Chronic in vivo optogenetic stimulation modulates Hebbian plasticity and spine density in the mouse hippocampus

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Abstract

Continuous excitation can trigger cell-wide homeostatic responses in neurons, altering membrane channels, promoting morphological changes and ultimately reducing synaptic weights. However, how synaptic downscaling interacts with classical forms of Hebbian plasticity is still unclear. In this study, we investigated whether chronic optogenetic stimulation of hippocampus CA1 pyramidal neurons in freely-moving mice could (a) cause morphological changes reminiscent of homeostatic scaling, and (b) lead to alterations in Hebbian plasticity. After 24 h of stimulation with 15-ms blue light pulses every 90 s, dendritic spine density was reduced in the CA1 region of mice expressing channelrhodopsin-2 (ChR2) when compared to controls. This protocol also impaired the induction of LTP and facilitated that of LTD in ex vivo slices obtained from ChR2-expressing mice immediately after the end of stimulation. Our results indicate that neuronal responses to prolonged network excitation can modulate subsequent Hebbian plasticity in the hippocampus.

Introduction

Hebbian forms of synaptic plasticity, such as long-term potentiation (LTP) and long-term depression (LTD), are believed to be fundamental for learning and memory. However, by inducing long-lasting changes in synaptic strength, these mechanisms can drive circuit saturation and destabilization if left unchecked (Abbott & Nelson, 2000). As a counterpart to input-specific Hebbian plasticity, thus, neurons can sense changes in their excitability and initiate global homeostatic regulations in synapses to restrain their activity to its usual physiological range, in a process known as synaptic scaling (Turrigiano, 2012). These homeostatic processes act to ensure network stability, providing a feedback that prevents unrestrained potentiation or depotentiation stemming from LTP and LTD (Vitureira & Goda, 2013).

This interplay is classically mediated by scaling-induced changes in receptor density, which compensate those brought upon by LTP and LTD; in this sense, however, homeostatic scaling could also affect subsequent induction of Hebbian plasticity itself. It has been proposed that synaptic scaling could modulate the threshold for LTP or LTD induction as a response to the changes in post-synaptic activity (Rabinowitch & Segev, 2008; Keck et al., 2017). This idea is supported by the fact that synaptic scaling and Hebbian plasticity, although operating under distinct plasticity rules, share several molecular pathways that converge to regulate synaptic morphology and membrane channel composition (Fernandes & Carvalho, 2016).

Moreover, there is experimental evidence that homeostatic plasticity induced by chronic neuronal inhibition can enhance subsequent LTP, as blockade of neuronal activity by tetrodotoxin (TTX) for 60 h in cultured mouse hippocampal slices was shown to increase the expression of AMPA and NMDA receptors at existing synapses. This prolonged inhibition was also able to create new synapses containing only NMDA receptors, which were recruited when LTP was induced at CA3-CA1 synapses, thus enhancing potentiation (Arendt et al., 2013). Another study demonstrated that acute deprivation of activity in hippocampal slices for only 3 hours by TTX and APV could also drive upscaling of spontaneous synaptic transmission. This brief period of homeostatic regulation was enough to facilitate LTP at CA3-CA1 synapses as well (Félix-Oliveira et al., 2014). Finally, in vivo suppression of neuronal activity in the mouse primary visual cortex, caused by 2 days of visual deprivation, was able to promote LTP and prevent LTD in layer 2/3 pyramidal neurons (Guo et al., 2012), suggesting that this protocol, known to induce homeostatic upscaling (Goel et al., 2011), was sufficient to slide the threshold between synaptic potentiation and depression.

Despite the evidence for enhancement of LTP by activity suppression, there is little data on the effects of continuous neuronal excitation and synaptic downscaling on Hebbian plasticity; nevertheless, there is reason to believe that these processes interact as well. As opposite mechanisms, up- and downscaling differ in their need for several signaling and trafficking elements, but both show modulation of regulatory pathways that are essential to synaptic potentiation and depression, such as structural morphology, glutamate transport and vesicle function (Turrigiano, 2012; Schanzenbächer et al., 2018). Furthermore, a recent study showed that 10 min of high-frequency optogenetic stimulation could induce homeostatic plasticity in organotypic hippocampal cultures, decreasing the number of excitatory spine synapses and increasing the number of inhibitory ones. This downscaling protocol also impaired memory recall and facilitated memory extinction in vivo when selectively applied to granule cells encoding a contextual fear memory (Mendez et al., 2018), although its effects on Hebbian plasticity were not explored.

To unveil the interaction between homeostatic response to overexcitation and Hebbian plasticity, as well as to establish a way to study the effects of synaptic downscaling in vivo, we developed a model using chronic optogenetic stimulation of pyramidal cells in the hippocampal CA1 region of freely-moving C57BL/6 mice. We then evaluated the effect of this intervention on neuronal morphology and synaptic plasticity in ex vivo brain slices.

Results and Discussion

We induced the expression of channelrhodopsin 2 (ChR2) in CA1 pyramidal neurons of the hippocampus by injecting a viral vector (AAV5-CaMKIIa-ChR2-eYFP). To verify opsin expression and functionality, we obtained ex vivo slices (Figure 1A-B) after 3 to 5 weeks and activated ChR2-expressing neurons by a blue light-emitting laser. Voltage-clamp recording was performed to verify the activation of these channels (Figure 1C). We also tested if a 15-ms pulse would enhance overall excitation in the CA1 circuitry. We chose this emission time because it would be theoretically sufficient for ChR2 to reach its maximum response (Lin et al., 2009) while minimizing neuronal damage caused by photostimulation. In extracellular recordings of field excitatory postsynaptic potentials (fEPSPs) in the stratum pyramidale, the mean number of spikes

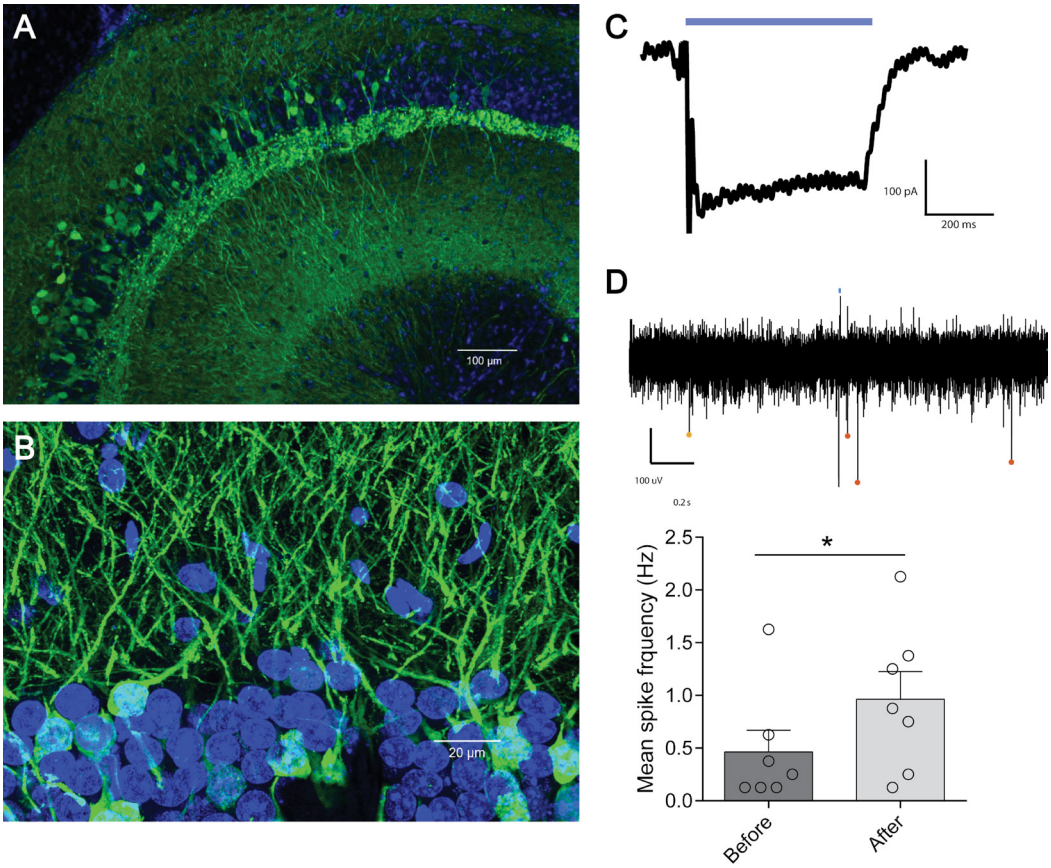

Figure 1 – Expression of ChR2 in the hippocampus. (A) Overview of ChR2-eYFP expression in hippocampal slices. ChR2 is associated with eYFP (green) and expressed under the CaMKII-α promoter, while nuclei are stained in blue with DAPI. (B) Expression in pyramidal cells of the CA1 region. (C) Qualitative example of a voltage clamp recording of a neuron expressing ChR2 in response to 473-nm light. (D) Field activity recorded during 1-sec intervals immediately before and after an acute 15-msec optogenetic pulse. Dots show an examples of spikes detected before (yellow) and after (orange) stimulation in a recording. Bars show mean number of spikes (± s.e.m) before and after stimulation across 5 trials per slice (n=7 slices; paired t test, p=0.013)

detected increased around twofold in the 1-second period following stimulation (Figure 1D; paired t test, p=0.013, n=7 slices).

To test the effects of chronic in vivo optogenetic stimulation, we applied 15 ms of blue light every 90 s during 24 h through a fiber-optic cannula implanted in the CA1 region of freely-moving mice expressing either ChR2 or an eYFP-only viral vector (AAV5-CaMKIIa-eYFP). Mice did not show any obvious change in behavior during this protocol. Immediately after chronic stimulation, mice were euthanized and hippocampal slices were used for either dendritic spine density assessment or ex vivo electrophysiology (Figure 2).

To visualize dendritic spines, we used confocal microscopy and anti-GFP/YFP immunocytochemistry. Mean spine density in the hippocampus did not differ between the control group that went through the stimulation protocol (eYFP 24h-stim) and home-cage mice expressing ChR2 without light stimulus (ChR2 naive). However, chronic stimulation of ChR2-expressing mice for 24 hours (ChR2 24h-stim) significantly reduced spine density (Figure 3; one-way ANOVA, p=0.002; Tukey's post-hoc: eYFP 24h-stim vs. ChR2 naive, p=0.945; eYFP 24h-stim vs. ChR2 24h-stim, p=0.005; ChR2 naive vs. ChR2 24h-stim, p=0.003; n=4 mice/group). Our results are comparable to previous studies using in vitro optogenetic excitation that showed reduced spine density in response to homeostatic regulation (Goold & Nicoll, 2010; Mendez et al., 2018). Thus, we believe that this protocol of ChR2 chronic activation is likely to be driving neuronal homeostatic scaling responses as well.

To evaluate the effects of chronic optogenetic excitation on Hebbian plasticity, we performed ex vivo electrophysiology on hippocampal slices from mice expressing ChR2-eYFP or eYFP alone after

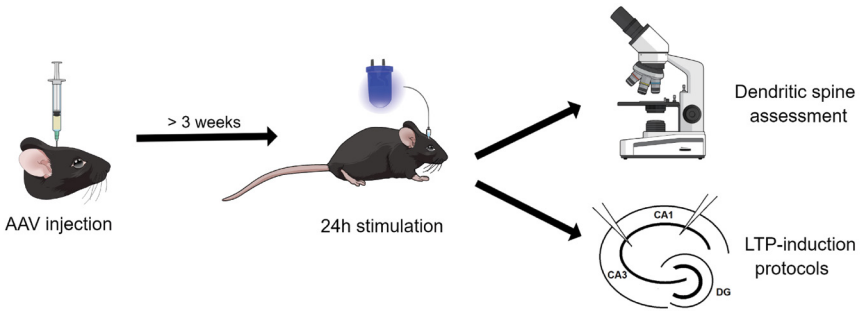

Figure 2 – Experimental protocol. Three weeks after injecting a viral solution in the ventral hippocampus to induce the expression of ChR2-eYFP or eYFP alone, we applied 15-ms light stimuli every 90 seconds during a 24 hour period. At the end of this period, hippocampal slices were either (a) fixed and processed in a confocal microscope after anti-GFP/YFP immunohistochemistry, or (b) used for LTP induction protocols, with recording of field evoked post-synaptic potentials (fEPSPs) in the stratum radiatum of CA1 in response to Schaffer collateral stimulation.

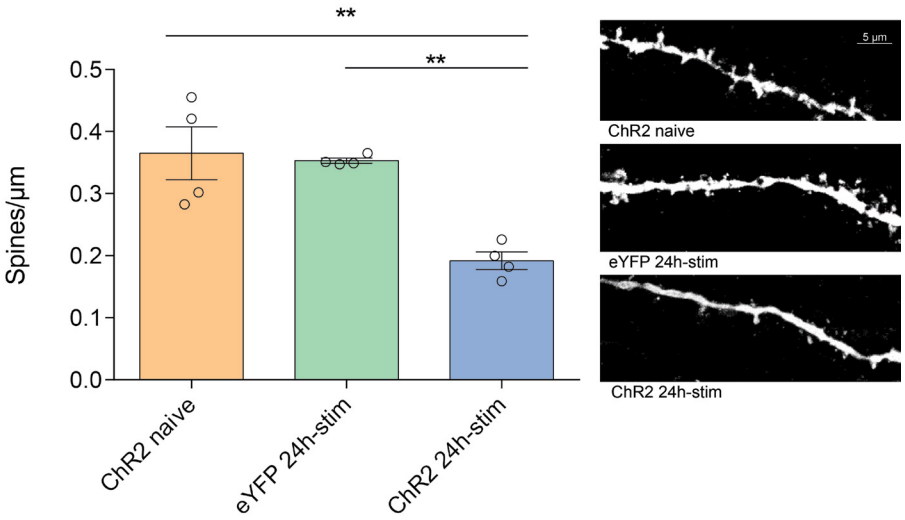

Figure 3 – Reduced dendritic spine density after chronic optogenetic excitation. Three weeks after injection of viral constructs containing ChR2-eYFP or eYFP alone, mice were optogenetically stimulated for 24h (eYFP 24h-stim and eYFP 24h-stim groups). After this period, they were perfused and CA1 dendritic spines were analyzed after anti-GFP/YFP immunocytochemistry. To control for the effect of viral expression, a group of ChR2-expressing mice that did not undergo optogenetic stimulation (ChR2 naive) was included. Left panel shows mean dendritic spine density (± s.e.m), while right panel shows illustrative examples for each group. (n = 4 animals/group; 1-way ANOVA, p=0.002; Tukey's post-hoc test: eYFP 24h-stim vs. ChR2 naive, p=0.945; eYFP 24h-stim vs. ChR2 24h-stim, p=0.005; ChR2 naive vs. ChR2 24h-stim, p=0.003).

the 24-h optogenetic stimulation protocol described above. fEPSPs were recorded in CA1 in response to Schaffer collateral stimulation, with baseline responses registered for 10 min using 200-ms pulses every 20s. We then applied a 20x theta-burst (TBS) protocol consisting of bursts of 4 pulses at 100Hz with 200-ms intervals to induce potentiation. This led to an increase in the fEPSP amplitudes in slices from the eYFP-only group, but not in those of chronically-stimulated mice (Figure 4A; Student's t test, p = 0.0004, n = 4 (ChR2 24h-stim) or 6 (eYFP 24h-stim) animals). To investigate if chronic optogenetic activation would change the response to weak presynaptic stimulation, we used a 2x TBS protocol that should not elicit any fEPSP amplitude increase in normal conditions. As expected, slices from chronically-stimulated eYFP animals did not show significant changes after this protocol; however, slices expressing ChR2 showed a surprising depotentiation in fEPSP responses (Figure 4B; Student's t test, p = 0.0083, n= 3 (eYFP 24h-stim) or 7 (ChR2 24h-stim) animals).

Our results thus indicate that chronic in vivo optogenetic stimulation can drive morphological changes characteristic of synaptic homeostatic scaling and modulate classical forms of Hebbian plasticity. To our knowledge, this is the first evidence of a direct effect of chronic neuronal stimulation and putative synaptic downscaling on Hebbian plasticity. Our protocol of chronic excitation was able both to disturb LTP and to facilitate LTD. These findings are consistent with the current hypothesis that synaptic scaling is able to change the threshold between potentiation and depression of neuronal connections (Keck et al., 2017).

Chronic increases in neuronal activity have been shown to influence several conditions, such as learning and memory (Mendez et al., 2018), neuropathic pain processing (Xiong et al., 2017) and the progression of Alzheimer's disease (Yamamoto et al., 2015). Thus, although the optogenetic excitation we used is a non-physiological approach to excitation, it can be a useful tool for the in vivo study of homeostatic neuronal regulation, a process that has mostly been studied in cultured neurons. Further studies are necessary to investigate the molecular mechanisms, boundary conditions and behavioral consequences of the plasticity changes caused by chronic in vivo stimulation of hippocampal neurons.

Detailed Methods

Animals and optogenetic stimulation

Experiments were performed on C57BL/6J mice of both sexes, aged 6-10 weeks, provided by the Transgenic Animal Lab (LAT), Federal University of Rio de Janeiro, Brazil and the Brain Institute, Natal, Brazil. All procedures were approved by the Institutional Ethics Committee for the Care and Use of Laboratory Animals of the Federal University of Rio de Janeiro (CEUA-CCS 024/15) and the Federal University of Rio Grande do Norte (Protocol 015.004/2017).

For stereotactic surgery, each animal was anesthetized with i.p. injections of ketamine (100 mg/kg) and xylazine (20 mg/kg). Viral solution was delivered unilaterally at 0.2 µl/min in two depth sites in the CA1 region of the ventral hippocampus (A.P. 2.8 mm; M.L. 2.6 mm; D.V. 3.0 and 3.5 mm; 0.75 µl/site) before the fiber-optic cannula was fixed. Vectors containing ChR2 under the promoter of the CaMKII-α gene (rAAV5/CaMKIIa-ChR2(H134R)-EYFP) or a control vector lacking the ChR2 sequence (rAAV5/CaMKIIa-EYFP) were obtained from the Vector Core Facility at the University of North Carolina.

After a period of 3 to 5 weeks after surgery, we applied a chronic stimulation protocol using 15-ms pulses

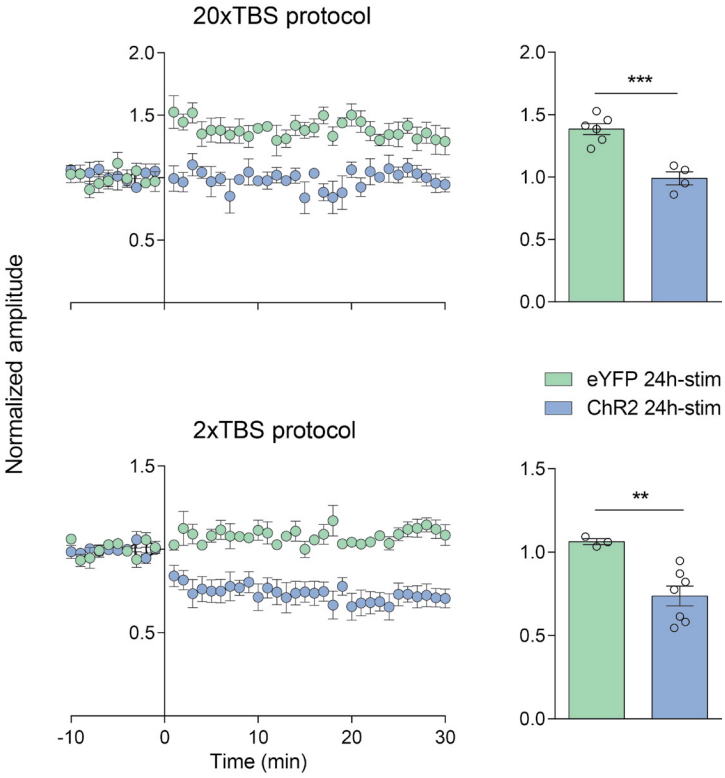

Figure 4 – Chronic optogenetic excitation modulates LTP and LTD. Left panels show mean ± s.e.m. of CA1 fEPSP responses for each group 10 min before and 30 min after different stimulation protocols on the Schaffer collaterals. Bars show mean amplitude change from baseline (± s.e.m.) over 30 min post-stimulation in each group. (A) LTP was induced in the control group after 20xTBS, increasing field amplitude response by about 40%. In slices from mice that underwent chronic optogenetic stimulation, no change was observed (n = 4 (ChR2 24h-stim) or 6 (eYFP 24h-stim) animals; Student's t test; p = 0.0004). (B) After a 2xTBS protocol, control slices did not change their fEPSP responses. However, LTD was induced in the slices of chronically stimulated mice. (n = 3 (eYFP 24h-stim) or 7 (ChR2 24h-stim) animals; Student's t test, p = 0.0083).

of blue light (473 nm) every 90 s during 24 hours on freely-moving mice. Immediately after this procedure, animals were euthanized as described below.

Dendritic spine density analysis

For analysis of dendritic spines, animals were anesthetized with ketamine (0.5-1g/kg) and perfused with 4% paraformaldehyde in 0.1M PBS. We obtained 150-µm-thick transverse hippocampal slices using a vibratome, and immunocytochemistry was performed on free-floating sections with sheep anti-GFP/YFP (1:700; AbD Serotec) and Alexa Fluor 488-conjugated donkey anti-sheep IgG (1:500; Thermo-Fischer). Labeled dendrites were imaged using a Leica TCS SPE confocal microscope with a 63x-objective, followed by 1.5-2.5x digital zoom. At least four different fields were digitally captured for each animal. Dendritic spines were counted in these captured images using ImageJ by an observer who was blinded to the experimental,groups, and the mean spine density of each field was calculated by dividing the spine number of all clearly visible dendrites by their total length. The average of all fields was used to compute the spine density for each mouse.

Electrophysiology

For electrophysiology, mice were euthanized by ketamine (0.5-1g/kg) and decapitation. Transverse 400-µm-thick hippocampal slices were obtained with a vibratome and maintained in artificial cerebrospinal fluid (ACSF: 124mM NaCl, 3.5mM KCl, 1.25mM NaH2PO4, 1.5mM MgCl2, 1.5mM CaCl2, 24mM NaHCO3 and 10mM glucose), constantly bubbled with 95% O2 and 5% CO2.

For qualitative demonstration of ChR2-mediated inward currents, borosilicate glass electrodes (resistance = 4–8 MΩ) were filled with an internal solution containing 130mM K-gluconate, 7mM NaCl, 2mM MgCl2, 2mM ATP, 0.5mM GTP, 10mM HEPES, 0.1mM EGTA, and pH was adjusted to 7.2 using KOH. Postsynaptic currents were obtained in voltage clamp at a holding potential of −60 mV.

For field spike measurements after ex vivo optogenetic stimulation, we recorded the extracellular fEPSP by placing ACSF-filled borosilicate glass electrodes in the CA1 stratum pyramidale of slices expressing ChR2. After recording fEPSPs for 1 s, a 15-ms light pulse was delivered and the recording was maintained for another 1 s. After 5 trials of this protocol for each slice, the mean number of spikes before and after stimulations was registered using the automatic threshold algorithm described by Quiroga et al. (2007).

For LTP experiments, extracellular fEPSP recordings were obtained by placing a concentric stimulation electrode at the stratum radiatum as previously described (Leão et al., 2012). A borosilicate glass pipette filled with ACSF was used to record Schaffer collateral fEPSPs at the CA1 region, 200–400µm away from the stimulation electrode. Stimulation strength was adjusted to obtain 50–60% of the maximum fEPSP amplitude, and 10 min of recordings were used to obtain a stable fEPSP baseline, with 200ms pulses delivered every 20 s. Stimulus-response curves were obtained by measuring fEPSP after each stimulation. Synaptic plasticity was induced by 2x-TBS or 20x-TBS (2 or 20 bursts of four pulses at 100 Hz spaced by 200 ms, respectively). After stimulation, recording of stimulus-response curves continued for 30 min, which were aggregated to measure the amplitude change from baseline. When more than one slice from the same animal successfully went through the same theta-burst protocol, the average amplitude change for that mouse was calculated.

Statistical Analysis

Data from all experiments were analyzed in GraphPad Prism 6. Comparisons between groups were performed using Student's t-test, paired t-test, or one-way ANOVA followed by Tukey's post-hoc test, with the specific test described for each result.

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