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Blockade of in vitro ictogenesis by low-frequency stimulation coincides with increased epileptiform response latency

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LOW-FREQUENCY STIMULATION, delivered through transcranial magnetic or deep-brain electrical procedures, can reduce seizures in patients with pharmaco-resistant epilepsy. A similar control of ictal discharges is exerted by low-frequency electrical stimulation in rodent brain slices maintained in vitro during convulsant treatment. By employing field and “sharp” intracellular recordings, we analyzed here the effects of stimuli delivered at 0.1 or 1 Hz in the lateral nucleus of the amygdala on ictal discharge generation in the entorhinal cortex (Corkin et al. 1990). This clinical evidence is supported by experimental data that were obtained from several in vitro preparations. Thus, following the demonstration that ictal discharge generation in the entorhinal cortex is depressed by repetitive stimuli delivered at 0.5–1.0 Hz (Barbarosie and Avoli 1997), similar results were reported to occur in parahippocampal, areas such as the amygdala (Benini et al. 2003) or the insular cortex (Sudbury and Avoli 2007), as well as in the neocortex (Schiller and Bankirer 2007).

We have recently found that, under control conditions, the latency of the synchronous oscillatory events generated by perirhinal cortex (PC) networks in response to single-shock electrical stimuli delivered in a portion of the lateral nucleus of the amygdala (LA) progressively increases when the stimulus interval is decreased from 10 to 1 s (Biagini et al. 2015). Clinical and experimental evidence indicates that these limbic structures play pivotal roles in mesial temporal lobe epilepsy (Avoli et al. 2013; Gloor 1997). The frequency-dependent changes in the latency of the PC responses induced by repetitive stimulation of the LA (Biagini et al. 2015) may indeed reflect an adaptive, depressant mechanism on network excitability that could also play a role in the ability of 1-Hz stimulation to reduce or abolish seizure-like events (Avoli et al. 2013). To test this hypothesis, we have used here focal repetitive electrical stimulation of the LA to establish whether the ability of 1-Hz repetitive stimulation to depress ictogenesis in the PC during application of the K+ channel blocker 4-aminopyridine (4-AP), in an in vitro brain slice preparation, is accompanied by changes in the latency of the stimulus-induced epileptiform responses, as well as whether these effects can be modulated by the GABA_B receptor antagonist CGP 55845.

METHODS

Male, young adult Sprague-Dawley rats (130–180 g; Charles River, St. Constant, QC, Canada) were decapitated under halothane anesthesia, according to the procedures established by the Canadian Council on Animal Care. All procedures were performed according to the protocols and guidelines approved by the McGill University Animal Care Committee and Canadian Council on Animal Care. All efforts were made to minimize animal suffering and the number of animals used. Horizontal brain slices (450 μm) were prepared and maintained in vitro as in our previous studies (Benini et al. 2003; Biagini et al. 2015; Kano et al. 2005). Artificial cerebrospinal fluid composition was as follows (in mM): 124 NaCl, 2 KCl, 1.25 KH2PO4, 2 MgSO4, 2 CaCl2, 26 NaHCO3, and 10 d-glucose. 4-AP (50 μM) and in some experiments 3-N-(1-(3,4-dichlorophenyl)ethyl)amino-2-(S-)hydroxypropyl-P-benzyl-phosphinic acid (CGP 55845, 4 μM) were bath applied. Chemicals were acquired from Sigma (St. Louis, MO) with the exception of CGP 55845, which was obtained from Tocris Cookson (Langford, UK). Surgical separation of the parahippocampal...
areas from the hippocampus proper was performed with a razor blade mounted on a micromanipulator at the beginning of the experiment to minimize the possible influence exerted by CA3-driven activity (see Fig. 1A).

Field potential and sharp-electrode, intracellular recordings were obtained with glass pipettes that were filled with artificial cerebrospinal fluid (tip resistance = 1.2–10 MΩ) and 3 M K-acetate (tip resistance = 70–120 MΩ), respectively. Microelectrodes were placed closely (≤500 μm) in the PC deep layers (i.e., 600–800 μm from the pia), while a stimulating tungsten electrode was positioned in the LA (Fig. 1A). Recording microelectrodes were connected to high-impedance amplifiers and the resistance compensation for the intracellular recording microelectrode was monitored throughout the experiment and adjusted as required. The fundamental electrophysiological parameters of the neurons included in this study were measured as follows: 1) resting membrane potential after cell withdrawal; 2) apparent input resistance and membrane time constant from the voltage change in response to a hyperpolarizing current pulse (100–200 ms, less than −0.5 nA); 3) action potential amplitude and duration from the baseline and at half-width, respectively. These electrophysiological characteristics (Table 1) were similar to those previously reported by D’Antuono et al. (2001) and Biagini et al. (2015). PC cells could be identified as regularly firing neurons when injected with pulses of intracellular depolarizing current (D’Antuono et al., 2001). PC cells could be identified as regularly firing neurons when injected with pulses of intracellular depolarizing current (D’Antuono et al., 2001).

Table 1. Fundamental electrophysiological properties of the PC neurons recorded intracellularly in the deep layers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Means ± SD</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>RMP, mV</td>
<td>−71.1 ± 4.9</td>
<td>14</td>
</tr>
<tr>
<td>R∞, MΩ</td>
<td>37.9 ± 5.6</td>
<td>14</td>
</tr>
<tr>
<td>τ, ms</td>
<td>16.2 ± 2.1</td>
<td>6</td>
</tr>
<tr>
<td>APA, mV</td>
<td>88.7 ± 6.9</td>
<td>14</td>
</tr>
<tr>
<td>APD, ms</td>
<td>1.3 ± 0.2</td>
<td>14</td>
</tr>
</tbody>
</table>

n, No. of neurons. RMP, resting membrane potential; R∞, input resistance; τ, time constant; APA, action potential amplitude; APD, action potential duration.
Field potential and intracellular signals were fed to a computer interface (Digitida 1200B, Axon Instruments, Union City, CA), and were acquired and stored by using the pClamp 8 software (Axon Instruments). Subsequent data analysis was made with the Clampfit 8 software (Axon Instruments).

A cutoff at 3 s, similar to what was previously chosen by Traub et al. (1996), was used to separate ictal from interictal epileptiform events. Single-shock stimuli (0.1 mA, 50–100 μs) in LA were delivered either randomly between 30 and 240 s after the termination of a spontaneous ictal discharge, or in a repetitive fashion at 0.1 or 1 Hz for periods of 6 min while keeping the stimulus strength constant throughout the session. Threshold stimulation strength was established in each experiment as that capable of consistently eliciting an ictal discharge when delivered during the random protocol; stimulation protocols were then performed at 0.1 and 1 Hz by employing such stimulation strength. Each series of stimuli was followed by a period of “no stimulation” lasting 12–20 min (Fig. 1B) with the exception of the experiments aimed at identifying the recovery dynamics of the changes induced by 1-Hz stimulation (schematic drawing in Fig. 3D).

Duration of the epileptiform events was obtained from the field recording trace by measuring the time between the first negative deflection and the full return to baseline (asterisk in Fig. 1C, Control). The latency of the stimulus-induced responses was measured from the stimulus artifact to the rising phase of the first action potential associated with the stimulus-induced response (start in Fig. 3Aa, 0.1-Hz column). Values under each experimental condition were obtained by averaging the latency of the intracellular responses that were generated during the stimulation session. Jitter of the stimulus-induced responses was established by measuring the difference between the latency of the earliest response and that of the longest response (see Fig. 3Ab, 1-Hz column).

Measurements in the text are expressed as means ± SD, and n indicates the number of neurons/experiments studied under each specific protocol. Data were statistically compared with one-way ANOVA, followed by Bonferroni corrections for multiple comparisons. Independent-samples t-tests and linear regressions were also applied when appropriate. Statistical analyses were performed in Matlab 7.9.0 (Mathworks, Natick, MA). The level of significance was set at P < 0.05.

RESULTS

Spontaneous and stimulus-induced epileptiform activity in the PC. Simultaneous field potential and intracellular recordings performed in 14 experiments from an equivalent number of brain slices, during continuous application of 4-AP, revealed the spontaneous occurrence of interictal (duration = 0.8–2.2 s; interval of occurrence = 18–34 s; arrowheads in Fig. 1Ca) and ictal (duration = 22–56 s; interval of occurrence = 146–375 s; dotted line in Fig. 1Ca) discharges. The mean values of the duration and interval of occurrence of the spontaneous ictal activity were 42.6 ± 10.3 s and 263.7 ± 82.3 s, respectively (n = 14). Both types of spontaneous epileptiform activity occurred regularly under control conditions (i.e., for at least 20 min before implementing any stimulation protocol). In addition, we found in these experiments a direct correlation between the length of the period between ictal discharges and their duration (r = 0.58, P < 0.05) (Fig. 1D). As reported in previous studies (Biagini et al. 2013; de Guzman et al. 2004), the onset of the ictal discharge recorded from the PC was characterized either by a series of three to four events that progressively increased in their rate of occurrence (Fig. 1Ca, arrows) or by a single, large amplitude transient (Fig. 2A, arrow in Control trace). Both types of onset evolved into a “tonic” phase that was followed by rhythmic depolarizations associated with action potential firing (i.e., the “clonic” phase) (Figs. 1C and 2A). Ictal discharges with similar electrographic characteristics could also be elicited by single-shock stimuli delivered in LA (Figs. 1Cc and 2A). When stimulating randomly 30–240 s after the termination of a spontaneous ictal event, we could identify a period of relative refractoriness that lasted several tens of seconds (compare epileptiform responses induced by single-shock stimuli in Fig. 1Cb, double arrows, and in Cc). The data obtained in eight experiments during such random stimulation, which are summarized in Fig. 1E, were characterized by a significant correlation (r = 0.94, P < 0.01).

LA repetitive stimulation and ictal discharge generation. Following a control period lasting over 20 min, repetitive stimulation was then implemented at either 0.1 or 1 Hz. As shown in Fig. 2A, ictal discharges continued to occur spontaneously during stimulation of the LA at 0.1 Hz, although some of the stimulus-induced epileptiform responses were similar to the interictal events occurring spontaneously (arrowheads in Fig. 2A, 0.1-Hz trace) or could be characterized by afterdischarge or failure (double arrowheads or open circles, respectively, in Fig. 2A, 0.1-Hz trace). Following a period of no stimulation (Fig. 2A, Poststimulus trace), repetitive stimuli were then delivered at 1 Hz. This procedure abolished ictal discharges in 12 of 14 experiments (Fig. 2A, 1-Hz trace), while it markedly reduced their duration and rate of occurrence in the two remaining cases. The responses generated by PC neurons during 1-Hz stimuli delivered to LA were characterized by interictal-like depolarizations associated with action potential firing lasting over 400 ms; however, response failures could also occur (open circles in Fig. 2A, 1-Hz trace). Spontaneous interictal and ictal discharges reappeared upon termination of the 1-Hz stimulation protocol (Fig. 2A, Poststimulus trace). Similar effects were seen when the stimulation protocols were inverted (i.e., first a period of stimuli at 1 Hz and then, after either “no” or randomly delivered single-shock stimulation, a period of consecutive stimuli at 0.1 Hz, see Fig. 1B).

The effects induced by repetitive stimulation at 0.1 and 1 Hz on the duration and rate of occurrence of spontaneous ictal discharges are summarized in Fig. 2B, while the length of time between the termination of the stimulus protocols at these two frequencies and the reappearance of the first spontaneous ictal discharge upon termination of the stimulation protocols are plotted in Fig. 2C. A significant effect of condition [Control, 0.1 Hz, 1 Hz, and poststimulus (PS)] was observed [F = 25.5, degrees of freedom (df) = 4, P < 0.01] and post hoc tests indicated that stimulation at 1 Hz induced a significant decrease in ictal discharge duration compared with all other conditions (P < 0.05) (Fig. 2B). Similar results were observed for the rate of occurrence of ictal discharges (F = 31.3, df = 4, P < 0.01), since stimulation at 1 Hz induced a significant decrease compared with all other conditions (P < 0.05) (Fig. 2B). Finally, the time before the reappearance of the first spontaneous ictal discharge upon termination of the 1-Hz stimulation protocol was significantly longer compared with the 0.1-Hz condition (P < 0.05) (Fig. 2C).

Next, we analyzed the latency of the responses generated by PC neuronal networks to LA stimuli delivered at 0.1 and 1 Hz. As illustrated in Fig. 3Aa, the first of a series of stimuli delivered at either 0.1 or 1 Hz induced responses with similar latencies. However, while successive stimuli at 0.1 Hz caused responses with latencies that were fairly constant from one
pulse to the next, those implemented at 1 Hz jittered by several tens of milliseconds (Fig. 3Ab). Specifically, during 1-Hz stimulation, the response latency to the first four to six stimuli of a series was similar, but then it progressively increased to attain a relatively steady value that was approximately the double of what was seen with the first stimuli. These findings are quantified for 14 experiments in the plot of Fig. 3B, which shows that the latency of the epileptiform responses induced by random stimulation (Control) as well as during the two PS periods or by 0.1-Hz stimuli had similar mean values (80 ms), while the latency of the responses generated during 1-Hz stimulation had mean values ~80 ms.

Statistical analyses revealed a significant effect of condition (Controls, 0.1 Hz, PS and 1 Hz) \( (F = 47.9, df = 4, P < 0.01) \), as well as that of the latency associated with the 1-Hz stimulation was significantly longer compared with all other conditions \( (P < 0.05) \) (Fig. 3B). These effects were mirrored by significant differences in the amount of jittering of the responses obtained under these different conditions \( (F = 101.03, df = 2, P < 0.01) \); post hoc tests showed that stimulation at 1 Hz induced a significant increase in the amount of jittering compared with all other conditions \( (P < 0.05) \) (Fig. 3C). The amount of jittering during stimulation at 0.1 Hz was, however, significantly lower compared with the control condition \( (P < 0.05) \). Therefore, these data indicate that the reduction and/or block of ictal discharges during repetitive stimulation at 1 Hz was accompanied by a robust increase in the latency of the stimulus-induced epileptiform responses. As illustrated in Fig. 3D, we established in 10 experiments the recovery time of the latency prolongation following termination of the 1-Hz stimulation. The increase in latency of the epileptiform responses induced by 1-Hz stimulation recovered to values similar to those seen under control (i.e., during periods when no stimulation or only random stimuli were applied) conditions within 20 s, once the stimulation frequency was changed to 0.1 Hz (\( F = 62.8, df = 2, P < 0.01 \), post hoc tests \( P < 0.05 \)).

Effects induced by blocking GABA_B receptor signaling. Since GABA_B receptors contribute to the frequency-dependent prolongation of the latency of the synchronous oscillatory responses recorded under control conditions in the PC during repetitive LA stimulation (Biagini et al. 2015), we analyzed the effects induced by the GABA_B receptor antagonist CGP 55845...
We have found in this study that: 1) ictal discharges induced by bath application of 4-AP in the PC are practically abolished during repetitive stimulation of the LA at 1 Hz; 2) this effect is associated with an increased latency of the epileptiform responses recorded in PC following each stimulus at 1 Hz; and 3) both changes recovered to prestimulus conditions upon arrest of the 1-Hz stimulation protocol. In addition, we have discovered that the control of ictal activity by 1-Hz stimulation and the concomitant latency increase are significantly reduced during pharmacological blockade of GABA_B receptors.

Repetitive LA stimulation at 1 Hz controls ictogenesis in the PC and prolongs response latency. As reported in adult brain tissue (Biagini et al. 2013; de Guzman et al. 2004), PC networks recorded in brain slices obtained from young animals generated interictal and ictal epileptiform discharges during application of 4-AP; the field potential and intracellular characteristics of these epileptiform events were similar to those of the responses recorded in PC following each stimulus at 1 Hz; and 3) both changes recovered to prestimulus conditions upon arrest of the 1-Hz stimulation protocol. In addition, we have discovered that the control of ictal activity by 1-Hz stimulation and the concomitant latency increase are significantly reduced during pharmacological blockade of GABA_B receptors.

DISCUSSION

Finally, we found that the progressive increase in latency of the responses to 1-Hz stimuli along with the jitter were not seen in the presence of CGP 55845 (Fig. 4, C and D). As a result, the mean latency of the responses to 1-Hz stimuli was significantly longer and the jitter was significantly higher during bath application of 4-AP compared with the GABA_B receptor blocker (F = 38.3, df = 5, P < 0.05, post hoc tests, P < 0.05) (Fig. 4C, P < 0.05). However, under CGP 55845, the latency of the responses was significantly longer during stimulation at 1 Hz compared with the control and PS condition (P < 0.05) (Fig. 4C). The jitter did not significantly differ between Control, stimulation at 1 Hz and PS (Fig. 4D).

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reported in several brain structures in the presence of this K⁺ channel blocker (see for review, Avoli and de Curtis 2011), and thus they will not be further discussed. In addition, single-shock electrical stimuli delivered in LA were capable of inducing in PC ictal discharges that closely resembled those occurring spontaneously. This evidence is in line with the well-known reciprocal innervation between the PC and LA (Paz and Pare 2013; Shi and Cassell 1999; von Bohlen und Halbach and Albrecht 2002) that underlies the highly correlated neuronal activity occurring in these two limbic structures under physiological conditions (Gloor 1997; Squire 1993). Our laboratory has also reported in two previous studies performed in this specific in vitro brain slice preparation that, under control conditions, LA and PC networks generate synchronously spontaneous or stimulus-induced oscillatory network-driven events (Biagini et al. 2015; Kano et al. 2005). Interestingly, following a spontaneous ictal event, the ability of PC networks to generate a similar ictal discharge in response to LA stimuli was characterized by a period of relative refractoriness that lasted over 200 s, a value that was similar to the mean interval of occurrence of the spontaneous ictal activity.

We have found here that 4-AP-induced ictogenicity in PC is depressed by repetitive stimulation of the LA at 1 Hz, but not when stimuli were delivered at 0.1 Hz. These results are in line with those obtained from previous in vitro studies in which it was shown that repetitive electrical stimuli delivered at 0.5–1.0 Hz can reduce and block ictal discharge generation in several limbic (Barbarosie and Avoli 1997; Barbarosie et al. 2002; Benini et al. 2003) and extralimbic (Schiller and Bankirer 2007; Sudbury and Avoli 2007) structures. In the last few years, several clinical investigations have proposed low-frequency stimulation, delivered through transcranial magnetic or deep-brain electrical procedures, as an alternative antiepileptic procedure to be implemented in epileptic patients who are refractory to conventional antiepileptic therapy (Tergau et al. 1999; Theodore and Fisher 2004; Vonck et al. 2002; Yamamoto et al. 2006). However, the real new finding of our study is that the control on ictal discharges in vitro is associated with a significant increase in the latency of the stimulus-induced responses, when values are compared with those elicited by stimuli delivered both at 0.1 Hz, or randomly within 30–240 s after the termination of a spontaneous ictal discharge.

A similar frequency-dependent change in latency during 1-Hz repetitive activation of PC neuronal networks has recently been reported by our laboratory for the responses induced by repetitive stimulation of the LA under physiological condition, i.e., in the absence of any convulsive drug (Biagini et al. 2015). Therefore, we are inclined to conclude that this adaptive, depressant mechanism may contribute to control network excitability and thus ictogenesis. Interestingly, and similar to what was identified in our laboratory’s previous “physiological” study (Biagini et al. 2015), the increased latency of the responses generated by PC networks recovered to prestimulation values within 20 s following arrest of the 1-Hz

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stimulation procedure. This evidence suggests that long-term depression of glutamatergic transmission, which is readily induced by 1-Hz stimuli (Cho et al. 2000; Park et al. 2006), is presumably not contributing to the frequency-dependent increase in latency as well as to the blockade of ictal activity reported in our study.

Contribution of GABA<sub>B</sub> receptors to ictogenesis control and stimulus-induced response latency prolongation. We have found that the ability of 1-Hz stimulation of the LA to abate ictal discharges generated by PC neuronal networks is markedly reduced by application of the GABA<sub>B</sub> receptor antagonist CGP 55845. In addition, this effect was associated with fading of the increased latency of the responses to repetitive 1-Hz stimuli along with their jittering. This evidence supports a causal relation between the anti-ictogenic effects of this stimulation procedure and the latency prolongation of the stimulus-induced responses. Moreover, these data are in agreement with those recently obtained by analyzing the latency increase of the responses generated by PC neurons during repetitive stimulation of the LA under physiological condition; we found in this study that CGP 55845 reduced significantly the frequency-dependent differences in latency prolongation (Biagini et al. 2015). Both presynaptic and postsynaptic GABA<sub>B</sub> heteroreceptors, which are likely to be repetitively activated during 1-Hz stimulation following GABA release from PC interneurons, are known to modulate neuronal excitability and thus to influence the latency of these responses (Brenowitz et al. 1998; Craig and McBain 2014; Kohl and Paulsen 2010; Scanziani 2000). A powerful feed-forward inhibitory network has been demonstrated in the PC following stimulation of both entorhinal cortex and neocortex (de Curtis and Paré 2004). It should, however, be mentioned that our findings contradict those obtained in a previous study in which it was shown that the ability of 1-Hz stimulation to control ictogenesis in the adult mouse entorhinal cortex was not influenced by the GABA<sub>B</sub> receptor antagonist CGP 35348 (Barbarosie et al. 2002); although species- and age-related differences should be taken into account, we are inclined to interpret this incongruity to reflect failure of this drug to antagonize effectively GABA<sub>B</sub> receptors.

Other synaptic and nonsynaptic mechanisms can, however, contribute to the ability of 1-Hz stimulation to increase the response latency and to abolish ictal discharge generation. For instance, modulatory depressant actions on neuronal excitability can result from the activation of pre- and postsynaptic adenosine A1 receptors (Haas and Sellbakh 2000), a receptor that has been implicated in the treatment of epilepsy (Swía, der et al. 2014). Moreover, one cannot exclude the active participation of intrinsic currents that are associated with voltage-, calcium-, or sodium-dependent afterhyperpolarizations (Abolafia et al. 2011; Sanchez-Vives et al. 2000; Schwindt et al. 1989; Spain et al. 1991). Interestingly, Gulledge et al. (2013) have recently identified a previously unappreciated role of sodium-potassium ATPase in regulating cortical excitability. All of these mechanisms, once activated by repetitive stimuli, could participate to decreasing epileptiform synchronization, as demonstrated in our study when PC networks are activated by LA inputs.

Extracellular alkalization is also known to occur during intense neuronal synchronization (Chesler and Kaila 1992; de Curtis et al. 1998), and this process can reduce gap-junction coupling (Church and Baimbridge 1991; Valiante et al. 1995); hence, one should expect as a result a decrease in excitability resulting in a prolongation of the time required for the neuronal recruitment induced by the stimulation procedures. In addition, it has been reported in the entorhinal cortex that repetitive stimulation, even during ionotropic glutamate receptor blockade, leads to a steady increase in extracellular K<sup>+</sup> concentration (Avoli et al. 2012). Elevations in extracellular K<sup>+</sup> concentration, which presumably result from synaptic and nonsynaptic mechanisms (Avoli and de Curtis 2011; Heinemann et al. 1986; Kaila et al. 1997; Viitanen et al. 2010), can paradoxically decrease the propagation of neural activity in axonal pathways along with the presynaptic release of neurotransmitters (Durand et al. 2010).

Conclusive remarks. Our findings reveal the ability of the 1-Hz stimulation in the LA to increase response latency and abolish ictal discharges in PC neuronal networks during 4-AP treatment. We have also found that this effect is mediated through a GABA<sub>B</sub> receptor-dependent adaptive mechanism that decreases neural network synchronization. Such findings await confirmation in vivo, following acute seizures induced by 4-AP or in animal models of refractory seizures. The modulation, through stimulation at 1 Hz, of neural activity in mesial temporal lobe structures known to play a role in ictogenesis may thus represent an alternative approach to the treatment of patients with seizures that are refractory to anti-epileptic drugs.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: T.K., Y.I., and M.D. performed experiments; T.K., Y.I., M.D., G.B., M.L., and M.A. analyzed data; Y.I., M.D., G.B., M.L., and M.A. drafted manuscript; Y.I., M.D., G.B., M.L., and M.A. edited and revised manuscript; M.D. and M.A. conception and design of research; M.A. interpreted results of experiments; M.A. prepared figures.

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Activity-dependent changes in perirhinal cortex excitability


