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GABAergic inhibition gates excitatory LTP in perirhinal cortex

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Abstract

The perirhinal cortex (PRh) is a key region downstream of auditory cortex (ACx) that processes familiarity linked mnemonic signaling. In gerbils, ACx-driven EPSPs recorded in PRh neurons are largely shunted by GABAergic inhibition (Kotak et al. 2015). To determine whether inhibitory shunting prevents the induction of excitatory long-term potentiation (e-LTP), we stimulated ACx-recipient PRh in a brain slice preparation using theta burst stimulation (TBS). Under control conditions, without GABA blockers, the majority of PRh neurons exhibited long-term depression. A very low concentration of bicuculline increased EPSP amplitude, but under this condition TBS did not significantly increase e-LTP induction. Since PRh synaptic inhibition included a GABA_B receptor-mediated component, we added a GABA_B receptor antagonist. When both GABA_A and GABA_B receptors were blocked, TBS reliably induced e-LTP in a majority of PRh neurons. We conclude that GABAergic transmission is a vital mechanism regulating e-LTP induction in the PRh, and may be associated with auditory learning.

Keywords

auditory cortex; perirhinal cortex; LTP; GABA_A receptor; GABA_B receptor

In rodents, learning-induced changes to perirhinal cortex (PRh) neurons generally result in responses to novelty and familiarity by increasing and decreasing firing rates, respectively (Ho et al. 2015). In the auditory domain, PRh neurons respond to species-specific ultrasonic vocalizations (Allen et al. 2007; Sadananda et al. 2008). These auditory-evoked responses are, in part, driven by afferent projections from auditory thalamus and cortex (Burwell and Amaral, 1998; Kimura et al. 2003; Kealy and Commins, 2011). The importance of PRh in mnemonic processing is revealed by lesions that disrupt learned behaviors involving auditory cues (Sacchetti et al. 1999; Lindquist et al. 2004; Bang and Brown, 2009; Kholodar-Smith et al. 2008). At a cellular level, excitatory long-term potentiation (e-LTP)

and depression (e-LTD) contribute to PRh plasticity associated with learning (Bilkey, 1996; Ziakopoulos et al. 1999; Cho et al. 2000; Perugini et al. 2012). In many brain regions, induction of e-LTP is facilitated by down-regulation of GABA_A and/or GABA_B receptor-mediated inhibition (Wigström & Gustafsson, 1986; Artola and Singer, 1987; Pacelli et al. 1989; Mott et al. 1990; Xie and Lewis, 1991; Mott and Lewis, 1991; Bear et al. 1992; Hirai et al. 1993; Kanter and Haberly, 1993; Brucato et al. 1996; Mondadori et al. 1996; Chapman et al. 1998; Hsu et al. 1999; Grover and Yan 1999; Stäubli et al. 1999; Kleschevnikov et al., 2004; Chan et al. 2006; Liu et al. 2014; Nomura et al. 2016). For example, the dentate-commissural pathway to the hippocampal CA1 region recruits powerful inhibition that occurs simultaneously with the excitatory drive. Although e-LTP is difficult to induce at in this pathway, local GABA_A receptor blockade permits e-LTP induction (Steward et al. 1990), suggesting inhibitory control of excitatory synaptic strength.

The gerbil PRh receives direct excitatory projections from auditory cortex, as revealed by *in vivo* anterograde tract tracing and *in vitro* ACx-evoked postsynaptic potentials (PSP) (Kotak et al. 2015). However, these evoked PSPs were significantly attenuated by GABAergic inhibition. Thus, we found that bicuculline treatment resulted in evoked PSPs reaching action potential threshold. We hypothesized that the strong inhibitory drive would prevent activity-dependent e-LTP. To test this idea, we used a very low concentration of BIC, and a GABA_B receptor antagonist to diminish the effect of inhibition. Under these conditions, we were able to reliably induce e-LTP in the majority of PRh neurons. Thus the expression of e-LTD or e-LTP is controlled by the magnitude of synaptic inhibition.

Gerbil (*Meriones unguiculatus*) breeding pairs were obtained from Charles River, and animals aged postnatal days (P) 17-68 were used in these experiments. All protocols were approved by the Institutional Animal Care and Use Committee at NYU. A previous publication describes the ACx-PRh brain slice preparation and auditory-recipient PRh synaptic properties (Kotak et al. 2015). Additionally, to validate that ACx-recipient PRh layer (L) 1 received a direct projection from the ACx, we used virally mediated tract tracing. Adult gerbils were deeply anesthetized and a craniotomy was made in the temporal bone at the level of core auditory cortex (Radtke-Schuller et al. 2016). The general approach was similar to that described in our previous report (Kotak et al. 2015). A glass electrode was loaded with adenovirus that expresses green fluorescent protein (GFP) in pyramidal cells under the control of a CaMKII promoter (AAV1-CaMKIIa-GFP; 1×10^{13} vg/ml; Penn Vector core), and 50 nL of virus was delivered at 300 μ m from the pial surface (Nanoject, Drummond Scientific). Similarly, we injected an adenovirus that expresses both channelrhodopsin 2 (ChR2) and mCherry under the control of a CaMKII promoter (AAV1-CaMKIIa-hChR2(H134R)-mCherry; 1×10^{13} vg/ml; Penn Vector core). Since these observations are confirmatory (Burwell and Amaral, 1998; Budinger et al., 2000; Kotak et al., 2015), we performed 4 injections. The stereotaxic injections were based on the gerbil brain atlas (Radtke-Schuller et al., 2016). The injections were made from the medial side using an angle of 45 degrees, with the rostrocaudal coordinate 2.45 mm rostral of lambda yielding an approximate mediolateral coordinate of 6 mm. The craniotomy was covered with sterile bone wax and surgical site was closed with vetbond surgery glue and the animal was allowed to recover for 3 weeks to permit expression prior to histological processing. All tissue was scanned with an Olympus VS120 slide scanner (Figure 1B-D).

ACx-PRh brain slices were obtained as described previously (Kotak et al 2015). A bipolar stimulating electrode was placed in layer (L) 4-5 of ACx, approximately 3 mm rostral to the PRh recording site. In separate recordings and all e-LTP experiments, a stimulating electrode was placed in ACx-recipient L1 of PRh, approximately 200 μm medial to the recording site. By choosing stimulation site to PRh L1 for e-LTP induction, we could record consistent and reliable EPSPs by low stimulus duration (100 μs) and intensity (less than 100 μA), higher firing by TBS and consistent e-LTP. In contrast, stimulation of ACx generally required 500 μs stimulus duration and 200-300 μA intensity, while the firing discharge by TBS was lower. All recordings were performed under whole-cell current-clamp conditions. The oxygenated artificial cerebrospinal fluid contained (in mM): 123 NaCl, 4 KCl, 1.2 KH_2PO_4 , 1.3 MgSO_4 , 24 NaHCO_3 , 15 glucose, 2.4 CaCl_2 , and 0.2 ascorbic acid. The pH was -7.3 after bubbling with 95% O_2 /5% CO_2 . The pipet solutions contained (in mM): 130 potassium gluconate, 5 KCl, 2 MgCl_2 , 2 ATP, 0.3 GTP, 0.6 EGTA, and 10 Hepes (pH=7.2). The calculated equilibrium potential for chloride was -70.6 mV, and the calculated equilibrium potential for potassium was -85.6 mV.

Control experiments were performed without pharmacological agents, and experiments tested the role of GABA_A and GABA_B receptors. In pilot experiments, we found that the GABA_A receptor antagonist, $(-)$ -bicuculline methbromide, 1S, 9R (BIC, Sigma, led to suprathreshold action potentials at doses ≥ 2 μM (Figure 2C). However, at a dose of 1.5 μM , BIC increased the amplitude of the ACx-evoked depolarizing PSP without it reaching action potential threshold. Furthermore, to block the GABA_B receptor-mediated inhibitory component, we added a GABA_B receptor antagonist, SCH-50911 (SCH, 10 μM , Tocris). Thus, all e-LTP experiments were conducted in the presence of 1.5 μM BIC and 10 μM SCH. TBS was applied at L1 and PRh pyramidal neurons were held at -50 mV during application of TBS. Pre and post-TBS EPSPs were recorded at a $V_{\text{HOLD}} = -65$ mV.

A modified theta burst stimulation (TBS) protocol was used to induce e-LTP (10 trains of 5 Hz; each train consisting of 10 pulses delivered over 2 sec intervals; this was repeated 15 times at 10 sec intervals, Figure 2E). TBS was chosen because it is effective at inducing excitatory synaptic plasticity in many areas of the CNS, including ACx (Larson and Munkácsy, 2015; Soutar et al. 2016). Prior to TBS, 10 sub-threshold baseline EPSPs were acquired at 30 sec intervals using a stimulus intensity at which evoked EPSPs were $\sim 50\%$ maximum amplitude. During TBS, the stimulus intensity was set at 3 times the pre-TBS intensity, and cells were held at -50 mV to facilitate spiking. Following TBS, cells were returned to a membrane potential of -65 mV, and the pre-TBS stimulus intensity was used to evoke EPSPs at 30 sec intervals for additional 30-40 min. Data were acquired using a Macintosh iMAC running on an Igor-based custom software (WaveMetrics, v4.04, SLICE) and analyzed offline with a separate Igor macro. Statistical analyses were performed using the SAS-based JMP 5.0.1 package.

In addition to the TBS protocol, we performed recordings from P17-41 neurons with high-frequency stimulation (HFS) of ACx. HFS comprised of 5 trains of 10 pulses delivered at 100 Hz repeated 5 times every 10 seconds. This was done in combination with several manipulations: HFS without any pharmacological treatment did not induced e-LTP ($n=11$). Although HFS can yield e-LTP when stimulating electrodes are placed just anterior or

posterior to the recording site (Bilkey 1996; Ziakopoulos et al. 1999), we did not observe ACx-evoked e-LTP with these stimulation parameters.

All data here were collected from PRh neurons, and each recording was obtained from a different brain slice preparation in gerbils aged postnatal days (P) 25 through 68 (n=46). Using virally mediated tract tracing from ACx, we found that ACx projected a dense terminal field in PRh. Figure 1A shows a schematic and a transverse section of the ACx injection site. Figure 1B shows the ACx terminal field within PRh at a lower magnification, and reveals dense arborizations in superficial and infragranular regions. Figure 1C shows a high power image that displays the dense ACx terminal arborizations within PRh L1, the site that we stimulated to induce e-LTP. To further validate the projection, we injected AAV-CaMKII-ChR2-mCh in ACx, and subsequently recorded light evoked excitatory postsynaptic potentials (EPSP) within PRh. Figure 1D shows a schematic of the AAV injection site, and the site at which blue light was used to activate ChR2-containing ACx fibers within PRh. Figure 1E shows a high power image of the PRh recording site both in brightfield (left, asterisk in whole-cell recording pipette) and fluorescence which reveals the ChR2-containing fibers. Figure 1F shows light-evoked EPSPs recorded in PRh. These observations are consistent with previous reports showing an ACx projection to PRh in the rat and the gerbil (Burwell and Amaral, 1998; Budinger et al., 2000; Kotak et al., 2015). Figure 1G illustrates the ACx-PRh brain slice preparation, along with the stimulation and recording sites. Figure 1H shows a recording from a PRh L1 neuron, and illustrates that ACx-evoked depolarizing PSPs were observed. Figure 1I shows a recording from an infragranular PRh neuron, and illustrates that L1-evoked depolarizing PSPs were observed.

Evoked depolarizing PSPs were small in the absence of GABA receptor antagonists (Figure 2A), and both GABA_A and GABA_B inhibitory components were revealed when the holding potential was shifted to -50 mV from -65 mV (Figure 2B). Our previous work showed that EPSPs could reach action potential threshold in the presence of 20 μ M BIC. Thus, we asked whether subthreshold EPSPs could be elicited in the presence of lower concentrations of BIC (15, 10, 5 and 2 μ M). However, at each of these doses, EPSPs reached threshold. Figure 2C shows one example recording obtained in the presence of 2 μ M BIC. The amplitude of the EPSP at the action potential inflection point was 24.1 ± 0.5 mV (n=16). In contrast, when a dose of 1.5 μ M BIC was bath applied, the evoked EPSP did not reach threshold, even with the addition of 10 μ M SCH (Figure 2D).

For LTP induction, we used a TBS protocol applied to ACx-recipient PRh L1. We chose to stimulate ACx-recipient L1 PRh for LTP experiments: (1) to circumvent polysynaptic pathway, and (2) to limit the variability in EPSP size. During TBS stimulation, neurons were held at -50 mV to augment PRh neuron discharge rate, which was necessary to induce e-LTP (Figure 2E). Prior to and after TBS, EPSPs were recorded at $V_{\text{HOLD}} = -65$ mV.

In control recordings without pharmacological agents (Figure 3A), e-LTP was only observed in only 2 of 13 recordings, and most neurons displayed excitatory long-term depression (e-LTD). To test whether e-LTP was prevented by GABAergic inhibition, we first assessed whether GABA_A receptor-mediated inhibition alone was involved. Figure 3B shows that a low concentration of BIC was not sufficient to reliably induce e-LTP. Only 5 of 18 PRh

neurons displayed e-LTP, and most neurons displayed e-LTD. We therefore performed a final set of experiments applying TBS in the presence of BIC (1.5 μ M) and 10 μ M SCH. Figure 3C shows that, under these conditions, e-LTP was reliably induced in 13 of 15 PRh neurons. The average change in PSP amplitude was significantly greater for the BIC+SCH group, as compared to either the Control or the BIC only groups (Control: $63 \pm 12\%$; BIC: $78 \pm 14\%$; BIC+SCH: $181 \pm 18\%$; 1-way ChiSquare test, followed by a Tukey-Kramer Honest Significant Difference post-hoc comparison of all means; $n=46$; $\chi^2=19.5$, $df=2$, $p<0.0001$).

PRh activity is associated with learning and mnemonic processing, and PRh lesions disrupt learned behaviors that involve auditory cues in rodents (Corodimas and LeDoux, 1995; Sacchetti et al. 1999; Lindquist et al. 2004; Bang and Brown, 2009a, 2009b; Plakke et al. 2009; Robinson et al. 2010; Gastelum et al. 2012). For example, PRh lesions can interfere with learning a task in which visual features determine whether target auditory stimuli are rewarded (Gastelum et al. 2012). A role for e-LTP during learning is supported by the observations that PRh neurons become more responsive to an auditory stimulus following conditioning (Furtak et al. 2007) or the presentation of a familiar visual stimulus (von Linstow et al. 2016). In fact, some forms of learning are diminished by blocking e-LTP (Warburton et al., 2005). A role for e-LTD during learning is supported by the observations that PRh neurons can become less responsive to familiar visual stimuli (Zhu et al. 1995; von Linstow Roloff et al. 2016). Although distinct cellular mechanisms have been identified for the induction of e-LTP and e-LTD (Banks et al. 2014), we sought to determine whether synaptic inhibition could serve as a gate between these two forms of excitatory synaptic plasticity. Our results suggest that strong synaptic inhibition biased plasticity towards e-LTD, whereas reduced inhibition permitted the induction of e-LTP (Figure 3).

Our findings are generally consistent with many studies showing that e-LTP is enabled or enhanced in the presence of GABA_A and/or GABA_B receptor blockers, as discussed above. In the absence of pharmacological agents, evoked PSPs were small and TBS stimulation generally induced e-LTD (Figure 3A). However, evoked PSPs were enhanced in the presence of a low concentration of BIC and SCH (Figure 2), and TBS generally induced e-LTP (Figure 3C). We acknowledge that the effect observed with 1.5 μ M BIC may have included sites other than the GABA_A receptor (Khawaled et al. 1999; Demuro et al. 2001). Our observations agree with findings from the hippocampal-septal pathway showing that e-LTP can be induced when both GABA_A and GABA_B receptors are blocked, but not when only GABA_A receptors are blocked (Hasuo and Akasu, 2001). Since GABAergic transmission is globally blocked by bath application of antagonists, ACx-mediated inhibition or inhibition driven by interceding regions between ACx and PRh such as ventroposterior ACx are also blocked (Kotak et al. 2015).

Under our experimental conditions, a reduction in GABA receptor-mediated inhibition was essential for e-LTP, implying that a disinhibitory mechanism operates in vivo to support learning. For example, a down-regulation of ACx synaptic inhibition is associated with auditory learning with either aversive or appetitive conditioning (Sarro et al. 2015). Thus it is possible that a cholinergic mechanism is responsible for inducing the diminished GABAergic inhibition that permits cortical plasticity (Froemke et al. 2007). Our results do

not rule out a different mechanism in which augmented inhibition supports excitatory long-term depression. In addition, our results do not rule out the participation of other neuromodulatory mechanisms. In fact, acetylcholine-dependent reduction of ACx inhibition is observed during associative fear learning (Letzkus et al. 2011). Our study provides a GABAergic mechanism by which these processes could permit the induction of e-LTP *in vivo*.

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Figure 1.

ACx projection to PRh and brain slice preparation. **A.** The schematic (top) shows the ACx injection site for anatomical tracing with AAV-CaMKII-GFP, and the transverse section (bottom) shows the injection site. **B.** A low magnification image of the dense ACx terminal arborization in PRh (dashed white rectangle indicates region shown in panel C). rhinal fissure, rf. **C.** A high power image of ACx arbors within PRh L1. **D.** The schematic shows the ACx injection site for AAV-CaMKII-ChR2-mCh, and the projection to PRh. **E.** Brightfield (left) and fluorescent (right) images are shown at the PRh recording site. ChR2-expressing ACx fibers are labeled with mCherry. Asterisk, recording pipette. **F.** Blue light (470 nm)-evoked EPSPs were recorded in PRh in response to ChR2-expressing ACx fibers.

G. The schematic shows the orientation of the ACx-PRh brain slice preparation (left) and the configuration of stimulation and recording sites (right). Medial geniculate nucleus, MG; hippocampus, Hipp. **H.** An ACx-evoked depolarizing PSP recorded in L1 of PRh. **I.** An L1-evoked PSP recorded in a PRh neuron. Note that optogenetically-evoked PSP amplitude was smaller than those recorded in response electrical stimulation.

Figure 2.

GABAergic inhibition shunts depolarizing EPSPs recorded in PRh. **A.** A PRh L1-evoked PSP recorded in a PRh neuron without GABA receptor antagonists. Note a delayed hyperpolarizing response (arrow) **B.** When the PRh neuron was held at -50 mV, both short-latency GABA_A and delayed GABA_B (arrow) IPSP components were revealed. Note the small depolarizing PSP immediately after stimulus onset is largely shunted. **C.** In the presence of 2 μ M BIC, the EPSP reached threshold to elicit an action potential. **D.** At a concentration of 1.5 μ M BIC and 10 μ M SCH, an evoked subthreshold EPSP could be recorded. **E.** An example of consecutive PRh neuron responses to L1-evoked TBS (arrowheads mark each stimulus pulse). Note that there was a gradual buildup of postsynaptic depolarization, leading to intense discharge in the PRh neuron. In this example, both BIC and SCH were in the bath.

Figure 3.

GABAergic inhibition gates the induction of e-LTP. **A.** Example evoked responses are shown before (black) and after (red) TBS in a control neuron without GABA receptor antagonists. The time course for e-LTD in this neuron is shown in the middle panel. The summary distribution of normalized PSP change in all control neurons (n=13) is shown to the right. Dashed line is the mean percent change. **B.** Example evoked responses are shown before (black) and after (red) TBS in a neuron treated with a low concentration of BIC. The time course for e-LTP in this neuron is shown in the middle panel. The summary distribution shows the PSP change in all neurons treated with low BIC (n=18), and indicates that most neurons displayed e-LTP. The blue arrow indicates a neuron with a percent change of 140%. **C.** Example evoked responses are shown before (black) and after (red) TBS in a neuron treated with both low BIC and SCH. The time course for e-LTP in this neuron is shown in the middle. The summary distribution shows the PSP change in all neurons treated with low BIC and SCH (n=15), and indicates that most displayed e-LTP. The blue arrows indicate 4 neurons with a percent change of 137, 174, 201, and 206%. The distribution was significantly greater than those shown in panels A and B (red asterisk).