LEARNING-INDUCED MODULATION OF THE GABA\textsubscript{B}-MEDIATED INHIBITORY SYNAPTIC TRANSMISSION: MECHANISMS AND FUNCTIONAL SIGNIFICANCE

Running title: Learning-induced enhancement of GABA\textsubscript{B} activity

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ABSTRACT

Complex olfactory-discrimination (OD) learning results in a series of intrinsic and excitatory synaptic modifications in piriform cortex pyramidal neurons that enhance the circuit excitability. Such over-excitation must be balanced to prevent runway activity while maintaining the efficient ability to store memories. We showed previously that OD learning is accompanied by enhancement of the GABA_A-mediated inhibition. Here we show that GABA_B-mediated inhibition is also enhanced after learning, study the mechanism underlying such enhancement and explore its functional role.

We show that pre-synaptic, GABA_B-mediated synaptic inhibition is enhanced after learning. In contrast, the population-average post-synaptic GABA_B-mediated synaptic inhibition is unchanged, but its standard deviation is enhanced. Learning-induced reduction in paired pulse facilitation (PPF) in the glutamatergic synapses interconnecting pyramidal neurons was abolished by application of the GABA_B antagonist CGP55845 but not by blocking GIRK channels only, indicating enhanced suppression of excitatory synaptic release via pre-synaptic GABA_B-receptors activation. In addition, the correlation between the strengths of the early (GABA_A-mediated) and late (GABA_B-mediated) synaptic inhibition was much stronger for each particular neuron after learning. Consequently, GABA_B-mediated inhibition was also more efficient in controlling epileptic-like activity induced by blocking GABA_A receptors.

We suggest that complex OD learning is accompanied by enhancement of the GABA_B-mediated inhibition that enables the cortical network to store memories while preventing uncontrolled activity.
INTRODUCTION

Rats that are trained in a particularly difficult olfactory-discrimination (OD) task demonstrate a dramatic increase in their capability to acquire memories of new odors, once they have learned the first discrimination task ('rule learning') (Saar et al., 1998; 1999). Such rule learning is accompanied by a series of pre and post–synaptic cellular modifications in layer II pyramidal neurons of the piriform cortex. Long-term enhancement occurs in the three components controlling neuronal activation; the excitatory synaptic drive mainly mediated by glutamate receptors (Saar et al., 1999; 2002, Knafo et al, 2005), the intrinsic neuronal excitability (Saar et. al 1998, 2001, Cohen-Matsliah et al., 2007), and synaptic inhibition mediated by GABA\textsubscript{A} receptors (Brosh and Barkai 2009). These modifications are widespread throughout the piriform cortex network; physiological and morphological modifications are found in most of the studied neurons (Saar et al., 1998, 1999; 2002, Saar and Barkai 2003; Knafo et al., 2005, Brosh an Barkai 2009). Similar large overall increase in synaptic and intrinsic excitability following various training paradigms were demonstrated in different brain structures (McKernan et al., 1997; Moyer et al., 1996; Oh et al., 2003; Sacchetti et al., 2001, 2004; Tye et al., 2008; Yin et al., 2009).

Since excitatory synaptic transmission and neuronal excitation are both profoundly enhanced by learning, the cortex may enter an over-excited state, during which epileptic-like activity propagates along the region (Chagnac-Amitai and Connors, 1989). Such hyper-excitable activity may prevent any efficient ability to store memories (see for example, Barkai et al. 1994, Hasselmo and Barkai 1995). Strengthening inhibition is a possible homeostatic mechanism for preventing the epileptic-like activity. Using theoretical analysis of network models (Golomb and Ermentrout, 2002), it has been shown that if the excitatory-to-excitatory synaptic
coupling is increased, a substantial enhancement in inhibition is needed to prevent the appearance of such epileptic discharge. Indeed, activity-induced enhancement of inhibitory synaptic transmission has been shown in several brain regions of the mammalian brain (Kano 1995, Grunze et al., 1996; Komatsu and Yoshimura, 2000; Holmgren and Zilberter, 2001). Both pre and post-synaptic mechanisms are implicated in the process. For example, activity-induced enhancement in the number of GABA_A receptor was shown in the hippocampus after kindling (Nusser et al., 1998) and enhanced GABA release was shown after BDNF application (Ohba et al., 2005). In a particularly interesting recent study, it was shown that learning of the eyeblink conditioning results with enhanced intrinsic excitability of hippocampal somatostatin-positive inhibitory neurons, that results enhanced inhibition onto CA1 pyramidal neurons (Mckay et al., 2013). We previously showed that olfactory-discrimination learning results with long-lasting enhancement of inhibitory synaptic transmission onto proximal dendrites of piriform cortex layer II pyramidal neurons (Brosh and Barkai, 2009). This enhancement is mediated by a strong hyperpolarizing shift in the reversal potential of GABA_A receptor-mediated inhibitory post synaptic potentials (IPSPs). OD rule learning also results in enhanced amplitude of spontaneous inhibitory synaptic events in piriform cortex pyramidal neurons (Saar et al., 2012). Moreover while enhanced synaptic inhibition is evident in most of the recorded neurons (Brosh and Barkai, 2009; Saar et al., 2012) some neurons receive an exceptionally large increase in GABA_A-mediated synaptic inhibition (Saar et al., 2012). Although GABA_B-mediated inhibitory synaptic transmission has been implicated in multiple forms of learning and memory (Bowery et al., 2002), the precise manner by which it is modulated to enhance learning and memory is yet to be explored. In the piriform cortex, GABA_B-mediated synaptic inhibition is a key player in modulation of neuronal and network activation, and it affects post-synaptic activity (Tseng and Haberly1988), as well as glutamatergic synaptic release (Tang and Hasselmo 1994).
Here we examine whether OD rule learning is also accompanied by modulation of GABA_B-mediated activity and explore the mechanisms underlying such modulation.

METHODS

Animal training

Subjects and apparatus: Age-matched young adult Sprague-Dawley male rats were used. Prior to training they were maintained on a 23.5 hr water-deprivation schedule, with food available ad libitum. Olfactory discrimination training protocol was performed daily on each trained and pseudo-trained rat in a 4-arm radial maze (figure 1A), with commercial odors that are regularly used in the food industry.

Training: Olfactory training consisted of 20 trials per day for each rat as previously described (Saar et al., 2001). In short, in each trial the rat had to choose between two odors (positive- and negative-cue) presented simultaneously. Rats designated to the trained group were rewarded upon choosing the positive cue. Rats in the pseudo-trained group were rewarded in a random fashion, upon choosing any odor. The criterion for learning was at least 80% positive-cue choices in the last 10 trials of a training day, as was previously used (Saar et al., 1999, 2001). Rats in the naive group were water restricted, but not exposed to the maze. Typically, 2-3 trained rats and 2-3 pseudo-trained rats were trained at the same training period, and all the rats in the trained group had to meet the criteria for the first pair of odors before all trained and pseudo-trained rats were exposed to a second pair of odors. Training for a new pair began only after training for the second pair was completed for all rats. Rats were trained with two pairs of odors to ensure rule learning.

As previously described (Saar et al., 1999, 2001), rats indeed learned the second pair of odors much faster than the first pair (7-8 days of training for the first
These data confirm our precious observation, that there are two phases of olfactory-discrimination learning, each with a distinct time course: a first phase during which the animal gradually acquires the appropriate behavioral strategy for completing the task (rule learning) and a second phase when the subject quickly acquires specific odor/reward associations (pair learning). Pseudo trained rats received that same number of runs.

Electrophysiological recordings

Animals were sacrificed five days after training completion, when olfactory learning-induced enhancement of excitatory synaptic transmission in the piriform cortex is at its peak (Saar et al. 1999). Experiments were done blind; the group affiliation of the rats (naive, trained, or pseudo-trained) was unknown to the person conducting the experiments and measurements. Rats were anesthetized with Pentobarbital (60 mg/kg), the brain was removed, and coronal brain slices of 400 μm of the posterior piriform cortex were cut as previously described (Saar et al, 1999). Brain slices were kept in oxygenated (95% O₂ + 5% CO₂) normal slice ringer (NSR), containing NaCl 124 mM, KCl 3 mM, MgSO₄ 2 mM, NaH₂PO₄ 1.25 mM, NaHCO₃ 26 mM, CaCl₂ 2 mM and glucose 10 mM.

Layer II pyramidal neurons receive inhibitory synaptic inputs from two sources; feed-forward (FF) inhibition is evoked by afferent inputs arriving to layer I via GABAergic neurons located at layer I, while feed-back (FB) inhibition is evoked by layer II pyramidal neurons via GABAergic neurons located in layer III. Inhibitory synaptic transmission is morphologically segregated; FF inhibition is terminated on the distal portion of the apical dendrite, while FB inhibition is terminated on the proximal dendrites (Suzuki and Bekkers, 2007). Consequently, DC current passed via the sharp recording electrode would be much more efficient in modifying the neuron's...
membrane potential at the locations where FB inhibition is generated. Thus, the stimulation electrode was placed just under layer II, at the border of layer III (figure 1B).

Intracellular current clamp recordings were performed in layer II pyramidal neurons using sharp microelectrodes with 4M K-Acetate containing electrodes. Membrane potential was shifted to different values by applying DC current via the recording electrode. Six to ten synaptic responses were evoked at by stimulating at a frequency of 0.05 HZ and digitally averaged to produce the measured response for each membrane potential. The reversal potential of the synaptic response was interpolated with a linear fit of the graph describing the synaptic response as the function of the membrane potential. In each cell, IPSPs were recorded in 5-6 different membrane potentials. The early, GABA_A-mediated (Tseng and Habelry, 1988), IPSP amplitude was measured at the first peak after stimulation, which usually occurred with a delay of 7-15 ms from synaptic stimulation. The late, GABA_B-mediated (Tseng and Habelry, 1988) IPSP amplitude was measured at the second peak after stimulation, in the trace recorded at the highest holding potential (figure 2A). The late IPSP peak occurred with a delay of 120-170 ms after stimulation.

For paired pulse facilitation (PPF) measurement, the stimulating electrode was placed in layer Ib of the piriform cortex, to stimulate the intrinsic fibers (figure 1B). Electrical stimuli were applied at 0.1 Hz. The amplitudes of the responses were measured from digital averaging of 10 consecutive responses. To standardize the intracellular recording conditions, stimulus intensity was adjusted so that the averaged amplitude of the PSPs in the recorded cell would be about 10 mV at V_m = -80 mV. Then, a pair of stimuli, with inter stimulus interval of 50 ms, were applied to evoke PPF.
To measure the velocity of propagation of paroxysmal discharges in disinhibited slices (Golomb and Amitai, 1997), two field potential recording electrodes (filled with 1 M NaCl, 6-8 M$^+$) were placed ~1.5-2.0 mm apart in layers II. The cortex was stimulated by 0.1-ms, 0.01- to 0.05-mA pulses at 0.1 Hz, delivered by stimulating electrode placed in layer II, at a distance ≥0.5 mm from the first recording electrode. Propagation velocity was calculated by measuring the difference in delay to onset of the epileptiform activity in the two recording sites and dividing it by the distance between these sites.

The GABA$\text{A}$ blocker, bicucullinemethobromide (BMB) (20 µM), the GABA$\text{B}$ blocker CGP55845 (1 µM), the AMPA receptors blocker DNQX (0.2-20 µM), NMDA receptors blocker APV (50 µM) and the GIRK channel blocker SCH23390 (3 µM) were applied via the perfusing solution.

**GABA$\text{B}$-mediated Vs GABA$\text{A}$-mediated conductance ratio**

To examine whether the conductance of the late IPSP is modified, we compared in each neuron the relations between the early and late IPSPs evoked by the same stimulus, by calculating the slopes of the graph describing the amplitudes of the IPSPs as a function of $V_m$ (figure 5B). The $\text{Early IPSP/Late IPSP slopes ratio}$ was used to explore conductance modifications.

**Immunoblot analysis**

Immunoblot analysis was performed as previously described (Brosh et al., 2006; Segev et al., 2013) The olfactory cortex was rapidly dissected (8 slices from each rat) in the same saline Ringer solution as used for brain slices, and immediately homogenized in 300 µl SDS*2 sample buffer (5 mMTris-HCl, pH 6.8; 10% glycerol; 2.3% sodium dodecyl sulfate (SDS) and 5% $\beta$-mercaptoethanol), boiled at 100 °C for 5 min, vortexed and stored at –80°C. For scaling purposes, we first identify the linear scale of
the different antibody-protein interaction, as defined by an increased protein concentration. Equal amounts from each sample (10μl) were separated on 7.5% polyacrylamide gel (SDS polyacrylamide gel electrophoresis (SDS-PAGE), 30mA) followed by blotting to a nitrocellulose membrane at 350 mA for 1 hour. Blots were blocked in freshly prepared Tris-buffered saline solution containing 0.1% Tween (TBST) with 5% dry milk and 5% bovine serum albumen BSA for GABA<sub>B</sub>R1 and β-actin respectively for 1 hour in room temperature. Then, the blots were incubated with primary antibody (GABA<sub>B</sub>R1 Abcam 1:1000 mouse; Actin 1:3000 goat, Santa Cruz) overnight at 4°C. After three washing steps, blots were incubated for 1 h in room temperature with secondary antibody coupled to horseradish peroxidase (HRP). After 3 more washing with TBST, proteins were visualized by enhanced chemiluminescence EZ-ECL (Biological Industries) and quantified using a CCD camera (XRS Bio-Rad) and Quantity One software. We then calculated the ratio between GABA<sub>B</sub>-R1 and β-actin for each rat.

**Statistical analysis**

Student's t-test was used to compare between two cell populations. Two-way ANOVA followed by pot-hoc t-test was used to evaluate the significance of difference between three and more cell populations. Two-way ANOVA was used to evaluate significance of difference between epileptiform wave's propagation velocities, by determining the differences between the trained and control groups prior to and after drug application and by determining the differences within each group prior to and after drug application. Linear regression was performed with Origin Lab software, using weighted least-square method to fit a linear model function to data. Comparison of cumulative frequency curves describing Early IPSP Vs late IPSP conductance ratio (figure 5D) were done using The Kolmogorov-Smirnov test.
RESULTS

As previously shown (Brosh and Brakai 209), the averaged reversal potential of early IPSP was significantly lower after learning (-78.0±5.1 mV, n=38 for trained compared to -73.0±6.6 mV, n=32 for naive and -73.0±6.4 mV, n=29 for pseudo-trained, p<0.001). Since neurons from pseudo trained and naïve rats had an identical averaged reversal potential for the early IPSP, these two groups were combined to a single control group (averaged value of 73.0±6.5, n=61). Such learning-induced reduction in the early IPSP reversal potential is apparent throughout the recorded neuronal population, as shown in the cumulative frequency graph (Figure 2B). In contrast, the averaged reversal potential of late IPSP was not modified by learning, and had the expected value, close to that reversal potential of the potassium ion (-86.8±6.2 mV, n=20 in trained compared to -86.2±8.7 mV, n=27 in controls).

Learning-induced enhancement in GABA<sub>B</sub>-inhibition potency to suppress excitatory synaptic release

GABA<sub>B</sub> receptors have been suggested to influence synaptic release via several mechanisms, which differ from the mechanism by which the GABAB-mediated late IPSP is generated. Inhibition of voltage-dependent calcium channels, opening of potassium channels and reducing vesicles priming are among these suggested pre-synaptic mechanisms (reviewed in Chalifoux and Carter, 2011). Thus, we next examined whether learning-induced enhancement in the potency of GABA<sub>B</sub>-mediated inhibition has a pre-synaptic component. Paired pulse facilitation (PPF) of excitatory synaptic responses between layer II pyramidal neurons is reduced for several days after OD learning (Saar et al., 1999). While these data may be interpreted as indirect evidence for enhanced glutamatergic synaptic release, it could also reflect the enhanced recruitment of feedback inhibition that reduces synaptic release via activation of pre-synaptic
GABA\textsubscript{B} receptors (Tang and Hasselmo, 1994). Specifically, if learning-induced reduction in PPF is caused by enhanced pre-synaptic inhibition that reduces the second synaptic response in the pair, the differences in PPF should be abolished in the presence of a GABA\textsubscript{B}-receptor blocker.

To test this hypothesis, PPF was evoked by stimulating the intrinsic synaptic pathway in layer Ib, interconnecting layer II pyramidal neurons (Saar et al., 1999, 2002), before and after GCP55845 application. PPF was significantly enhanced by CGP in neurons from trained rats (1.37±0.14, n=10 before CGP compared to 1.58±0.29, n=8 in CGP, P<0.05). In contrast, PPF in neurons from control rats was not affected by CGP (1.54±0.25, n=15 before CGP compared to 1.44±0.17, n=9 in CGP). Consequently, the difference in averaged paired pulse facilitation values in neurons from control and trained rats (P<0.05, see also Saar et al., 1999, Cohen-Matsliah et al. 2008) was abolished in the presence of the GABA\textsubscript{B} blocker (figure 3). CGP application did not affect the amplitude of the first PSP in the pair.

Learning-induced modifications in GABA\textsubscript{B}-mediated post-synaptic inhibition

OD learning does not result with modulation of the late IPSP reversal potential (figure 2B). However such learning-induced modulation can potentially be expressed also by modifications in the late IPSP conductance or by changes in the distribution of the strength of synaptic inputs onto different pyramidal neurons.

To examine whether such modifications occur, we compared in each neuron the relation between the evoked early and late IPSPs. To evoke pure IPSPs, these recordings were made in the presence of DNQX (10 uM) and APV (20 uM). The amplitudes of the evoked IPSPs at different membrane potentials, and the slope of the graph describing these amplitudes as a function of Vm, do not carry significant information regarding the synaptic strength on their own, since these values are
determined to a great extent by the intensity of the stimulus applied to evoke the 
synaptic response. However, since for a given post-synaptic neuron both IPSPs are 
evoked by the same synaptic stimulation (figure 2A), the slopes of the graphs 
describing the amplitudes of the synaptic responses versus the membrane potentials in 
which the responses were evoked (see figure 4B) can be used to explore potential 
modifications in the relations between the early and late IPSPs conductances, in each 
particular cell (see methods). Figure 4(A, B) shows an example of such comparison; 
the two synaptic responses, evoked by the same stimulation, were recorded at several 
different membrane potentials (figure 4A). Subsequently, a graph describing the 
synaptic potentials as a function of the membrane potentials was constructed for the 
early and late IPSPs (figure 4B). The ratio of slopes of the two graphs represents the 
relation between the early and late synaptic conductances.

The averaged calculated relation between the two synaptic conductances is not 
modified on average by learning (figure 4C). However, a more detailed analysis 
indicates that learning has an effect on the ratio between synaptic strengths for each 
particular neuron; figure 4D shows cumulative frequency histograms of synaptic 
conductance ratios in trained rats and controls. While the averaged ratio value is not 
modified after learning, the curve of the trained neurons differs from the curve of 
control neurons' in their extremes values; the curve describing the ratios distributions 
in the trained rats is more uniform, indicating that the conductance ratio between the 
early and late synaptic responses is more uniform in the pyramidal cell population in 
trained rats. The Kolmogorov-Smirnov test indicates that the curves are significantly 
different (p=0.035). The difference between the two groups in the synaptic strengths' 
ratios is also apparent when comparing the averaged standard deviations of the slopes
ratios, which is considerably smaller for trained neurons (1.27), compared with controls (2.03) (figure 4E).

**GABA$_B$-receptor level is not modified after learning**

We next examined the effect of learning on the expression level of the GABA$_B$ channels. Using type specific antibodies and western blot analysis (see under methods), we measured the expression level of the GABA$_B$R1 channels in the piriform cortex of the different groups. We did not detect any changes in the expression level of the receptor, normalized to the levels of b-actin, following learning (figure 4F). These results suggest that learning-induced modulation of the late synaptic inhibitory transmission is mediated by a mechanism other than increasing the number of the GABA$_B$ receptors.

**Learning-induced enhanced correlation between the strengths of the two GABA-mediated IPSPs**

To further explore the possibility that the strengths of the early and late IPSPs are more correlated in neurons from trained rats, we measured the amplitudes of the two synaptic responses in each neuron, at membrane potential of –60 mV. At this holding potential large amplitude early and late IPSPs are generated, but the cell is not depolarized to the point where uncontrolled action potential firing occurs. While there was no apparent correlation between the amplitudes of the early and late IPSPs in neurons from control rats, such a strong correlation was observed in neurons from trained rats (figure 5). These data suggest that the strength of the early and late IPSPs is redistributed after learning, to match each other in each particular neuron.

**GABA$_B$-mediated control of over excitation is enhanced after learning.**

To examine the functional role of learning-induced enhancement in GABA$_B$-mediated inhibition, we examined its effect on the cortical network activity in the
absence of GABA_A-mediated inhibition. When GABA_A receptors are blocked, an epileptiform propagating wave can be readily evoked in piriform cortex slices by electrical stimulation. The velocity of the epileptic activity propagation can be gradually reduced by application of increasing concentrations of the AMPA receptor blocker DNQX (figure 6A). We examined the velocity of the epileptiform wave propagation in slices from trained and control rats. Notably, although AMPA-dependent synaptic transmission is greatly enhanced after learning (Saar et al., 1999, 2002; Knafo et al., 2005), the velocity of propagation did not differ between slices from trained and untrained animals (p=0.45, F=0.92) (figure 6B). However, when the GABA_B blocker CGP55845 (1 µM) was applied, a marked difference was observed between the two groups. CGP did not significantly modify the propagation velocity in slices from control rats (p=0.26, F=1.48), although the epileptic wave could be evoked at higher DNQX concentrations (see response at 1 µM DNQX). In contrast, propagation velocity in slices from the trained rats was strongly enhanced for the higher DNQX concentration ([DNQX] ≥0.8 µM) (p=0.023, F=3.71) (figure 6C). Subsequently, in CGP the propagation velocity differed significantly between slices from trained and control rats (p=0.009, F=5.39). These data indicate that GABA_B-mediated synaptic inhibition may be enhanced after learning in a manner that enables it to counter balance the enhancement in excitatory synaptic transmission.

**Learning-induced modulation of GABA_B-mediated inhibition is not dependent on GIRK-channel activation**

GABA_B-dependent post synaptic inhibition is mediated via the G protein-gated inwardly rectifying potassium (GIRK) channels (Luscher et al., 1997). To further examine if learning-induced modulation of GABA_B effect on PPF is indeed mediated by suppression of synaptic release, we blocked the GABA_B-mediated IPSP by applying the GIRK-channel blocker SCH23390, thus isolating the pre-synaptic effect of CGP. SCH23390 did not modify PPF in neurons from naïve (1.39±0.1 before
and 1.39±0.18 after SCH23390(n=7)) and from trained rats (1.26±0.0.08 before and 
1.24±0.15 after SCH23390 (n=7)) (figure 7 A, B). SCH23390 also did not affect the 
propagation velocity of the epileptiform activity in 6 out of 6 tested slices, although it 
induced late synaptic responses (figure 7C). These findings suggest that learning-
induced modulation of GABA<sub>B</sub>-mediated mediation is the result of its pre-synaptic 
effect.
DISCUSSION

We have previously shown that olfactory-discrimination learning is accompanied by enhancement in intrinsic neuronal excitability, excitatory synaptic transmission, and GABA$_A$-mediated fast inhibitory synaptic transmission, throughout the piriform cortex pyramidal cell population (Saar et al., 1998, 2002; Broshand Barkai 2009, Saar et al., 2012). While activity-induced enhancement in GABA$_A$-mediated inhibitory synaptic transmission has been shown in several brain regions of the mammalian brain (Grunze et al., 1996; Komatsu and Yoshimura, 2000; Holmgren and Zilberter, 2001), plasticity of the GABA$_B$-mediated synaptic inhibition has been less studied. Here we show that after OD learning GABA$_B$-mediated pre-synaptic transmission is also enhanced, whereas the standard deviation, but not the strength of post-synaptic transmission is increased.

Learning-induced modulation of GABA$_B$-dependent pre synaptic inhibition

Reduced PPF has been reported after different forms of learning (Mckernan and Shinnick-Gallagher, 1997, Saar et al., 1999), and is commonly interpreted as indication for enhanced synaptic release, in accordance with the residual calcium theory (Katz and Miledi, 1968; Wu and Saggau, 1994). An inter stimulus interval of 50 ms was chosen since at this particular ISI the PPF of excitatory evoked synaptic responses in the intrinsic fibers reaches its maximal value, and the difference in PPF values between trained and control neuronal is at the highest (Saar et al., 1999).

Notably, at this ISI there's also a notable effect of the late IPSP on the second excitatory response in the pair (Saar et al., 1999 and figure 7A). We show that while blocking GABA$_B$-activation strongly affects PPF in neurons from trained rats, blocking only the post-synaptic GABA$_B$-activated potassium channels (GIRK channels) does not have such an effect. These data support the notion that OD
learning-induced PPF reduction results from GABA\textsubscript{B}-mediated pre-synaptic inhibition, which is enhanced to the point where it reduces glutamtergic release. One potential advantage of pre-synaptic inhibition over post synaptic inhibition is that presynaptic inhibition would be very efficient in blocking inputs from very specific sources. Notably, the GABA\textsubscript{B} blocker did not modify the first evoked PSP in a pair. This lack of effect can be explained by the delay to onset of the GABA\textsubscript{B}-mediated pre-synaptic inhibition, which is too slow to affect the first fast EPSP (Tseng and Haberly, 1998).

It has been previously shown that GABA\textsubscript{B}-receptors mediate inhibition of synaptic release through several mechanisms, such as modulation of pre-synaptic Ca\textsuperscript{2+} channels and activation of potassium channels (Marshall 2008, Tang and Hasslemo 1994, Otmakhova and Lisman 2004, Chalifoux and Carter 2010, 2011). Activity-induced GABA\textsubscript{B}-dependent plasticity was previously attributed to activation of pre-synaptic receptors of inhibitory neurons (Ivenshitz and Segal, 2006).

It is currently difficult to speculate on the source of GABAergic input to presynaptic terminals of layer II axons. There are several classes of GABAergic interneurons in the piriform cortex, distinguished based on morphology and laminar location (Suzuki and Bekkers 2007); Horizontal cells which are located in layer Ia with long dendrites spreading in parallel to the cortical surface. Multipolar cells (without dendritic spines) lie within Layer II and III, mediating classic inhibitory feedback functions. A class of small bipolar or bitufted cells with somata in layer IIa and dendrites extending into both Layers I and III. Neurogliaform cells are likely candidate for generating the presynaptic inhibition in response to stimulus applies at layer Ib, since they are especially common in this layer (Suzuki and Bekkers, 2010a,b). Such neurogliaform cells have been shown to generate widespread volume
transmission, and thus can inhibit the release of glutamate from axon terminals located remotely from the GABA release site (Capogna and Pearce 2011).

Notably, the GABA<sub>B</sub>-receptor density is not increased after learning, suggesting that learning-induced enhancement of presynaptic inhibition is mediated via a different mechanism. Indeed, OD learning-induced PPF reduction has been shown to be dependent on persistent activation of the ERK and PKC second messenger systems (Cohen-Matsliah et al., 2008). Thus, the hypothesis that GABA<sub>B</sub>-dependent modulation of synaptic release is mediated by these second messenger systems is well worth pursuing.

Learning-induced modulation of GABA<sub>B</sub>-dependent post synaptic inhibition

Unlike the early IPSP, the averaged reversal potential of the late IPSP was not modified after learning, and has the expected value for potassium current. Also, the averaged relative strength of the late IPSP (compared to the strength of the early IPSP) is not modified after learning. These results well agree with our previous that the ratio between the decay rates of the second and the first EPSP, evoked by pairs of stimuli, is not modified (Saar et al., 1999).

However, the range of the early IPSP / late IPSP slope values is reduced after learning, indicating that the strength of the GABA<sub>A</sub> and GABA<sub>B</sub>–mediated synaptic inhibition is more correlated in neurons from trained rats. Measurements of the amplitudes of the early and late IPSPs in each neuron lend further support to this notion; while no apparent correlation exists between the amplitudes of the two IPSPs in neurons from control rats, a highly significant correlation is apparent in neurons from trained rats. These findings suggest that the strength of the late synaptic inhibition is redistributed in the piriform cortex neurons after learning in a manner that might particularly increase synaptic inhibition onto a sub-group of neurons. Since
GABA\textsubscript{A}-mediated inhibition is enhanced after OD learning in throughout the pyramidal cell population, and mostly in a sub group of neurons (Saar et al., 2012), it is likely that GABA\textsubscript{B}-mediated inhibition undergoes a similar change. Importantly, a recent study shows that following classical conditioning of the eyeblink response, hippocampal somatostatin-positive inhibitory interneurons are more excitable, and thus their inhibitory synaptic inputs onto pyramidal neurons are enhanced (McKay et al., 2013). Such enhanced GABA release was not observed in our present and previous (Saar et al., 2012) studies. Notably, such enhanced release is attributed to decrease in the small conductance (SK) channel-mediated potassium current which affects repetitive spike firing. In our study, synaptic inhibition was evoked by a single or double synaptic stimulation. Thus it is plausible that such a presynaptic effect also exists in the piriform cortex after OD-learning and would be exposed under different stimulation protocols.

**Preventing epileptiform activity by pre-synaptic GABA\textsubscript{B}-mediated inhibition**

Our previous results (Brosh and Barkai 2009) showed that GABA\textsubscript{A}-mediated inhibition is enhanced following olfactory-discrimination rule learning. Here we show that even if GABA\textsubscript{A}-mediated inhibition is completely blocked, epileptiform discharges propagate at about the same velocities in piriform cortex slices from trained and control rats, with the same concentrations of DNQX (Fig. 6B). Since AMPA-mediated excitatory-to-excitatory connectivity is enhanced after OD rule learning (Saar et al., 2002), another mechanism should balance the increase in excitability and tendency towards epilepsy caused by the global enhancement of excitation among excitatory neurons (Golomb and Amitai 1997, Golomb and Ermentrout, 1999, 2002; Pinto et al., 2005). We did not find an average increase of post-synaptic GABA\textsubscript{B}-mediated inhibition. Furthermore, even if such increase exists,
the large delay and slow rise time of post-synaptic GABA-B-mediated IPSPs (Kim et al., 1997) would make it too slow to affect the propagation of epileptic-like activity; the discharge would pass through the cortical network before this slow inhibition develops.

Here we show that pre-synaptic GABA-B-mediated inhibition is enhanced after rule learning and that blocking GABA-B-receptors by CGP enables epileptic-like activity to propagate at higher DNQX concentrations (i.e., lower excitation conductance). These data suggest that GABA-B-mediated pre-synaptic suppression of excitatory synaptic release has a key role in controlling hyper-excitability in the cortical network, which is likely to develop as result of learning-induced increase in synaptic and intrinsic excitation.

To conclude, our data show that the strength of GABA-B-mediated synaptic inhibition is enhanced for days after learning, by two different mechanisms. Pre-synaptic inhibition is more potent in controlling evoked glutamate release from pyramidal neurons, while post synaptic inhibition appears to be redistributed between neurons to match the strength of the fast synaptic inhibition, thereby generating particularly strong synaptic inhibition on specific neurons. Together, such combined pre and post-synaptic enhancements enable GABA-B-inhibition to efficiently control hyper excitability, enabling the cortical network to maintain its functionality in storing long-term memories.
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Authors Contribution

AK, NOG, LJ and DS collected analyzed and interpreted data

DG and EB contributed to concept and design of experiments

All authors contributed to writing the article

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FIGURES LEGENDS

Figure 1: Description of the olfactory maze and brain slice preparation

A. Schematic description of the olfactory maze. An electronic ‘start’ command opens randomly two out of eight valves (V), releasing a positive-cue odor (P) into one of the arms and a negative-cue odor (N) into another. Eight seconds later, the two corresponding guillotine doors (D) are lifted to allow the rat to enter the selected arms. Upon choosing the arm containing the positive-cue odor arm, reaching the far end of an arm (90 cm long), the rat body interrupts an infrared beam (I, arrow) and a drop of drinking water is released from a water hose (W) into a small drinking well. A trial ends when the rat interrupts a beam, or in 10 seconds, if no beam is interrupted. A fan is operated for 15 seconds between trials, to remove odors.

B. Schematic illustration of the piriform cortex in a coronal brain slice and the experimental procedure. Intracellular recordings were performed from cell bodies in layer II. Feed forward inhibition (FF) inputs are terminated on the distal apical dendrite, while feedback inhibition (FB) is terminated on cell bodies and proximal dendrites of the cell (Suzuki and Bekkers 2007). For activation of inhibitory synaptic inputs, electrical stimuli were applied to the border between layers II and III, in the presence of the glutamatergic AMPA and NMDA receptors blockers. To evoke excitatory synaptic potentials and PPF measurements, electrical stimuli were applied to layer Ib, where intrinsic excitatory synaptic axons are mostly located. Such stimulation also excite neurogliaform cells, which are especially common in layer Ib (Suzuki and Bekkers 2010 a, b), which may subsequently suppress excitatory synaptic release by activating presynaptic GABA_B-receptors on layer II pyramidal neurons (Tang and Hasselmo, 1994) (see discussion).
Figure 2: Olfactory-learning induced modification of the early IPSP reversal potential.

B. Example of intracellular recording of the early and late IPSPs in a layer II pyramidal neuron. The synaptic potential of the early IPSP was measured at the first peak of the synaptic response, and the potential of the late IPSP at the second peak of the same response. Numbers on the left of the traces note the holding membrane potential. These measurements were then used to calculate the reversal potential of the responses (see for example figure 5B).

B. A cumulative frequency graph comparing the reversal potentials of the early and late IPSPs in neurons from control versus trained rats. Each point represents the calculated reversal potential in one cell. The curve of the early IPSP reversal potential in the trained group is shifted smoothly leftwards, compared with the curve of the control group, as previously reported (Brosh and Barkai 2009). The curves describing the reversal potential distribution of the late IPSP in the control and trained groups are overlapping.

Data were taken from 18 control and 12 trained rats.
Figure 3: Learning-induced PPF reduction is dependent on GABA\textsubscript{B}-receptors

A. PPF was measured in response to two stimuli, with inter stimulus interval of 50 ms. Neurons were recorded at membrane potential of -80 mV. Each trace is a digital average of ten responses to stimuli applied at 0.1 Hz. While CGP has no effect on PPF in a neuron from a naive animal (left trace), it increased PPF in a neuron from a trained rat (right trace).

B. As previously reported (Saar et al., 1999), the averaged PPF in neurons from trained rats was significantly lower compared to average in neurons from naive and pseudo-trained. CGP application enhanced PPF in the trained group only, as result of which the difference between the trained and the control groups was abolished. PPF in NSR was measured in 15 neurons from 10 control rats, and 10 neurons from 8 trained rats. PPF in CGP was measured in 10 neurons from 8 control rats, and 8 neurons from 6 trained rats. Values represent mean ± SE.
Figure 4: learning-induced modification in the late IPSP

A. Example of early and late IPSPs (taken from a naïve neuron), recorded at several holding potentials. IPSPs were measured at the latency of the first and second peaks (dashed lines), as appeared in the most depolarized recording potential.

B. The reversal potentials of the two synaptic responses shown in A were determined by the linear regression line, describing the amplitude of the synaptic potential as a function of the membrane holding potential. The ratios of the slopes of the two lines can be used to measure the conductance ratio of the two IPSPs, as they are generated simultaneously in the recorded neuron by the same stimulation. The slopes ratio (early IPSPVs late IPSP) in this particular neuron is 4.5.

C. The averaged slope ratio is not modified after learning. IPSPs were recorded in 27 neurons from 20 control rats, and 21 neurons from 15 trained rats. Values represent mean ± SE.

D. Cumulative frequency distributions of the slope ratios, each point represents the EarlyIPSP/Late IPSP slopes ratio in one cell. Although the averaged value of the ratios did not change after learning, the curve describing the ratios in neurons from trained rats does not entail the low and high extreme values observed in neurons from the control group.

E. The averaged standard deviations of the slopes ratios, is markedly reduced after learning.

F. The expression of GABA<sub>B</sub>-receptor protein level is not modified by learning.

Top: Representative immunoblots for GABA<sub>B</sub>-receptor protein prepared from the piriform cortex of naïve (N) and trained (T) animals.

Bottom: Protein expression level of the GABA<sub>B</sub>-receptor is not modified by olfactory learning. For each channel type, the protein level is normalized to the average value for actin. Summarized data are presented as mean O.D. ± SD, n=12 for naïve rats and n=9 for trained rats.
Figure 5: The correlation between the amplitudes of the early and late IPSPs is modified by learning.

A. Top: lack of correlation between the early late and IPSPs amplitudes in neurons from control rats. Examples for synaptic responses taken from neurons in which the early IPSP is particularly low (C1) and high (C2) (see arrows indicating these neurons in figure 6B) are shown. Note the similarity of the amplitudes of the late IPSPs in these cells. Bottom: A strong correlation between the early late and IPSPs amplitudes in neurons from trained rats. Examples for synaptic responses taken from neurons in which the early IPSP is particularly high (T1) and low (T2) (see arrows indicating these neurons in figure 5B) are shown. Note the difference in the amplitudes of the late IPSPs in these cells.

Synaptic responses were recorded at holding potential of -60 mV.

B. In neurons from control rats, the amplitudes of the two inhibitory synaptic responses show no correlation (r=0.14, p=0.51). In sharp contrast, after learning a highly significant correlation between the amplitudes of the early and late IPSPs is apparent (r=0.73, p=0.001). Each point represents the synaptic responses in a neuron, recorded at holding potential of -60 mV. Note that such a significant correlation is not dependent on point T2 (r=0.59, p=0.008 without this particular point).

Data were taken from 10 control (n=26 neurons) and 8 trained (n=20 neurons) rats.
Figure 6: learning-induced enhancement of late the IPSP potency to restrict the propagation of epileptic activity.

A. Example of epileptic response evoked in a piriform cortex slice by blocking GABA$_A$-mediated synaptic inhibition. Traces show two field potentials recorded simultaneously with two electrodes placed in layer II, with a distance of 2 mm between them. Epileptiform activity was evoked by stimulating in layer II (see methods). Numbers on the left note the concentration of the AMPA-receptors blocker, DNQX, add to the medium solution. As previously shown (Golomb and Amitai, 1997), with increasing concentration of DNQX the amplitudes of the epileptic responses are gradually reduced and their latencies are gradually increased.

B. In the presence of the GABA$_A$ blocker (BMB) only, the averaged velocity at which the epileptic wave advances between the two recording sites is similar in slices from control and trained rats for all DNQX concentrations.

C. When the GABA$_B$ blocker (CGP), is added to the medium solution, the averaged velocity at which the epileptic wave advances is higher in slices from trained rats, when exposed to high DNQX concentrations.

Data were recorded from 5 slices taken from 5 control rats and 6 slices taken from 6 trained rats. Values represent mean ± SE.
Figure 7: Learning-induced modulation of GABA\textsubscript{B}-receptors mediated inhibition is not dependent on GIRK channels

A. PPF was measured in response to two stimuli, with inter stimulus interval of 50 ms. Neurons were recorded at membrane potential of -80 mV. Each trace is a digital average of ten responses to stimuli applied at 0.1 Hz. The GIRK channel blocker SCH23390, did not affect response's amplitude and the PPF in the naïve (upper trace) and a trained (lower trace) neurons. However, note that in both neurons the decay time of the second responses in increased, as would be expected from reduced GABA\textsubscript{B}-mediated post synaptic conductance (see Saar et al., 1999).

Each trace is a digital average of ten responses to stimuli applied at 0.1 Hz. The GIRK channel blocker SCH23390, did not affect response's amplitude and the PPF value on .

B. In the presence of SCH23390, the averaged PPF in neurons from naïve and trained rats remained in the same averaged values as before drug application. PPF was measured before and after SCH23390 application in 7 neurons from 7 naïve rats, and 7 neurons from 7 trained rats. Values represent mean ± SE.

C. Propagation of an epileptic is shown in the presence of the GABA\textsubscript{A} blocker and DNQX (0.8 µM) only (control) and after SCH23390 application (GIRK blocker). The propagation velocity is not modified by the GIRK-channel blocker. However, note that late multi-synaptic responses occur after SCH23390 application, indicating that the blocker increases the neuronal excitability (dotted arrow).
recording electrode

stimulating electrodes

For PPF experiments

For pure IPSPs experiments

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7