Rhythmic brain stimulation reduces anxiety-related behavior in a mouse model based on meditation training

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Meditation training induces changes at both the behavioral and neural levels. A month of meditation training can reduce self-reported anxiety and other dimensions of negative affect. It also can change white matter as measured by diffusion tensor imaging and increase resting-state midline frontal theta activity. The current study tests the hypothesis that imposing rhythms in the mouse anterior cingulate cortex (ACC), by using optogenetics to induce oscillations in activity, can produce behavioral changes. Mice were randomly assigned to groups and were given twenty 30-min sessions of light pulses delivered at 1, 8, or 40 Hz over 4 wk or were assigned to a no-laser control condition. Before and after the month all mice were administered a battery of behavioral tests. In the light/dark box, mice receiving cortical stimulation had more light-side entries, spent more time in the light, and made more vertical rears than mice receiving rhythmic cortical suppression or no manipulation. These effects on light/dark box exploratory behaviors are associated with reduced anxiety and were most pronounced following stimulation at 1 and 8 Hz. No effects were seen related to basic motor behavior or exploration during tests of novel object and location recognition. These data support a relationship between lower-frequency oscillations in the mouse ACC and the expression of anxiety-related behaviors, potentially analogous to effects seen with human practitioners of some forms of meditation.

meditation | anxiety | optogenetics | theta | mouse

One month of integrated mind body meditation (1), a form of mindfulness meditation, has been shown to reduce self-reported anxiety as measured by the Profile of Mood States, reduce the stress hormone cortisol, increase ventral anterior cingulate cortex (ACC) activity, and change the white matter pathways surrounding the ACC as measured by increased fractional anisotropy in diffusion tensor imaging studies (1–4). How might a purely mental exercise such as paying attention to the present moment work to produce these changes in behavior and brain connectivity (5)?

Some of these changes, including those to white matter, were hypothesized to be related to the finding that meditation can increase frontal theta activity even when the person is at rest (6). The theta activity may increase the number of active oligodendrocytes leading to increased myelination, thereby improving connectivity between the ACC and other limbic areas. Moreover, theta activity in humans has been uniquely correlated with glucose metabolism in the ACC (7). Because the ventral ACC connects to the amygdala and is thought to regulate its activity (8), these changes in the brain could result in the reduced anxiety found following meditation training.

To test this idea, we developed a mouse model in which various frequencies of oscillatory activity were induced in the ACC using optogenetics. Because mindfulness meditation has often been associated with reductions in anxiety and negative affect (9), we examined exploratory behavior in the light/dark box. In this ethological model of anxiety, time in the light versus dark sides is thought to be a joint effect of novelty (favoring exploration of the light side) and anxiety (favoring the dark) (10). Novel object and location recognition memory also were tested as a measure of cognitive change (11) because improved attention and memory also have been reported following meditation training (9).

To drive rhythmic activity across the ACC, we used optogenetic control of parvalbumin- expressing interneurons (PV-INs). Because PV-INs provide broad and potent inhibitory control over pyramidal cells, their activation or inactivation can control global levels of activity very effectively (12, 13). We used a PV-Cre driver line together with either a Cre-dependent Archaerhodopsin-2 (Arch) line (14) or Cre-dependent Channelrhodopsin-2 (ChR2) line (15) to provide cell type-specific hyperpolarization or depolarization, respectively. Three crosses were bred: (*i*) PV-Arch, in which light reduces PV-IN firing, thus stimulating global ACC excitatory activity; (*ii*) PV-ChR2, in which light increases PV-IN firing, thus suppressing global ACC excitatory activity; and (*iii*) homozygous PV-PV, to control for nonspecific light effects. For each cross, behavior also was assessed in control mice receiving no light delivery (no-laser condition). We reasoned that increased rhythmic output from the ACC would improve ACC connectivity and thus reduce anxiety and improve cognitive performance. Comparisons of PV-ChR2 and PV-PV mice enabled us to address the relative importance of rhythmic stimulation versus suppression of neural activity as well as any nonspecific laser effects.

Results

Here we evaluated the behavioral effects of optogenetically inducing rhythms in the mouse ACC. Rhythmic increases in spiking

Significance

Meditation training has been shown to reduce anxiety, lower stress hormones, improve attention and cognition, and increase rhythmic electrical activity in brain areas related to emotional control. We describe how artificially inducing rhythmic activity influenced mouse behavior. We induced rhythms in mouse anterior cingulate cortex activity for 30 min/d over 20 d, matching protocols for studying meditation in humans. Rhythmic cortical stimulation was followed by lower scores on behavioral measures of anxiety, mirroring the reductions in stress hormones and anxiety reported in human meditation studies. No effects were observed in preference for novelty. This study provides support for the use of a mouse model for studying changes in the brain following meditation and potentially other forms of human cognitive training.

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activity were induced in PV-Arch mice ($n = 34$) by delivering pulses at 1 Hz (200 ms per pulse), 8 Hz (5 ms per pulse), or 40 Hz (5 ms per pulse). Rhythmic decreases were induced in PV-Chr2 mice ($n = 27$) at the same frequencies. Light pulses also were presented to PV-PV mice ($n = 15$) lacking either optogenetic effector. Behaviors (Fig. S1) were quantified both before (Pre) and after (Post) a 4-wk protocol of rhythmic light delivery. Behavior from each cross was compared with a control group of mice ($n = 25$, drawn from all three crosses) that underwent the same Pre and Post behavioral assessments without exposure to rhythmic light pulses.

Effects on Neural Activity. Two groups received equivalent total illumination (1 Hz and 40 Hz, 200 ms per pulse), and two groups received equivalent pulse durations (8 Hz and 40 Hz, 5 ms per pulse) to account for those factors when assessing the effects of each manipulation. Mean responses at each frequency are illustrated in Fig. 1. Recordings were from histologically verified ACC neurons (Fig. S2*A*). Analyses compared (*i*) mean response amplitude to the light pulse, i.e., baseline versus response intervals (Fig. 1) and (*ii*) cumulative changes in spiking output with rhythmic light exposure (Fig. S2*B*).

In PV-Arch mice ($n = 2$), rhythmic light pulses induced robust phase-locked increases in putative pyramidal neuron firing at each frequency (Fig. 1 and Table S1). In PV-ChR2 mice ($n = 2$), rhythmic light pulses induced phase-locked suppression in spiking activity at each frequency (Fig. 1 and Table S1). No effects were detected in neuronal activity of PV-PV mice ($n = 4$) (Fig. 1 and Table S1). Therefore, our method of inducing rhythmic activity was successful and specific to mice expressing either Arch or ChR2.

To determine if any of the differences found in behavior were caused by changes in overall spiking output rather than by rhythms, we examined the cumulative change in activity over a 5-min pulse interval compared with a 5-min baseline period (Fig. S2*B*). In PV-Arch mice, rhythmic stimulation at 40 Hz produced a cumulative increase in spiking output during the pulse interval (*SI Results, Effects on Neural Activity*). In PV-ChR2 mice, rhythmic suppression at both 1 Hz and 40 Hz produced a cumulative decrease in activity.

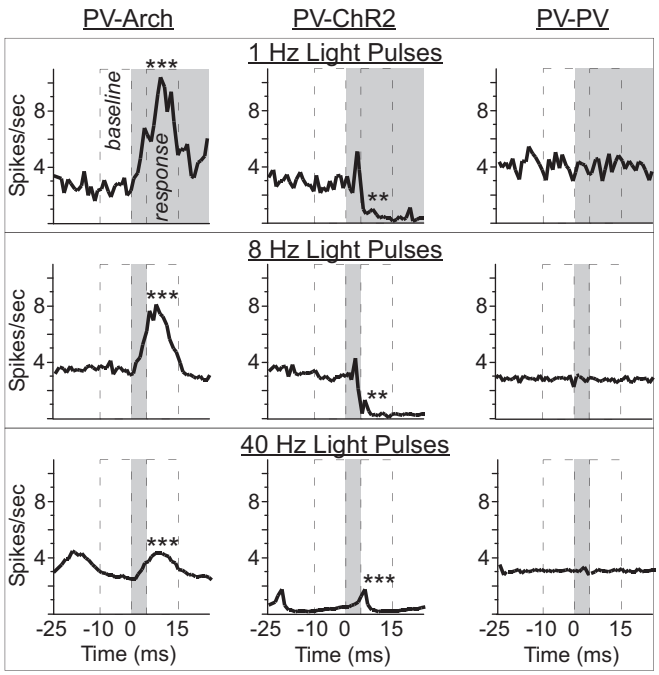

Fig. 1. Mean responses of PV-Arch, PV-ChR2, and PV-PV mouse pyramidal neurons to light pulses delivered at 1 Hz, 8 Hz, and 40 Hz. Light pulses elicited robust phase-locked increases in spiking activity in ACC neurons of PV-Arch mice ($n = 2$) and decreases in neurons of PV-ChR2 mice ($n = 2$) at each frequency, based on comparison of the 10-ms intervals before (baseline) and after (response) light onset. No light effects were seen in cells from PV-PV mice ($n = 4$). In the peristimulus time histograms, gray regions indicate the period of illumination, beginning at time 0 ms. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Behavior.

Light/dark box. The light/dark box is viewed as an ethological model of anxiety (10), placing into competition the drives to remain safe and to explore novel environments. The apparatus has two compartments (Fig. S1), one dark and enclosed, the other well-lit with transparent walls and an open top. Following 2 min of habituation in the dark, a barrier between the two sides was removed, enabling the mouse to explore the light side freely. Light-side exploration is typically reduced in anxious mice (10, 16). We held novelty constant by giving all groups the same exposure to the experimental box, so differences among groups following the laser treatment should reflect their relative anxiety. We analyzed the time to the first entry into the light side (latency), the total number of entries into the light side (entries), the time elapsed during exploration of the light side (duration), and the total number of vertical rears (verticals) for PV-Arch ($n = 34$), PV-ChR2 ($n = 27$), PV-PV ($n = 15$), and no-laser control mice ($n = 25$) before (Pre) and after (Post) the 4-wk protocol.

ANOVAs revealed differences in exploratory behavior (Table S2) between Pre and Post assessments that differed across groups. Effects for time (Pre × Post) were highly significant for all measures (all comparisons $P < 0.0001$). Each group exhibited significant decreases for duration, total entries, and verticals during the Post versus the Pre assessment (Table S3), representing a reduction in exploratory behavior. Significant interactions (time × group) were seen for duration [$F(3,97) = 5.81$, $P = 0.001$] and total entries [$F(3,97) = 3.6$, $P = 0.02$], and a trend was seen for latency ($P = 0.10$), indicating that the reduction in exploratory behavior differed in extent among groups. Interactions also were seen when analyzing by frequency (time × frequency), with significant effects for latency [$F(11,89) = 2.37$, $P = 0.01$], total entries [$F(11,89) = 3.53$, $P = 0.0004$], and duration [$F(11,89) = 3.61$, $P = 0.0003$] and a strong trend for verticals ($P = 0.051$). These decreases in exploratory behavior presumably resulted from the apparatus no longer being novel, reducing the impetus for exploration.

During the Pre assessment, light-side exploration was similar among groups (Fig. S3 and Table S2). During the Post assessment, however, PV-Arch mice that received rhythmic cortical stimulation made more light-side entries, scored higher for duration, and performed more verticals than mice from the other three groups (Fig. 2, *SI Results, Behavior*, and Table S2), including no-stimulation controls (no-laser group) and mice receiving rhythmic suppression of cortical activity (PV-ChR2 group). The latency to first light-side entry was largely similar among groups, although PV-PV mice were significantly delayed compared with PV-Arch mice ($df = 47$, $t = 2.2$, $P = 0.04$). Therefore, PV-Arch mice exhibited greater exploratory behavior following the 4-wk protocol.

The differences seen with PV-Arch mice during the Post assessment were frequency specific (Fig. 3 and *SI Results, Behavior*). Compared with the no-laser control group, mice exposed to 8-Hz pulses consistently scored higher for total entries, duration, and verticals. An increase in duration was also seen for mice exposed to 1-Hz pulses. The mice exposed to 40-Hz pulses were similar to the no-laser controls. These data demonstrate that increased exploration by PV-Arch mice during the Post assessment was associated specifically with cortical stimulation at 1 Hz and 8 Hz.

No frequency-specific effects were seen for PV-ChR2 or PV-PV mice relative to no-laser controls. Surprisingly, differences between frequencies were seen in the PV-PV group (Fig. 3, and *SI Results, Behavior*). PV-PV mice exposed to 1-Hz pulses exhibited more entries than mice exposed to 8-Hz pulses, in part because the

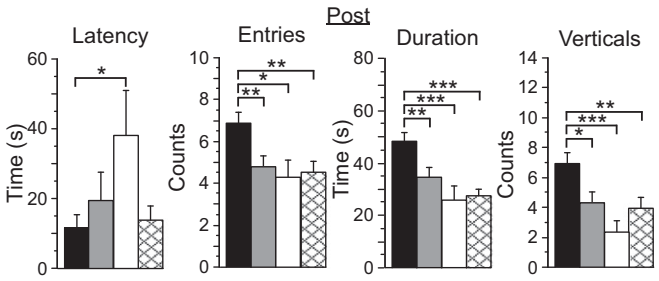

Fig. 2. Exploration differs significantly among groups following the induction of oscillatory activity. PV-Arch mice (n = 34, black bars) exhibited significantly reduced latency and more total entries into the light side of the light/dark box, explored the light side for longer (duration), and exhibited more vertical rears than mice from the PV-ChR2 (n = 27, gray bars), PV-PV (n = 15, white bars), and no-laser (n = 25, cross-hatched bars) groups. Error bars indicate SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

mice exposed to 1-Hz pulses did not show a decline in entries compared with the Pre assessment (entries Pre: 8.1 ± 1.1; Post: 7.9 ± 1.4). PV-PV mice exposed to 1-Hz and 40-Hz pulses both scored higher for duration and verticals than mice exposed to 8-Hz pulses.

Motor output. To assess basic motor output, we examined movement through a uniformly illuminated cylindrical arena (Fig. S1). Quantified behaviors included total distance traveled (distance), mean run speed (speed), total time immobile, number of immobile periods, and number of rotations. The results are summarized in Fig. 4 and Table S4.

ANOVAs revealed highly significant effects for time (Pre × Post) for all measures (all comparisons $P < 0.0001$) except rotations. Significant interactions (time × group) were observed for distance [$F(3,97) = 2.91$, $P = 0.04$], mean speed [$F(3,97) = 3.1$, $P = 0.03$], time immobile [$F(3,97) = 4.5$, $P = 0.005$], and total immobile periods [$F(3,97) = 6.0$, $P = 0.0009$]. When separated by frequency (time × frequency), significant interactions were seen for time immobile [$f(11,93) = 1.95$, $p = 0.04$] and total immobile periods [$F(11,93) = 2.4$, $P = 0.01$], with trends for distance ($P = 0.10$) and mean speed ($P = 0.08$).

During the Pre assessment, behavior in the arena was comparable among PV-Arch, PV-ChR2, PV-PV, and no-laser mice (Fig. S4). Following the 4-wk protocol, PV-Arch, PV-ChR2, and no-laser mice all demonstrated significant decreases in distance traveled and mean speed and increases in immobile periods and time immobile (all t test comparisons yielding $P \leq 0.0003$; see Table S4 for mean data). No-laser mice made fewer rotations ($df = 24$, $t = 2.3$, $P = 0.03$), with trends toward a similar reduction for PV-Arch and PV-ChR2 mice ($P = 0.18$ and $P = 0.06$, respectively). The changes also were similar in magnitude, with no significant between-group differences observed for any of the arena behaviors. These changes indicate (i) that optogenetic manipulation of ACC activity had no subsequent effect on spontaneous motor output and (ii) reduced exploration of the arena that, as with the Post light/dark box assessment, likely reflects the loss of novelty.

In contrast to the other three groups, the arena behavior of PV-PV mice during the Post assessment was almost identical to that seen 4 wk earlier during the Pre assessment (Table S4), resulting in the group differences illustrated in Fig. 4. During the Post arena assessment, PV-PV mice exhibited greater total distance traveled and greater mean speed than PV-Arch, PV-ChR2, and no-laser mice (Fig. 4 and *SI Results, Behavior*). PV-PV mice also exhibited fewer immobile periods but more time immobile (Fig. 4 and *SI Results, Behavior*).

What might account for the differences in PV-PV motor output? As noted above, PV-PV mice behaved similarly during Pre and Post assessments, in contrast to PV-Arch, PV-ChR2, and no-laser mice. When the data were broken down by frequency, no significant differences were observed between Pre and Post assessments for any behavior for PV-PV mice exposed to1 Hz, 8 Hz, or 40 Hz pulses and no-laser mice. This result cannot be attributed simply to the lower number of mice in each PV-PV group, because the SEs observed at each frequency for each behavior Pre and Post are comparable to those for the PV-Arch, PV-ChR2, and no-laser groups (each of which dis have more mice per group). Therefore, the difference in PV-PV motor output during the Post assessment appears to be partly attributable to a difference specific to that cross, rather than to an effect of light. However, during the Post assessment, PV-PV mice exposed to 1-Hz pulses did score higher than no-laser mice in distance traveled and mean speed (Fig. 5 and *SI Results, Behavior*), indicating that an effect of laser cannot be ruled out for some differences in PV-PV motor output.

Novel object and novel location recognition. To assess attention, basic cognition, and short-term memory, we quantified novel object and novel location recognition behavior (Fig. S1). During initial exposure, mice explored a pair of objects (familiar) in the same arena in which motor output data were collected. Next, mice explored one familiar and one novel object to assess object recognition memory. Finally, mice explored the same objects but with the familiar object moved to a novel location. This sequence is illustrated in Fig. S1. The time spent exploring each object was compared as a measure of preference for one object/position over another.

ANOVAs revealed a significant effect for time (Pre × Post) for each object/location in assessments 3–5 (most comparisons

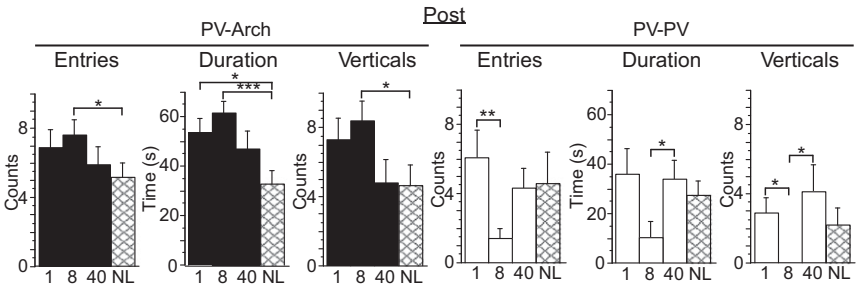

Fig. 3. Increased exploration in PV-Arch mice was associated with 1-Hz and 8-Hz cortical stimulation. Compared with PV-Arch no-laser mice (NL, n = 11, cross-hatched bars, left three panels), mice exposed to 8-Hz pulses (n = 13, black "8" bars) scored higher for total entries into the light side of the light/dark box, total duration of light-side exploration, and total vertical rears. Additionally, mice exposed to 1-Hz pulses (n = 13, black "1" bars) scored higher for duration. No differences were seen between PV-Arch exposed to 40-Hz pulses (n = 11, black "40" bars) and the no-laser control group (data not shown). For PV-ChR2 mice, no differences relative to the no-laser control group were seen at any frequency. For PV-PV mice, no differences relative to the no-laser control group were seen at any frequency (n = 5, cross-hatched bars, right three panels), although some differences were seen for mice exposed to 1-Hz pulses (n = 5, white "1" bars) and 40-Hz pulses (n = 5, white "40" bars) relative to mice exposed to 8-Hz pulses (n = 5, white "8" bars). Error bars indicate SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

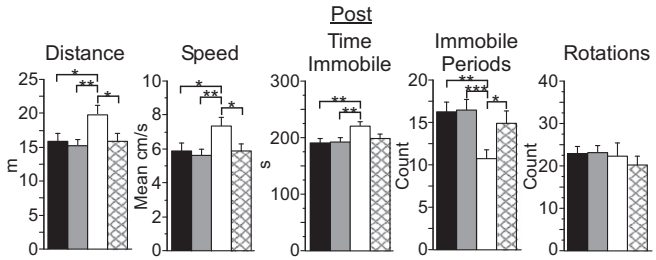

Fig. 4. PV-PV mice exhibit distinct arena behavior following the 4-wk protocol (Post). PV-PV mice (n = 15, white bars) scored higher for total distance traveled, mean speed, and total time immobile and had fewer immobile periods than mice from the PV-Arch (n = 34, black bars), PV-ChR2 (n = 27, gray bars), and no-laser (n = 25, cross-hatched bars) groups. Error bars indicate SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

$P < 0.0001$; novel location $F = 5.8$, $P = 0.02$), reflecting the general decrease in the time spent exploring the objects during the Post session (Table S5). A significant interaction (time × group) was detected for exploration of both objects during the test of object recognition memory [familiar object: $F(3,97) = 6.4$, $P = 0.0005$; novel object: $F(3,97) = 3.6$, $P = 0.02$]. An interaction also was seen when sorting by frequency (time × frequency) during this test [familiar object: $F(11,89) = 3.3$, $P = 0.0008$; novel object: $F(11,89) = 2.2$, $P = 0.02$]. No interactions (by group or by frequency) were detected for either object during the test of location recognition memory.

During the Pre assessment, PV-Arch, PV-ChR2, and no-laser mice all recognized the novel object (Fig. S5 and Table S6), whereas PV-Arch, PV-ChR2, and PV-PV mice all recognized the novel location. PV-PV mice and no-laser mice failed to exhibit object recognition and location recognition, respectively, although in both cases a modest preference for the novel stimulus was observed.

During the Post assessment, PV-Arch, PV-ChR2, and no-laser mice explored the two initial exposure objects comparably (Table S5), whereas PV-PV mice were biased toward the object serving as the familiar stimulus in the subsequent object recognition test. However, as with PV-Arch and PV-ChR2 mice, PV-PV mice exhibited a significant preference for the novel object, indicating that the bias seen during initial exposure did not interfere with, or spuriously account for, novel object recognition (Table S6). No-laser mice failed to exhibit significant object recognition behavior following the 4-wk protocol, although a strong trend was observed ($P = 0.051$) (Fig. 6). All four groups exhibited robust location recognition behavior. Comparisons of the "strength" of novelty preference, either as a ratio of novel/familiar or simple difference between novel and familiar exploration times, yielded little evidence of group effects. The ratio for novel object preference was greater for PV-PV mice than for no-laser mice ($df = 38$, $t = 2.2$; $P = 0.04$). The difference in novel location preference was greater for no-laser mice than for PV-ChR2 mice ($df = 50$, $t = 2.2$, $P = 0.03$). No other differences in the strength of preference for either object or location novelty were observed.

Discussion

In previous work it was found that integrated mind body meditation training (20 sessions of 30 min each) reduced measures of negative affect such as self-reported anxiety (1). Such training also has been found to increase rhythmic activity in the ventral ACC and improve connectivity in white matter tracts related to the ACC (1, 3, 4). In the current study we examined how inducing rhythmic activity at different frequencies in the mouse ACC influenced a range of behaviors, including light/dark box exploration (an assay of anxiety), motor output in the open arena, and novel object and location recognition (tests of attention and short-term memory). Each behavior was measured before and after 4 wk of rhythmic manipulation of ACC activity. All mice, including the non-stimulated controls, exhibited changes in behavior following the 4-wk protocol consistent with an overall reduction of exploration on the second occasion of being tested compared with the first occasion. However, we found that light/dark box exploration by PV-Arch mice that underwent rhythmic stimulation remained elevated compared with mice receiving rhythmic suppression or no manipulation. In contrast, behavior of PV-Arch mice in the open arena was comparable to each of the other groups, as was object exploration and performance during the tests of recognition memory. Given the absence of (*i*) a motor phenotype (e.g., hyperactivity), (*ii*) any generalized changes in exploratory behavior, or (*iii*) evidence of cognitive impairment or inability to recall past experience, the data suggest that rhythmic cortical stimulation may facilitate a reduction in anxiety. This finding supports our hypothesis that increased output from the ACC would be critical to behavior change. Nonetheless, we recognize that the light/dark box behavior is complex and that other tests of anxiety, such as extinction of conditioned fear, should be examined in future studies to help further constrain the nature of the effects observed here.

The greater light/dark box exploration seen in PV-Arch mice depended on the frequency of stimulation (Fig. 3). Stimulation at 8 Hz was associated with the most exploration. The effects of 1-Hz stimulation were in the same direction as with 8-Hz stimulation but were less robust. Stimulation at 40 Hz produced little or no difference from no-light stimulation, indicating that the effects on behavior were related to frequency rather than to the cumulative increase in spiking output. The absence of a behavioral effect with rhythmic suppression in PV-ChR2 mice confirms the importance of phase-locked increases in spiking activity rather than simply the induction of oscillations, per se. Overall, these data support our prediction that 8-Hz (theta) stimulation would be most effective, but the effect was not completely specific because 1-Hz stimulation produced similar results.

What factor(s) might underlie the possible decreased anxiety exhibited by PV-Arch mice following 1-Hz and 8-Hz stimulation? According to some views, the ACC serves to regulate limbic areas to which it is connected (8, 17). It is reasonable to propose that altering the strength and/or efficiency of those connections could influence behavior. Learning is associated with changes in synaptic transmission that may be induced artificially with 1-Hz and 8-Hz stimulation (18, 19). Learning also induces structural changes in white matter (20–23). Recently, it was shown that these white matter changes likely result from the formation of new myelin-producing oligodendrocytes (24). Our stimulation of cortex at 1 Hz and 8 Hz may drive the generation of new oligodendrocytes, resulting in increased myelination of fibers linking the ACC with

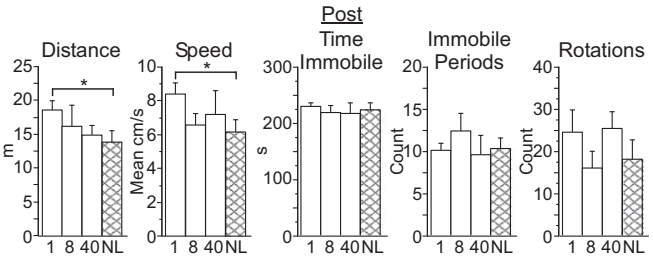

Fig. 5. PV-PV mice exposed to 1-Hz pulses show greater distance traveled and mean speed in the arena following the 4-wk protocol (Post). Although PV-PV mice exposed to 8-Hz (n = 5, "8" bars) or 40-Hz (n = 5, "40" bars) pulses did not differ from the no-laser control group (n = 5, cross-hatched bars), the differences in distance and speed between the mice exposed to 1-Hz pulses (n = 5, "1" bars) and no-laser mice (NL, cross-hatched bars) indicate that a light-specific effect cannot be ruled out in PV-PV mice. Error bars indicate SEM. *P < 0.05.

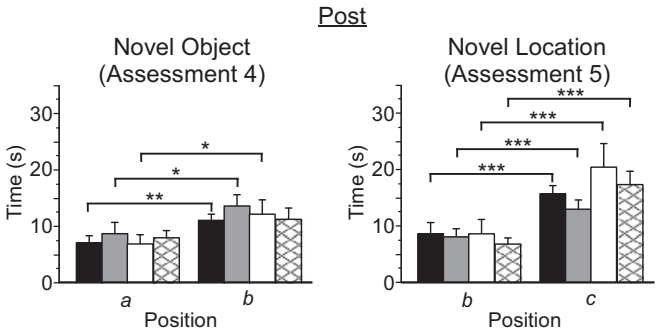

Fig. 6. Rhythmic manipulation of cortical activity did not alter object or location recognition memory. Mice were tested for novel object and novel location recognition memory before and after the 4-wk protocol. Greater exploration times are interpreted as novelty recognition. Following the 4-wk protocol (Post), PV-Arch ($n = 34$, black bars), PV-ChR2 ($n = 27$, gray bars), and PV-PV ($n = 15$, white bars) mice all recognized the novel object; a strong trend toward object recognition ($P = 0.051$) was also exhibited by no-laser mice ($n = 25$, cross-hatched bars). All four groups exhibited robust location recognition. *a*, *b*: positions corresponding to familiar and novel objects, respectively, during the object recognition test; *b*, *c*: positions corresponding to the familiar and novel locations, respectively, during the location recognition test. Error bars indicate SEM. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

other limbic areas and consequently reducing anxiety-related behaviors. The effects of rhythmic cortical stimulation on light/dark box exploration are consistent with ventral ACC involvement in regulating anxiety, fear, and stress (25–29), and chronic exposure to anxiety/stress induces changes in neurotransmission and long-term potentiation in the rodent ACC (26, 30, 31). Validation of the hypothesis linking 1- to 8-Hz cortical stimulation with increased myelination and decreased anxiety will require quantifying the generation of new oligodendrocytes and the density of myelin in mice that undergo stimulation.

Demonstrating that rhythmic cortical stimulation had no effect on motor output in the arena was essential for the interpretation of the light/dark box results. The ACC is reciprocally connected with primary and secondary motor areas and is often associated with motor processes (32–35). In the mouse, secondary motor cortex is situated immediately lateral to the ACC and likely was exposed to light, albeit to a lesser degree, during the 4-wk protocol. Any hyper-locomotor activity or other motor phenotype during the Post assessment would have complicated interpretation of the light/dark box exploration data. The absence of any such phenotype confirms the specificity of the effect to a reduction of anxiety-associated behavior.

The absence of an effect on recognition memory did not match our expectation based on previous observations of improved cognition with meditation (1, 2). However, it fits with the finding that meditation training, in comparison with relaxation, increased activity in the ventral rather than the dorsal ACC. This part of the ACC is connected with limbic areas and is thought to control mainly emotional activity (8). Moreover, it is not inconsistent with the broader literature. In rodents and humans, the ACC is active during object exploration and exposure to novelty (36–43). However, it is unclear whether the ACC is necessary for recognition memory (44–46). Whether the stimulation protocol used here would improve performance on more complex cognitive tasks or behaviors known to be dependent on the ACC remains to be determined.

We consider some hypotheses for the results obtained with the PV-PV animals. First, the Pvalb-IRES-Cre animals were originally generated by insertion of an internal ribosome entry site (IRES)-Cre cassette into the 3′ UTR of the parvalbumin (Pvalb) locus (47). Although this strategy effectively mimics the exact expression of a gene, endogenous expression of the target locus could be disrupted. That said, defects in endogenous parvalbumin expression that could account for our results have not been reported (48). Second, the PV, Arch, and ChR2 mouse lines, although all maintained on a C57BL6/J background, have been bred separately and thus could have undetected spontaneous mutations that have become fixed (genetic drift) that could result in physiological and behavioral differences. In light of these possibilities, and given the absence of an effect on spiking activity in PV-PV mice with light delivery, we feel the line-specific no-laser controls are more relevant to the effect of pulse frequency.

In previous work it was found that meditation training reduced measures of negative affect such as self-reported anxiety (1). Meditation training also has been found to increase activity in the ventral ACC and to improve connectivity in white matter tracts related to the ACC (1, 3, 4). Our current results show that rhythmic stimulation of frontal cortical activity in an animal model can produce some of the affective changes found in humans. It is important to note that our previous studies of human meditation used only one form of mindfulness training, and that other forms of meditation have been associated with rhythmic changes in other brain regions. For example, a study of experienced Buddhist meditators found increases in gamma frequency rhythms in parietal–occipital cortical regions (49). Nonetheless, although there is no reason to believe that theta stimulation accounts for all the effects of meditation, it may prove useful to have a plausible account of how meditation and lower frequency oscillations could lead to such physical changes in the brain as altered white matter (3, 4, 6). Future studies may examine the brain mechanisms that support these behavioral changes in our mouse model. It also will be possible to explore experimental manipulation of frontal theta activity by less invasive external stimulation in humans as a further test of the idea that frontal theta activity may be the effective mechanism in changing white matter following meditation.

Methods

All studies were conducted with protocols approved by the University of Oregon Institutional Animal Care and Use Committees in compliance with National Institutes of Health guidelines for the care and use of experimental animals.

Mice. We assessed the effects of rhythmic suppression or stimulation of cortical activity in mice expressing the light-gated ion channel Channelrhodopsin-2 or the proton pump Archaerhodopsin in parvalbumin-positive inhibitory interneurons. Parents were homozygous for Pvalb-IRES-Cre (PV; 008069; The Jackson Laboratory), Rosa-CAG-LSL-ChR2(H134R)-EYFP-WPRE (ChR2; 012569; The Jackson Laboratory), or Rosa-CAG-LSL-Arch-GFP-WPRE (Arch; 012735; The Jackson Laboratory). Optogenetic suppression of the ACC was performed using offspring heterozygous for both PV and ChR2 alleles (PV-ChR2). Optogenetic stimulation was performed using offspring heterozygous for both PV and Arch alleles (PV-Arch). Mice homozygous for Pvalb-IRES-Cre (PV-PV) were used to control for nonspecific effects of pulsed light delivery. Data were collected from both male and female mice aged 8–12 wk at the time of surgery. A total of 47 PV-Arch, 38 PV-ChR2, and 25 PV-PV mice were included in the behavioral and single-neuron recording experiments.

Surgery. To study the behavioral effects of rhythmic cortical activity manipulations, mice were implanted with a two-by-two array of optic fibers (200-μm diameter) overlying each hemisphere of the ACC. For recording single-neuron activity, a four-tetrode array with a single 200-μm optic fiber was implanted in the ACC.

Behavioral Data Acquisition and Stimulus Delivery. Mice were run through a sequence of five 5-min assessments both before and after a 4-wk protocol (for a detailed description, see *SI Methods* and Fig. S1. The only changes before (Pre) and after (Post) the 4-wk protocol were in the set of objects used and their positions on the arena floor relative to the visual cues during assessments 3–5 (i.e., one set of objects was used before the 4-wk protocol, and another set was used after the protocol). For optogenetic manipulations, 520-nm wavelength modules were set to 9.5 mW for PV-Arch mice, and 445-nm wavelength modules were set to 6.3-mW for PV-ChR2 mice. The effective spread for each wavelength at these intensities is 1.5 mm in diameter (14, 50, 51).

Assessments 1 and 3–5 were hand-scored separately by at least two viewers blind to experimental condition. Assessment 2 was quantified by the commercial tracking software package Any-maze (Stoelting Co.). All analyses of behavioral data were performed using the statistical analysis program StatView. ANOVAs were used to assess effects of time (Pre versus Post) and interactions by group or frequency. Paired *t* tests were used to assess within-group differences following the 4-wk protocol. Unpaired *t* tests were used for between-group comparisons and for within-group comparisons for frequency effects versus no-laser controls (for details, see *SI Methods*).

Single-Neuron Recording and Analysis. Single-neuron data were analyzed during a 5-min baseline interval followed by a 5-min pulsed-light interval for each frequency. Data were collected from each mouse at 1-Hz, 8-Hz, and 40-Hz pulse frequencies. For PV-PV mice, data were collected using either 445-nm or 520-nm wavelength light to control for nonspecific wavelength effects. Analyses were limited to well-isolated putative pyramidal neurons [see *SI Methods* for interneuron exclusion criteria and cluster isolation metrics (52–54)]. Mean firing rates were calculated for each 5-min interval and were analyzed using paired *t* tests. To identify significant phase-locked changes in firing rate associated with the laser, paired *t* tests compared spiking activity during a 10-ms response interval 6–15 ms following the onset of each laser pulse with a 10-ms baseline interval immediately preceding each pulse (the 5-ms interval following the pulse was excluded to control for potentially confounding photoelectric artifacts). Unpaired *t* tests were used for between-frequency and between-group comparisons. Between-group comparisons for the size of the change were calculated using a simple difference between response and baseline firing rates.

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