology.

AUTHOR CONTRIBUTIONS

SC acquired all the data and participated in experimental design. FC analyzed the data. FC and SC interpreted the data and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Transient Receptor Potential Vanilloid 1 Function at Central Synapses in Health and Disease

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The transient receptor potential vanilloid 1 (TRPV1), a ligand-gated nonselective cation channel, is well known for mediating heat and pain sensation in the periphery. Increasing evidence suggests that TRPV1 is also expressed at various central synapses, where it plays a role in different types of activity-dependent synaptic changes. Although its precise localizations remain a matter of debate, TRPV1 has been shown to modulate both neurotransmitter release at presynaptic terminals and synaptic efficacy in postsynaptic compartments. In addition to being required in these forms of synaptic plasticity, TRPV1 can also modify the inducibility of other types of plasticity. Here, we highlight current evidence of the potential roles for TRPV1 in regulating synaptic function in various brain regions, with an emphasis on principal mechanisms underlying TRPV1-mediated synaptic plasticity and metaplasticity. Finally, we discuss the putative contributions of TRPV1 in diverse brain disorders in order to expedite the development of next-generation therapeutic treatments.

Keywords: vanilloid receptor, endocannabinoids, synaptic function, brain, neurotransmision

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INTRODUCTION

TRPV1 is a nonselective ligand-gated cation channel predominantly expressed in peripheral endings of small diameter nociceptive primary afferent neurons, where its activation is associated with nociception and pain sensation including thermal hyperalgesia (Caterina et al., 2000; Davis et al., 2000; Brederson et al., 2013). This homotetrameric receptor can be activated by a wide range of stimuli, including heat, changes in pH, exogenous compounds such as capsaicin (Caterina et al., 1997) and endogenous lipid ligands including endovanilloids/endocannabinoids such as anandamide (AEA) (Ross, 2003; Toth et al., 2009). Also, TRPV1 channels are located on the central endings of nociceptive neurons in the spinal cord that make synaptic contact with second order neurons (Caterina et al., 1997; Hwang et al., 2004). Pharmacological activation of presynaptic TRPV1 channels modulates glutamatergic transmission by increasing the frequency of miniature excitatory postsynaptic currents (mEPSCs) and by inhibiting evoked EPSCs (Tognetto et al., 2001; Baccei et al., 2003; Medvedeva et al., 2008).

Although first identified and cloned in peripheral afferent fibers (Caterina et al., 1997), accumulating evidence using diverse experimental approaches indicates that TRPV1 is also expressed in different brain regions including prefrontal cortex, amygdala, brainstem,

hypothalamus, cerebellum and the hippocampal formation (Sasamura et al., 1998; Mezey et al., 2000; Sanchez et al., 2001; Roberts et al., 2004; Toth et al., 2005; Cristino et al., 2006; Starowicz et al., 2008; Kauer and Gibson, 2009; Huang et al., 2014; Kumar et al., 2018). While brain TRPV1 is seemingly expressed at lower levels than the peripheral counterparts (Cavanaugh et al., 2011), its activation has been linked to different forms of synaptic plasticity and metaplasticity. Moreover, TRPV1 likely modulates anxiety, fear and panic responses *in vivo* (Almeida-Santos et al., 2013; Aguiar et al., 2014; Terzian et al., 2014) and also the severity of neurological and cognitive disorders, including epilepsy, depression and Alzheimer's Disease (Chen et al., 2017; Balleza-Tapia et al., 2018; Wang et al., 2019, 2020a; Du et al., 2020). However, the mechanisms by which brain TRPV1 regulates synaptic function and behavior remains far from being completely understood.

PRESYNAPTIC TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 CHANNELS IN THE BRAIN

Capsaicin, the pungent ingredient in hot chili peppers, acts as an exogenous TRPV1 agonist, and it has been shown to enhance transmitter release in the spinal cord (Gamse et al., 1979; Baccei et al., 2003; Kim et al., 2009), supporting the presence of TRPV1 on presynaptic nerve terminals (Tominaga et al., 1998; Cortright et al., 2007). Consistent with this idea, TRPV1 has also been reported at excitatory presynaptic terminals in various brain regions, including the striatum, substantia nigra, hypothalamus, hippocampus, dorsolateral periaqueductal gray and medial prefrontal cortex (Karlsson et al., 2005; Xing and Li, 2007; Musella et al., 2009; Anstotz et al., 2018; Zhang et al., 2020). In these regions, its pharmacological activation facilitated spontaneous excitatory postsynaptic current (EPSC) frequency but not amplitude, consistent with a presynaptic site of action. In the hypothalamus, hippocampus and entorhinal cortex, TRPV1 activation also increased the frequency of spontaneous inhibitory postsynaptic currents (IPSCs; Banke, 2016). These results support a presynaptic localization of TRPV1 channels in glutamatergic and γ-aminobutyric acid (GABA) ergic nerve terminals, whose activation presumably increases intracellular calcium, resulting in enhanced synaptic transmission.

Although seemingly contradictory, TRPV1 activation has also been shown to reduce the release of GABA and glutamate (Gibson et al., 2008; Lu et al., 2017; Zhang et al., 2020). In the hippocampus, TRPV1 mediates a form of long-term depression (LTD) at excitatory inputs onto GABAergic interneurons in the CA1 area of the hippocampus (Gibson et al., 2008). Such synaptic plasticity is dependent on presynaptic TRPV1 that are activated by the postsynaptically produced arachidonic acid metabolite 12-HPETE. At the presynaptic site, TRPV1 opening likely permeates calcium and subsequent activation of calcium-sensitive pathways to persistently depress transmitter release (Jensen and Edwards, 2012; **Figure 1**). It is noteworthy that a similar form of presynaptic LTD, involving a TRPV-like channel, has also been described in invertebrates (Yuan and Burrell, 2010, 2013), suggesting evolutionary conservation of this synaptic regulatory mechanism.

POSTSYNAPTIC TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 CHANNELS IN THE BRAIN

TRPV1 also mediates postsynaptic forms of LTD (Chavez et al., 2010; Grueter et al., 2010; Puente et al., 2011), strongly suggesting that these channels are also present postsynaptically. In the dentate gyrus and nucleus accumbens, repetitive stimulation of glutamatergic afferents triggers TRPV1-mediated LTD of excitatory synaptic efficacy through a mechanism that involves metabotropic glutamate receptor (mGluR) signaling and clathrin-dependent endocytosis of the α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate-type glutamate receptors (AMPARs; Chavez et al., 2010; Grueter et al., 2010). In the nucleus accumbens, mild pharmacological activation of muscarinic M1 receptors can also trigger persistent excitatory depression that requires TRPV1 (Neuhofer et al., 2018). It remains to be determined whether M1 receptors can play a role in activity-dependent TRPV1-mediated LTD. Interestingly, in the extended amygdala, TRPV1-mediated LTD selectively required mGluR5 but not mGluR1 (Puente et al., 2011), suggesting that not all G-protein coupled receptors can equally activate TRPV1 at synapses. Notably, TRPV1 activation also induces clathrin-dependent internalization of type A GABA receptors ($GABA_A$Rs), showing a preference for somatic over dendritic compartments of dentate granule cells (Chavez et al., 2014). Because TRPV1 may be expressed in distinct intracellular compartments (Gallego-Sandin et al., 2009; Dong et al., 2010; Samie and Xu, 2011), it is possible that this synapse-specificity of TRPV1-mediated suppression of inhibition arise from channel expression in specific subcellular domains. Moreover, it remains unclear how TRPV1 can be endogenously activated to modulate GABAergic synaptic plasticity.

Although diverse forms of TRPV1-mediated LTD can be induced in the hippocampus (**Table 1**, i.e., CA1 vs dentate gyrus), TRPV1 channels themselves may be acting in the same way (**Figure 1**). For instance, calcium influx may be triggered by activation of either pre- or postsynaptic TRPV1, resulting in the recruitment of calcium-sensitive enzymes such as calcineurin (also known as protein phosphatase 2B), the predominant calcium/calmodulin-dependent serine/threonine phosphatase that maintains the appropriate phosphorylation status of many ion channels present at presynaptic and postsynaptic sites (Huang et al., 2021). Indeed, several forms of pre and postsynaptic forms of TRPV1-mediated LTD require calcineurin (Gibson et al., 2008; Chavez et al., 2010; Grueter et al., 2010; Yuan and Burrell, 2010, 2013; Jensen and Edwards, 2012). Thus, it is possible that TRPV1 may initiate an important negative feedback via calcineurin in response to increased neuronal activity. Further investigations are required to determine whether calcineurin is the main actor in TRPV1-mediated LTD. The presence of multiple phosphorylation sites in TRPV1 implies possible regulatory actions by different kinases, including calcium

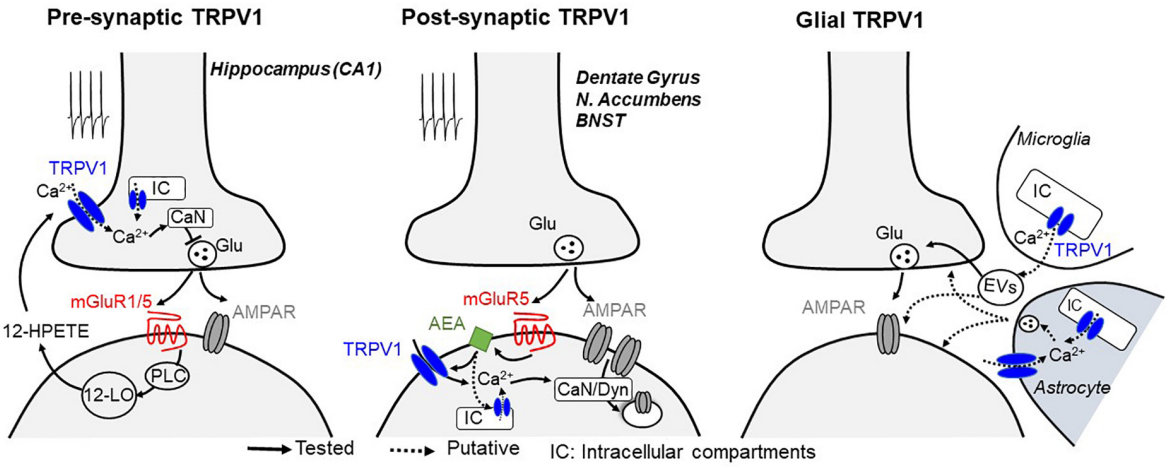

FIGURE 1 | Mechanisms described for TRPV1-mediated plasticity at central synapses. Glutamate release during synaptic stimulation activates Gq-coupled mGluRs, leading to the activation of phospholipase C (PLC) and downstream calcium-regulated enzymes, which likely triggers the biosynthesis of lipids including 12-HPETE (presynaptic TRPV1-LTD, *left*) or AEA (postsynaptic TRPV1-LTD, *middle*), two endocannabinoid/endovanilloid known to activate TRPV1 receptors. At presynaptic terminals, activation of TRPV1 on plasma membrane or intracellular compartments (IC) leads to a calcium (Ca^{2+})-dependent long-term depression of synaptic vesicle release, whereas when located at postsynaptic compartments, TRPV1 activation promotes a long-lasting, clathrin- and dynamin (Dyn)-dependent endocytosis of AMPA receptors. Notably, stimulation of calcium-sensitive phosphatase calcineurin (CaN) resulting from TRPV1 activation is likely to be a common mechanism to promote both pre and postsynaptic long-term changes of synaptic efficacy. TRPV1 channels are also expressed in glial cells (*right*), where its activation can modulate synaptic efficacy presumably through the production and release of inflammatory mediators through microglia-derived extracellular vesicles (EVs) or by Ca^{2+}-dependent release of gliotransmitters from astrocytes.

TABLE 1 | TRPV1-mediated synaptic plasticity in the mammalian brain.

Brain area	Synapse type	Synaptic plasticity	Induction protocol	Expression mechanism	Endogenous agonist	References
Hippocampus	Excitatory inputs to GABAergic interneurons	LTD	HFS	Presynaptic Ca^{2+} Calcineurin	Endovanilloid 12-HPETE	Gibson et al., 2008 Jensen and Edwards, 2012
Dentate gyrus	medial perforant path to dentate granule cells excitatory inputs	LTD	1Hz Paired Protocol	Postsynaptic Ca^{2+} Calcineurin AMPA receptor endocytosis	Anandamide	Chavez et al., 2010
Nucleus accumbens	Excitatory inputs to D2+ neurons	LTD	LFS	Postsynaptic AMPA receptor endocytosis	Anandamide	Grueter et al., 2010
Amygdala, stria terminalis	Excitatory inputs	LTD	LFS	Postsynaptic	Anandamide	Puente et al., 2011
Superior colliculus	Excitatory inputs*	LTD	HFS	Presynaptic?	Endovanilloid 12-HPETE	Maione et al., 2009

*LFS, low frequency stimulation; HFS, high frequency stimulation; LTD, long-term depression. *Transiently in juvenile but not in adult.*

calmodulin, protein kinase A and C (Rosenbaum et al., 2004; Rosenbaum and Simon, 2007) as well as Src kinase (Jin et al., 2004). Their contribution to TRPV1-mediated plasticity remains unclear.

GLIAL TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 CHANNELS IN THE BRAIN

Modulation of synaptic transmission could also be the result of activation of TRPV1 in glia (**Figure 1**). For instance, proinflammatory molecules such as the bioactive phospholipid lysophosphatidic acid has been shown to activate TRPV1 by directly binding to its C-terminal (Nieto-Posadas et al., 2011). While microglial TRPV1 is primarily localized in mitochondrial but not plasma membranes (Miyake et al., 2015), its activation indirectly enhances glutamatergic transmission in cortical neurons (Marrone et al., 2017), presumably through the production and release of inflammatory mediators (Miyake et al., 2015; Marrone et al., 2017; Kong et al., 2019). TRPV1 may also be expressed in astrocytes (Toth et al., 2005; Marinelli et al., 2007), although most of the recent evidence suggests that this is induced in response to brain injury. For example, TRPV1 activation in substantia nigral astrocytes produces the ciliary neurotrophic factor, which prevents the active degeneration of dopaminergic neurons in rat models of Parkinson's disease (Nam et al., 2015). Hypoxic ischemia

also reportedly triggers the expression of astrocytic TRPV1 and consequent release of pro-inflammatory cytokines from astrocytes to neighboring neurons in epileptogenesis (Wang et al., 2019). However, whether TRPV1 in astrocytes can modify synaptic function and plasticity in neurons under physiological conditions remains unclear. It is also important to note that capsaicin has been reported to exert direct effects on voltage-sensitive ion channels (Hagenacker et al., 2011; Yang et al., 2014; Pasierski and Szulczyk, 2020) and thus may modulate neurotransmitter release in a TRPV1-independent manner in cases where TRPV1 antagonists or knockouts were not assessed. For example, in the lateral amygdala (LA), capsaicin reportedly modifies LTD through activation of TRPM1 (Gebhardt et al., 2016). Last but not least, activation of postsynaptic TRPV1 may induce the production of calcium dependent retrograde signaling molecules to suppress neurotransmitter release (see below).

INTERACTION BETWEEN TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 AND THE ENDOCANNABINOID SYSTEM

Within the hippocampus, inhibition of TRPV1 dramatically reduces glutamatergic input to oriens-lacunosum-moleculare (OLM) interneurons (Hurtado-Zavala et al., 2017) and can block presynaptic LTD of excitatory synapses at inhibitory GABAergic interneurons but not pyramidal neurons (Gibson et al., 2008). Notably, this TRPV1-dependent presynaptic modulation of synaptic plasticity is similar in many ways to that reported for endocannabinoid (eCB)-mediated LTD that involves the activation of presynaptic type 1 cannabinoid (CB1) receptors (Castillo et al., 2012; Katona and Freund, 2012). For instance, in both forms of LTD, postsynaptic mGluRs are involved in promoting the production of a lipid messenger that then acts retrogradely to suppress transmitter release in a long-term manner. In postsynaptic TRPV1-LTD, mGluR activation promotes the production of anandamide (AEA), a major endocannabinoid (Chavez et al., 2010; Grueter et al., 2010; Puente et al., 2011). In this case, AEA likely acts directly on postsynaptic TRPV1 to induce endocytosis of AMPARs expressed on dendritic spines, reducing their number and consequently the long-term reduction in excitatory synaptic strength. Given the ability of eCBs like AEA to activate TRPV1, it has been suggested that TRPV1 may act as an ionotropic eCB receptor (De Petrocellis et al., 2017; Muller et al., 2018; Storozhuk and Zholos, 2018) with central neuromodulatory effects that either mimic or oppose those exerted by CB1 receptors.

The factors that determine whether eCBs act on TRPV1 or CB1 receptors to regulate synaptic transmission remain unclear. Interestingly, in the bed nucleus of stria terminalis, L-type calcium channel activation in dendrites leads to production and secretion of 2-arachidonoylglycerol (2-AG) that acts on presynaptic CB1 receptors in short-term depression, whereas AEA secretion following mGluR5 activation acts on postsynaptic TRPV1 in LTD (Puente et al., 2011). Additionally, other eCBs such as N-arachidonoyl-dopamine (Huang et al., 2002), can also activate both TRPV1 and CB1R, producing opposing actions on synaptic transmission (Marinelli et al., 2007). Moreover, it has been proposed that postsynaptic TRPV1 activation by AEA can regulate the magnitude of 2-AG-mediated tonic inhibition of perisomatic GABAergic transmission in CA1 pyramidal neurons (Lee et al., 2015), putatively through TRPV1-induced reduction of 2-AG levels (Maccarrone et al., 2008).

Spike-timing-dependent long-term potentiation (tLTP) in the striatum and neocortex, important for acquiring new associative memories, requires both TRPV1 and CB1 receptors (Cui et al., 2015, 2018). Notably, capsaicin activation of TRPV1 alone in the neocortex suppressed excitatory transmission (Cui et al., 2018). The mechanisms underlying TRPV1 and CB1 receptor co-dependency and their conversion from depressive to potentiating effects remain to be investigated. The signaling pathways downstream of TRPV1 and CB1 receptor activation may be dynamically inter-regulated. Indeed, the lack of TRPV1 reportedly modifies the expression and localization of different components of the eCB system, causing a shift from CB1 receptor-mediated LTD to LTP in the dentate gyrus (Egana-Huguet et al., 2021). While these results suggest an ability of eCBs to act through both CB1 and TRPV1 receptors, the factors that determine directionality of synaptic plasticity and functional consequences in behavior need to be determined.

TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 IN METAPLASTICITY

TRPV1 activity can alter the inducibility of long-term synaptic plasticity (**Table 2**). In the entorhinal cortex, LTP does not occur unless TRPV1 is blocked (Banke, 2016). Similarly, activation of TRPV1 with capsaicin attenuates the magnitude of LTP in the LA (Zschenderlein et al., 2011). Given the ability of TRPV1 activation to induce endocytosis of AMPARs, it is possible that the simultaneous expression of TRPV1-mediated LTD occludes or opposes LTP under these conditions (**Figure 2**, top). In contrast, TRPV1 knockout mice show deficits in LTP at the Schaffer collateral–commissural pathway to CA1 hippocampal neurons (Marsch et al., 2007), which can be rescued by activating OLM neurons with nicotine (Hurtado-Zavala et al., 2017). Moreover, these TRPV1 deficient mice reportedly show impaired hippocampal-dependent learning, fear conditioning and anxiety, effects that cannot be explained by alterations in nociception. Additionally, capsaicin exposure in wildtype mice enhances CA1 LTP and spatial memory (Li et al., 2008; Bennion et al., 2011), potentially by decreasing inhibition via TRPV1-mediated iLTD (**Figure 2**, bottom). Hippocampal TRPV1 activation also enables spatial memory retrieval under stressful conditions and rescues LTP in slices derived from swim-stressed mice (Kulisch and Albrecht, 2013). Although these findings support a role for brain TRPV1 in regulating synaptic function and behavior, it remains unclear how TRPV1 is able to bidirectionally modulate metaplasticity.

TABLE 2 | TRPV1 metaplasticity in the mammalian brain.

Brain area	Synapse type	Effects	Induction protocol	References
Hippocampus	Schaffer collateral to CA1	Reduced LTP	HFS	Marsch et al., 2007
	Schaffer collateral to CA1	Enhanced LTP and reduced LTD	HFS-LFS	Li et al., 2008
	Schaffer collateral to CA1	Enhanced LTP	TBS-HFS	Bennion et al., 2011
Entorhinal cortex	Excitatory Inputs to layer II/III	TRPV1 blockade enables LTP	HFS	Banke, 2016
Lateral amygdala	Cortical Excitatory inputs	Depressed LTP in ether	HFS	Zschenderlein et al., 2011
		Enhanced LTP in isoflurane	HFS	
		Depressed LTP in ether		Kulisch and Albrecht, 2013
		Blocked stress-induced impairment of LTP		
Dorsolateral striatum	Excitatory inputs	Co-dependent CB1-TRPV1 LTP	STDP	Cui et al., 2015
Neocortex	Excitatory inputs	Co-dependent CB1-TRPV1 LTP	STDP	Cui et al., 2018

LFS, low frequency stimulation; HFS, high frequency stimulation; TBS, theta-burst stimulation; STDP, spike-timing dependent plasticity; LTP, long-term potentiation; LTD, long-term depression.

TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 MIGHT REGULATE HIPPOCAMPAL DEVELOPMENT/CIRCUIT MATURATION

In addition to regulating synaptic function, a role for TRPV1 in development is indicated by the ability of capsaicin to activate these channels in hippocampal Cajal-Retzius cells (Anstotz et al., 2018). These cells are a major source of the extracellular matrix protein reelin, which is essential for development (Del Rio et al., 1997; Frotscher, 1997). Indeed, Cajal-Retzius cells can powerfully excite GABAergic interneurons of the molecular layer in the dentate gyrus via TRPV1 activation (Anstotz et al., 2018), supporting the idea that TRPV1 may shape layer-specific interneuron connectivity in hippocampal development. Interestingly, the density of Cajal-Retzius cells decreases during postnatal development, raising that possibility that functional TRPV1 receptors may drive this progressive reduction by calcium-dependent apoptosis. Consistent with this idea, in the mouse hippocampus, it has been suggested that the expression of both the messenger (mRNA) and the protein are low in the early stages (<P15), increasing from the eighth postnatal week and then decreasing towards postnatal week 16 (Huang et al., 2014). Whether changes in the expression and activation of TRPV1 in early stages of development ($P < 20$) play a role in circuit maturation remain unclear. Notably, TRPV1 is transiently expressed in retinal afferents to principal neurons in the superior colliculus, where excitatory synapses onto them show a form of activity-dependent LTD that is dependent on TRPV1 in juvenile but not adult animals (Maione et al., 2009).

TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 IN MENTAL DISORDERS AND NEUROLOGICAL DISEASES

TRPV1 has been linked to numerous neurological and neuropsychiatric disorders, with channel desensitization and blockade as primary therapeutic objectives (Edwards, 2014; Weng et al., 2015; Singh et al., 2019; Escelsior et al., 2020; Wang et al., 2020b; Allain et al., 2021; Asth et al., 2021; Zhou et al., 2021; Akhilesh et al., 2022). Emerging evidence indicates that TRPV1 expression is increased following diverse forms of stress (Li et al., 2008; Rubino et al., 2008; Kulisch and Albrecht, 2013; Saffarzadeh et al., 2015; Garami et al., 2020; Aghazadeh et al., 2021) to likely drive maladaptive responses. For example, chronic exposition to heat can upregulate TRPV1 channels in the brain, probably by the generation of reactive oxygen species (Aghazadeh et al., 2021), but the functional effect of this expression in regulating neuronal function has not been studied. Interestingly, TRPV1 antagonists have been shown to induce hyperthermia in human clinical trials (Garami et al., 2020), suggesting that TRPV1 channels may play a role in regulating core body temperature. The central mechanisms are unknown but may involve TRPV1 channels in the hypothalamus (Iida et al., 2005; Sharif-Naeini et al., 2008; Sudbury and Bourque, 2013; Zaelzer et al., 2015; Molinas et al., 2019). Notably, TRPV1 blockers act through different mechanisms depending on animal model, specifically targeting proton-activated TRPV1 domains in rodents, but heat and proton-activated TRPV1 regions in humans (Garami et al., 2020). In neuropathic pain, nerve damage alters synaptic circuits in the spinal cord, brainstem and cortex, involving neurons and glial cells, that leads to persistent amplification of pain signals (Giordano et al., 2012; Tsuda et al., 2017; Arribas-Blazquez et al., 2019). It is unclear whether heightened TRPV1 function in these cell types contribute to such plasticity. However, in support of this idea, activation of microglial TRPV1 enhances glutamatergic neurotransmission and the presence of neuronal TRPV1 increases action potential firing, which could enhance cortical activity and be crucial for the emotional alteration in chronic pain (Marrone et al., 2017).

Moreover, TRPV1 may tonically modulate emotional defensive responses such as anxiety, fear and panic (Millan, 2003; Mcnaughton and Corr, 2004; Terzian et al., 2009; Canteras et al., 2010; Casarotto et al., 2012; Aguiar et al., 2015; Gobira et al., 2017). Stress conditioning is accompanied by augmented TRPV1 levels in both the hippocampus and LA (Kulisch and Albrecht, 2013; Navarria et al., 2014). Intracranial injection of TRPV1 antagonists or its genetic deletion in these limbic brain regions promotes anxiolytic and antidepressant states as well as decrease cue and contextual

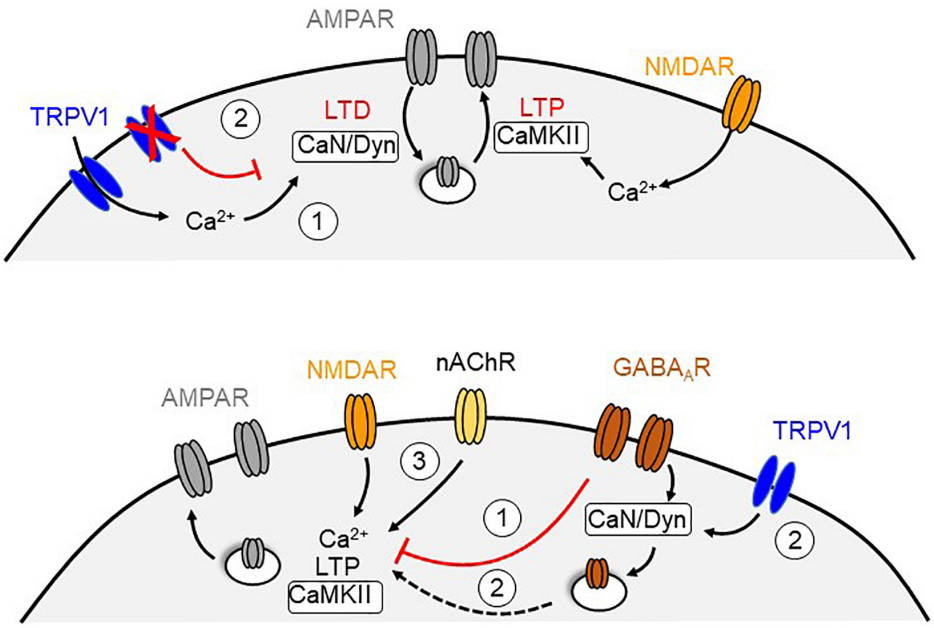

FIGURE 2 | Potential mechanisms for TRPV1-dependent metaplasticity at central synapses. *Top,* Calcium influx through TRPV1 channels triggers a form of postsynaptic LTD that require endocytosis of AMPARs, which may counterbalance the level of N-methyl-D-aspartate receptor (NMDAR) dependent LTP (1). In contrast, blockade or genetic deletion of TRPV1 channels recovers NMDAR-mediated LTP that may be due to the absence of TRPV1-LTD (2). *Bottom,* NMDAR-dependent LTP can be reduced by activation of $GABA_AR$s (1). Activation of TRPV1 channels could triggers a calcineurin (CaN) and dynamin (Dyn)-dependent endocytosis of $GABA_AR$s that remove the inhibitory suppression of the LTP (2). Exogenous activation of nicotinic acetylcholine receptors (nAChR) may oppose the influence of GABAergic inhibition and rescue LTP in the absence of TRPV1 likely by increasing calcium levels (3).

fear conditioning (Kasckow et al., 2004; Marsch et al., 2007; Rubino et al., 2008; Aguiar et al., 2009; Terzian et al., 2014; Wang et al., 2017). It has been proposed that blocking TRPV1 may be antidepressive by consequently increasing serotonin levels (Manna and Umathe, 2012). On the other hand, some reports indicate that activation of TRPV1 rescued stress-induced impairment of hippocampal LTP and spatial memory retrieval (Li et al., 2008). In contrast, activation of TRPV1 in control conditions suppresses LTP in LA by modulation of the nitric oxide system (Zschenderlein et al., 2011). More work is needed to understand how the blockade or deletion of TRPV1 promotes anxiolytic and anti-depressive states, while its overexpression rescues cognitive deficiencies caused by stress.

Interestingly, TRPV1 expression is reduced in a transgenic model of Alzheimer's Disease (AD; Du et al., 2020), which is a neurodegenerative disorder characterized by memory and learning deficits. Disrupted TRPV1-mediated synaptic plasticity and metaplasticity may be involved, but this remains a matter of debate. For instance, TRPV1 activation or reinsertion prevents the shift in excitatory/inhibitory balance and restores normal brain oscillatory activity (Balleza-Tapia et al., 2018), rescues hippocampal LTP and improves memory performance (Chen et al., 2017; Du et al., 2020; Wang et al., 2020a, 2021). However, a recent report suggests that the absence of TRPV1 rescues memory deficits in AD (Kim et al., 2020). The diverse influences of TRPV1 channels in pathological condition may reflect cause or consequence of brain disorders. More time-lapse studies are needed to fully understand the exact contribution of TRPV1 in disease etiology and pathophysiology.

CONCLUSION AND FUTURE DIRECTIONS

Accumulating evidence indicate that, in addition to its well-known role in modulating pain transduction, TRPV1 seems to play an important role in regulating brain synaptic transmission and plasticity. TRPV1 can be expressed both presynaptically and postsynaptically, and depending on the specific synapse, it can either increase or decrease neurotransmission. The impact of glial TRPV1 function on central synapses requires further investigation. Given the ubiquitous expression of TRPV1 throughout the brain, it is not surprising that TRPV1 has been implicated in several neurological and psychiatric disorders such as epilepsy, anxiety, and depression as well as drug-addiction disorders (Edwards, 2014; Singh et al., 2019; Escelsior et al., 2020; Allain et al., 2021; Asth et al., 2021; Zhou et al., 2021). Future work will expand our appreciation of the role and the exact mechanism underlying TRPV1-mediated changes in synaptic transmission and plasticity at central synapses in health and disease. For example, it is unknown whether TRPV1-mediated plasticity is present at GABAergic synapses and how it influences circuit function. It is also unclear under what conditions AEA acts on TRPV1 or CB1 receptors to modify synaptic function and

behavior. Whether glial TRPV1 can be activated by AEA or other vanilloids is unclear as well. Moreover, different neuromodulators including serotonin, histamine, or prostaglandins are known to stimulate TRPV1 in the periphery (Cesare et al., 1999; Premkumar and Ahern, 2000; Vellani et al., 2001). Whether neuromodulation regulates brain function and behavior in a TRPV1-dependent manner remains unknown.

AUTHOR CONTRIBUTIONS

AEC and CQC conceived of the general ideas presented in this review and supervised the work. CA-G and RM contributed equally to all sections and helped with figure and table construction. All authors made significant contributions to the information content and writing of this final manuscript.

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