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Cortically projecting basal forebrain parvalbumin neurons regulate cortical gamma band oscillations

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Cortical gamma band oscillations (GBO, 30–80 Hz, typically ∼40 Hz) are involved in higher cognitive functions such as feature binding, attention, and working memory. GBO abnormalities are a feature of several neuropsychiatric disorders associated with dysfunction of cortical fast-spiking interneurons containing the calcium-binding protein parvalbumin (PV). GBO vary according to the state of arousal, are modulated by attention, and are correlated with conscious awareness. However, the subcortical cell types underlying the state-dependent control of GBO are not well understood. Here we tested the role of one cell type in the wakefulness-promoting basal forebrain (BF) region, cortically projecting GABAergic neurons containing PV, whose virally transduced fibers we found apposed cortical PV interneurons involved in generating GBO. Optogenetic stimulation of BF PV neurons in mice preferentially increased cortical GBO power by entraining a cortical oscillator with a resonant frequency of ∼40 Hz, as revealed by analysis of both rhythmic and nonrhythmic BF PV stimulation. Selective saporin lesions of BF cholinergic neurons did not alter the enhancement of cortical GBO power induced by BF PV stimulation. Importantly, bilateral optogenetic inhibition of BF PV neurons decreased the power of the 40-Hz auditory steady-state response, a read-out of the ability of the cortex to generate GBO used in clinical studies. Our results are surprising and novel in indicating that this presynaptically inhibitory BF PV input controls cortical GBO, likely by synchronizing the activity of cortical PV interneurons. BF PV neurons may represent a previously unidentified therapeutic target to treat disorders involving abnormal GBO, such as schizophrenia.

Significance

When we are awake, purposeful thinking and behavior require the synchronization of brain cells involved in different aspects of the same task. Cerebral cortex electrical oscillations in the gamma (30–80 Hz) range are particularly important in such synchronization. In this report we identify a particular subcortical cell type which has increased activity during waking and is involved in activating the cerebral cortex and generating gamma oscillations, enabling active cortical processing. Abnormalities of the brain mechanisms controlling gamma oscillations are involved in the disordered thinking typical of neuropsychiatric disorders such as schizophrenia. Thus, these findings may pave the way for targeted therapies to treat schizophrenia and other disorders involving abnormal cortical gamma band oscillations.


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reduced cortical activation following BF lesions was correlated with the extent of PV neuronal loss (17). Interestingly, BF GABAergic projections to cortical GABAergic interneurons containing PV (24, 25) have been demonstrated, although direct BF PV→cortical PV connections remain to be shown. When considered together, the physiology and anatomical connections of BF GABAergic/PV neurons suggest that they are ideally positioned to control cortical GBO (Fig. 1A). However, this hypothesis has not been tested directly. Thus, here we tested the effect of selective excitation of BF PV neurons on the cortical EEG and the effect of selective inhibition on the 40-Hz auditory steady-state response (ASSR), a test of the ability of the cortex to generate a prominent increase at 40 Hz in the ChR2 group. Power spectra of 5-s prestimulation (Fig. S3) are shown is the average of data from five animals, with each dot representing power at each frequency the EEG was averaged, and the ratio of the power at each frequency ±2 Hz during the 5-s stimulation epoch to the 5-s prestimulation epoch was calculated. Fig. 2A shows a grand average of the five animals for the ratio of power during stimulation to the prestimulation power at the same frequency (geometric mean and SEM). A one-way repeated-measures ANOVA showed significant differences among the frequencies (F = 6.498, df = 6, P < 0.001). Compatible with the visual impression in Fig. 2A, the power of the EEG decreased at frequencies closest to it (30 and 50 Hz) was significantly different from pre-stimulation baseline (paired t test, P < 0.05), but the power of other frequencies did not differ significantly from prestimulation baseline. The preferential enhancement of EEG power at and near 40 Hz in our experiments suggests that BF PV stimulation was not simply driving cortical EEG at any frequency but instead was entraining a cortical oscillator with a resonant frequency of ∼40 Hz. Four additional observations supported this interpretation. First, the EEG power response at a stimulation frequency of 40 Hz increased gradually over time during the course of stimulation, indicative of entrainment of the oscillation (Fig. 2B; r = 0.675, P < 0.001, linear fit; shown is the average of data from five animals, with each dot representing power at 40 ± 5 Hz for successive 200-ms epochs). A statistically significant increase was observed only at 40 Hz (Fig. S2). Second, the relative power of the harmonic response was significantly higher with 20-Hz stimulation than with 30-Hz stimulation (n = 5; t = 3.445, df = 8, P = 0.009, unpaired t test) (Fig. 2C, D, and E), as would be expected if the oscillator is tuned at 40 Hz. Third, the oscillation in response to 40-Hz stimulation developed over time and persisted for four or more 40-Hz cycles following cessation of stimulation (Fig. 2F), another feature expected of an oscillator. Stimulation at other frequencies, e.g., 10 Hz, also induced 40-Hz oscillations that persisted at the end of the stimulation and lasted longer than those at the stimulation frequency (Fig. S3), as is consistent with 40 Hz being the resonant frequency. Finally, in a separate group of mice (n = 3), nonrhythmic stimulation of BF PV in a frequency band spanning the natural firing frequencies of BF GABA neurons during wakefulness and REM sleep (20–60 Hz) (20) preferentially enhanced cortical power at 40 Hz (Fig. S4).

Validation of Selective Optogenetic Excitation of BF PV Neurons. Several lines of evidence supported our conclusion that selective optogenetic stimulation of BF PV neurons was responsible for the entrainment of cortical gamma oscillations. First, in mice with a high GBO response (>4-fold power increase relative to

**Results**

**Optogenetic Excitation of BF PV Neurons Preferentially Enhances Cortical Power at 40 Hz.** To determine if optical stimulation of BF PV neurons affects cortical GBO activity (Fig. 1B), mice constitutively expressing Cre-recombinase in PV neurons (PV-Cre mice) received unilateral BF injections of double-floxed adeno-associated viral (AAV) vectors expressing channelrhodopsin2 (ChR2) and enhanced YFP (EYFP) fusion protein (7, 8). Subsequently, transduced BF PV neurons were stimulated in vivo at 40 Hz with a 5-s train of 10-ms light pulses (Fig. 1 C, E, and G and Fig. S1). Control mice received the same optical stimulation but without prior viral injection (Fig. 1 D, F, and H). In each mouse this optical stimulation protocol was repeated 20 times, and the EEG traces were averaged; a grand average for ChR2-expressing mice (n = 5) and control mice (n = 3) is presented in Fig. 1. Strikingly, in ChR2-expressing mice, time-frequency analysis (Fig. 1C) and power spectra (Fig. 1G) of averaged EEG traces (Fig. 1E) all showed a pronounced increase in 40-Hz power during optical stimulation. In contrast, controls did not show this response (Fig. 1 D, F, and H).

These results showed that 40-Hz stimulation of BF PV neurons strongly modulates the cortical EEG at 40 Hz. However, it was not clear from these experiments whether BF PV neurons have a specific role in controlling GBO or simply drive cortical activity at the stimulation frequency. Thus, we next examined if there were preferred BF stimulation frequencies for eliciting a cortical response. The BF was optically stimulated at seven frequencies within the physiological firing range of BF GABAergic neurons (2, 10, 20, 30, 40, 50, and 60 Hz) (20) in time-of-day-matched trials in which each stimulation frequency was given 20 total times in a pseudorandom order for each mouse, independent of ongoing behavioral state. For each mouse and each frequency the EEG was averaged, and the ratio of the power at each frequency to prestimulation baseline (paired t test, P < 0.05), but the power of other frequencies did not differ significantly from prestimulation baseline.
BF PV neurons selectively transduced with ChR2-EYFP project onto cortical PV interneurons, suggesting apposition. (D) Anti-PV immunohistochemistry shows AAV-EYFP control injections selectively transduce PV somata in the BF. (E–G) Anti-GFP-stained BF PV fibers transduced with ChR2-EYFP (green) innervated layers II-III and V of the somatosensory cortex, and fiber varicosities formed appositions onto cortical PV interneurons (red). (H) Low-magnification image. (I) High-magnification (100x) confocal immunofluorescence 3-stack image (36 optical sections, 1-μm thickness) of the boxed area in G. Arrowheads indicate colocalization of BF PV fibers and cortical PV interneurons, suggesting apposition. (J) High-magnification of the boxed area in H showing one 1-μm optical section. Apposition of the BF PV fiber to the cortical PV interneuron, suggestive of a synaptic contact, was confirmed by the presence of a yellow (merge of red and green)-labeled axonal varicosity (arrowheads) on the PV cell in the orthogonal planes of the x-z (Upper) and y-z (Right) projection images. (Scale bars: 1 mm in A, 500 μm in B, 50 μm in C, 100 μm in F and G, and 10 μm in H and I.)
significantly as the stimulation frequency was increased (Fig. S6C). Thus, the gradually diminishing cortical EEG response in response to stimulation at frequencies above 40 Hz is not caused by an inability of BF PV neurons to follow higher frequency stimulation. Our data also raise the question of whether the BF itself has a preferred resonant frequency of 40 Hz or whether the GBO results from a 40-Hz cortical oscillator that is entrained by BF input. Local field potential data recorded ipsilateral to the BF stimulation site support the latter possibility, because they did not show a preferred 40-Hz response in the BF (n = 3) (Fig. S6 D–F).

**Cholinergic Neurons Are Not Required for the Cortical 40-Hz Response Mediated by Stimulation of BF PV Neurons.** Our immunohistochemical experiments revealed a dense plexus of ChR2-EYFP–labeled fibers in the BF (Fig. S3). Thus, effects of BF PV stimulation could be mediated through interaction with other BF neurons, in particular, cholinergic neurons (32). To determine if BF cholinergic neurons are required for increased cortical GBO produced by BF PV stimulation, we bilaterally injected the selective cholinergic toxin minirine-p75NTR-saporin (mu p75-saporin) (33) into the lateral ventricles and tested the cortical response 3–4 wk after saporin-induced destruction of BF cholinergic neurons. Successful lesioning of cholinergic neurons was confirmed by immunohistochemistry for the selective marker of cholinergic neurons, choline acetyltransferase (CHAT) (Fig. S7A). Cell counting indicated an extensive (70.2 ± 2.1%, n = 4) lesion of BF cholinergic neurons, a percentage consistent with the literature reports using mu p75-saporin (33). These extensive lesions did not impair the ability of BF PV optogenetic stimulation to facilitate cortical GBO (control, 15.49 ± 6.43 fold-increase in power at 40 Hz; saporin-lesioned, 12.06 ± 1.95 increase, n = 6; n.s., Mann–Whitney u test) (Fig. S7B).

**BF PV Neuronal Control of Cortical GBO Is Likely Mediated by Direct Cortical Projections.** In addition to the cortical fiber labeling observed following transduction of BF PV neurons (Fig. 3 G–I), labeled fibers also were seen densely innervating the thalamic reticular nucleus (TRN), a known projection site of BF PV neurons (34). The strong projection of BF PV neurons to the TRN suggested that this could also be a pathway that modulates cortical GBO via entrainment of thalamic relay neurons. Therefore, we tested whether optogenetic stimulation of TRN PV neurons would mimic the effect of stimulation of BF PV neurons. However, in contrast to the effect of stimulation of BF PV neurons, bilateral stimulation of ChR2-transduced TRN PV neurons preferentially enhanced cortical GBO at 1 Hz, consistent with the known role of TRN neurons in the generation of spindle (8–14 Hz) activity during NREM sleep (n = 4) (Fig. S8). Thus, the most parsimonious explanation for the BF PV control of cortical GBO is via their direct projections to cortical PV interneurons.

**Bilateral Inhibition of BF PV Neurons Impaired the 40-Hz Auditory Steady-State Cortical Response.** To assess further a physiological role of BF PV neurons in the control of cortical GBO, we tested the effect of BF PV inhibition on the ASSR, a paradigm commonly used to test GBO in clinical studies of schizophrenia (5) and anesthesia (27). In this paradigm, presentation of a train of auditory clicks elicits a steady-state oscillation in the EEG recorded above auditory and frontal cortices (5, 26). In our ASSR paradigm, mice were exposed to 1-s trains of auditory stimuli at 40 Hz with parameters similar to those used in ASSR studies of schizophrenia patients (5). To inhibit BF PV neurons, AAV vectors with Cre-dependent expression of the inhibitory opsin, Archaeorhodopsin from Halorubrum strain TP009 (ArchT) (35), and a fluorescent marker of expression (AAV-ArchT-GFP) were bilaterally injected into the BF of PV-Cre mice (n = 8) (Fig. 4 A and Fig. S9A).

Under control conditions each 1-s 40-Hz train elicited an auditory evoked potential, associated with a pronounced low-frequency response recorded in the frontal EEG electrode, followed by a sustained steady-state 40-Hz response (Fig. 4B). In the same animals the mean power at 40 ± 2 Hz was calculated during the 1-s click train with and without bilateral ArchT inhibition of BF PV neurons. For the ArchT trials, laser light (532 nm) was applied continuously, beginning 2 s before the first trial and continuing throughout the block of trials. When BF PV neurons were bilaterally inhibited by ArchT stimulation, the power of the 40-Hz EEG response recorded above frontal cortex was reduced by 33.1 ± 4.4% (ASSR alone, 0.52 ± 0.10 μV²; ASSR + ArchT, 0.34 ± 0.06 μV²; P < 0.05, n = 8, Wilcoxon signed-rank test) (Fig. 4 B–D), indicating that inhibition of BF PV neurons reduced the ability of the cortex to generate GBO in response to a sensory stimulus. Post hoc histological examination of BF slices from AAV-ArchT-GFP–injected animals (n = 8) (Fig. S9A) revealed an extensive plexus of transduced neurons/fibers in the BF. As with injections of AAV-Chr2-EYFP (Fig. 3 G–I), extensive cortical fiber labeling was observed, particularly in layers II–III and V (Fig. S9B). In vitro whole-cell patch-clamp recordings confirmed that optical activation of ArchT caused a strong hyperpolarization and inhibited BF PV neuronal discharge (Fig. S9 C–E).

**Discussion**

Here we found that selective optogenetic excitation of BF PV neurons preferentially enhanced EEG power in the gamma range. Both rhythmic and nonrhythmic stimulation of BF PV neurons led to entrainment of a frontal cortex oscillator tuned at ~40 Hz. Conversely, optogenetic inhibition of BF PV neurons reduced the power of the 40-Hz ASSR. Together, these results suggest a role for BF PV projection neurons in the state-dependent control of cortical GBO activity, most likely mediated by a direct projection of BF PV neurons to cortical PV interneurons.

Previous anatomical tracing and unit recording studies (cited in the Introduction) had hinted at a possible role for BF GABA/PV neurons in control of cortical GBO. Until now, however, this
Evidence for BF PV Entrainment of a Cortical Gamma Band Oscillator.

The preferential enhancement of EEG power at and near 40 Hz by optogenetic stimulation of BF PV neurons suggests the increase in cortical EEG power was caused not by a passive response to optogenetic stimulation but rather by an entrainment of a cortical oscillator with a resonant frequency of ~40 Hz. Similar conclusions regarding the existence of a 40-Hz cortical oscillator were reached by Gray and Singer (1) in their study of GBO in visual cortex and Cardin and colleagues (8) using optical stimulation of fast-spiking cortical PV interneurons.

In our study, four additional observations supported this interpretation. First, the EEG power response at a stimulation frequency of 40 Hz increased gradually over time during the course of stimulation, indicative of entrainment of the oscillator. Second, the relative power of the harmonic response was significantly higher with 20-Hz stimulation than with 40-Hz stimulation. Third, examination of the oscillation in response to 40-Hz stimulation at a millisecond timescale revealed the oscillation both increased in time and persisted for several cycles following cessation of stimulation. Finally, nonrhythmic stimulation at frequencies (20–60 Hz) approximating the natural firing frequencies of BF GABA neurons during wakefulness/REM sleep (20) significantly enhanced cortical power only at 40 Hz. Recent studies using transcranial magnetic stimulation in humans have shown that the frontal cortex has a preferential response to gamma band stimulation (37), which is reduced in schizophrenic patients (38). As shown by our anatomical data (Fig. 3 and Fig. S9), BF GABA/PV neurons may have a privileged access to this cortical oscillator through their projections to cortical PV interneurons.

ChR2-EYFP and ArchT-GFP are targeted specifically to the membrane and distribute throughout the neuron, allowing the analysis of axonal projections. Previous anterograde tracing experiments in rats showed that GABAergic BF neurons project to the neocortex and preferentially target cortical interneurons, containing PV and somatostatin (24, 25). Here, in the mouse, fibers of BF PV neurons, labeled with ChR2-EYFP or ArchT-GFP, innervated both deep and superficial layers of the neocortex, and varicosities apposed cortical PV interneurons, suggestive of a direct BF PV→cortical PV synaptic connection. In addition, there were strong projections to the TRN. However, stimulation of TRN PV neurons preferentially enhanced cortical EEG power at 10 Hz. Thus, the direct cortical projection is a more likely mediator of BF PV control of cortical GBO.

Previous studies suggested a prominent role for BF cholinergic neurons in the control of cortical activation (32, 39, 40). However, noncholinergic BF neurons are likely to be equally or more important. Selective lesions of cholinergic neurons using IgG192-saporin have relatively minor effects on the cortical EEG (17, 19). In contrast, ibotenate lesions of BF which preferentially target noncholinergic neurons cause more profound reductions in cortical low-voltage fast activity than cholinergic lesions alone (17, 19). Here, we found that saporin lesions of BF cholinergic neurons, resulting in a 70% loss of this cell type, did not significantly affect the ability of BF PV stimulation to induce a cortical 40-Hz response, suggesting that cholinergic neurons are not required. However, under normal conditions, cholinergic and GABA/PV neurons likely work together to control cortical activation and GBO activity because cholinergic neurons strongly excite identified BF GABA and PV neurons in vitro (41).

Further strong evidence supporting our conclusion that BF PV neurons control cortical GBO came from our loss-of-function experiment using ArchT to inhibit BF PV neurons bilaterally. These experiments showed a dramatic reduction in the 40-Hz ASSR. Validation experiments confirmed that ArchT transduction was targeted correctly to the BF and that light activation of ArchT inhibited BF PV neurons. Cortical fiber labeling was observed following ArchT-GFP transduction, similar to our findings with ChR2-EYFP, suggesting a direct BF PV→cortical PV projection as a likely mediator of the effect.

Functional and Clinical Implications of BF PV Modulation of Cortical GBO.

Spontaneously occurring gamma oscillations vary according to behavioral state, being higher during wakefulness than during NREM sleep (12) and reduced in anesthesia (13). Similarly, the ASSR power is reduced by 50% during sleep (28) and is abolished by deep anesthesia (42). Although other parts of the ARAS, such as the thalamus (3), likely are important, our results suggest that BF PV neurons are involved in state-dependent control of GBO. Juxtacellularly identified BF PV neurons in anesthetized rats increased their firing rate with cortical activation caused by tail pinch (23). Our preliminary recordings from two optically identified BF PV neurons in mice confirmed that their discharge is highest in states that show more GBO activity, i.e., wakefulness and REM sleep. Furthermore, our results showed that stimulation of BF PV neurons increases GBO, and inhibition was targeted correctly to the ASSR. GBO are strongly reduced or abolished by deep anesthesia (42). Although other parts of the ARAS, such as the thalamus (3), likely are important, our results suggest that BF PV neurons are involved in state-dependent control of GBO. Juxtacellularly identified BF PV neurons in anesthetized rats increased their firing rate with cortical activation caused by tail pinch (23).

The BF also plays a critical role in sleep homeostasis (39). Adenosine levels rise in the BF as cortical PV neurons are activated in states of wakefulness (39). In vitro, glutamatergic inhibition of BF PV neurons is inhibited by adenosine (45). Thus, adenosinergic inhibition of BF PV neurons may contribute to the reduced cortical GBO associated with drowsiness (46).

The BF is one of the earliest brain areas to be affected in Alzheimer’s disease, and abnormalities of cortical oscillations are a feature of this disease (4, 47). Atrophy of BF cholinergic neurons has been widely replicated both in this disease and in normal aging (48). Cholinergic neurons normally excite BF GABA/PV neurons (41), and loss of BF GABA/PV neurons has been noted in aged mice which have accumulation of extracellular amyloid plaques and episodic-like memory impairments (49). Thus, reduced activity caused by the loss of cholinergic neurons or the direct loss of BF GABA/PV neurons may be partially responsible for cortical oscillation abnormalities and associated cognitive impairments in aging and Alzheimer’s disease.

With respect to schizophrenia, one of the most widely replicated findings is a reduced power of the 40-Hz ASSR (5, 29). Cortical PV neurons show reductions in GAD67 and PV expression in schizophrenia (11). BF PV neurons are derived from the same developmental pathway as cortical PV interneurons (50) and thus may be affected also. Unfortunately, to date, no postmortem study has examined BF PV neurons in schizophrenia. Our findings suggest that modulating the activity of BF PV neurons may be an effective strategy to restore abnormal GBO in schizophrenia.
Conclusions
Our results showing that BF PV neurons regulate and entrain cortical GBO have important implications for our understanding of the physiological and pathophysiological control of arousal.

Methods
PV-Cre mice (strain 008069; Jackson Laboratories) received BF injections of AAV-Chr2-EYFP or AAV-Arch-GFP followed by implantation of optical fibers. Cortical EEG recordings, immunohistochemistry, and in vitro recordings were performed as described previously (21, 41, 44). Details of the experimental procedures are provided in SI Materials and Methods. All experimental procedures conformed to US Veterans Administration, Harvard University, and US National Institutes of Health guidelines. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the VA Boston Healthcare System.

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