Prefrontal cortical mechanisms underlying delayed alternation in mice

Mark A. Rossi,1 Volodya Y. Hayrapetyan,1 Benjamin Maimon,1 Krystal Mak,1 H. Shawn Je,2 and Henry H. Yin1

1Department of Psychology and Neuroscience and Department of Neurobiology, Center for Cognitive Neuroscience, Duke University, Durham, North Carolina; and 2Program in Neuroscience and Behavioral Disorders, Duke-National University of Singapore Graduate Medical School, Singapore

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Prefrontal cortical mechanisms underlying delayed alternation in mice. J Neurophysiol 108: 1211–1222, 2012. First published April 25, 2012; doi:10.1152/jn.01060.2011.—The prefrontal cortex (PFC) has been implicated in the maintenance of task-relevant information during goal-directed behavior. Using a combination of lesions, local inactivation, and optogenetics, we investigated the functional role of the medial prefrontal cortex (mPFC) in mice with a novel operand delayed alternation task. Task difficulty was manipulated by changing the duration of the delay between two sequential actions. In experiment 1, we showed that excitotoxic lesions of the mPFC impaired acquisition of delayed alternation with long delays (16 s), whereas lesions of the dorsal hippocampus and ventral striatum, areas connected with the PFC, did not produce any deficits. Lesions of dorsal hippocampus, however, significantly impaired reversal learning when the rule was changed from alternation to repetition. In experiment 2, we showed that local infusions of muscimol (an agonist of the GABAA receptor) into mPFC impaired performance even when the animal was well trained, suggesting that the mPFC is critical not only for acquisition but also for successful performance. In experiment 3, to examine the mechanisms underlying the role of GABAergic inhibition, we used Cre-inducible Channelrhodopsin-2 to activate parvalbumin (PV)-expressing GABAergic interneurons in the mPFC of PV-Cre transgenic mice as they performed the task. Using whole cell patch-clamp recording, we demonstrated that activation of PV-expressing interneurons in vitro with blue light in brain slices reliably produced spiking and inhibited nearby pyramidal projection neurons. With similar stimulation parameters, in vivo stimulation significantly impaired delayed alternation performance. Together these results demonstrate a critical role for the mPFC in the acquisition and performance of the delayed alternation task.

THE PREFRONTAL CORTEX (PFC) is widely thought to be involved in higher cognitive functions, as shown by studies in primates (Caster et al. 2004; Fuster 1973; Tanji and Hoshi 2008; Walker et al. 2009; Wang et al. 2004; Warden and Miller 2010) and in rodents (Farovik et al. 2008; Horst and Laubach 2009; Taylor et al. 2003; Uylings et al. 2003). Damage to the PFC commonly produced deficits. Lesions of dorsal hippocampus, however, significantly impaired reversal learning when the rule was changed from alternation to repetition even when the animal was well trained, suggesting that the mPFC is critical not only for acquisition but also for successful performance. In experiment 3, to examine the mechanisms underlying the role of GABAergic inhibition, we used Cre-inducible Channelrhodopsin-2 to activate parvalbumin (PV)-expressing GABAergic interneurons in the mPFC of PV-Cre transgenic mice as they performed the task. Using whole cell patch-clamp recording, we demonstrated that activation of PV-expressing interneurons in vitro with blue light in brain slices reliably produced spiking and inhibited nearby pyramidal projection neurons. With similar stimulation parameters, in vivo stimulation significantly impaired delayed alternation performance. Together these results demonstrate a critical role for the mPFC in the acquisition and performance of the delayed alternation task.

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devices such as Alzheimer’s disease (Cole et al. 2011; Fernando and Robbins 2011; Goldman-Rakic and Selemon 1997; Huntley and Howard 2010; Insel and Fernald 2004; Sigurdsson et al. 2010). In recent years, the mouse has become a major model organism for the study of such disorders in particular and of cognition and behavior in general, and those mouse models provide powerful genetic tools that allow us to dissect the underlying molecular, cellular, and circuit mechanisms. As altered prefrontal cortical functioning may lead to cognitive deficits associated with neuropsychiatric and neurodegenerative disorders, understanding the underlying molecular mechanisms of these cognitive deficits is critical for the development of successful treatment. However, because of the lack of valid functional assays, it remains unclear whether the mouse mPFC plays a role similar to that of its rat and primate counterparts. Consequently, despite rapidly growing interest in the mouse mPFC as a result of the development of numerous models of psychiatric disorders with impaired prefrontal function (Belforte et al. 2010; Kellendonk et al. 2006; Yizhar et al. 2011), its function remains poorly understood.

In this study we assessed the functional role of the mouse mPFC, using a novel delayed alternation task in which task difficulty can be manipulated by changing the imposed delay between presses. Using operant conditioning methods (Fan et al. 2012; Yin 2009; Yu et al. 2009), we trained mice to press one lever and a different lever after a predetermined delay for food rewards. This task introduces a delay between two actions in a behavioral sequence and a simple “switching” rule (AB or BA), so that the mouse must remember which action it just completed to choose the next action correctly (Mizumori et al. 1987). We found that selective lesions of the mouse mPFC, but not NAc or rostral-dorsal hippocampus, disrupted delayed alternation performance at long delays. Temporary local inactivation of mPFC with the GABAA receptor agonist muscimol also impaired performance even after the mice had been well trained, suggesting that mPFC is critical for both acquisition and performance of delayed alternation. Finally, by injecting Cre-inducible channelrhodopsin-2 (ChR2) into mPFC of mice with selective expression of Cre recombinase in parvalbumin (PV)-expressing GABAergic interneurons, we found that stimulation of mPFC PV-expressing interneurons also impaired delayed alternation performance. Together this set of experiments demonstrates a critical role for the mPFC in acquisition and performance of the delayed alternation task.

MATERIALS AND METHODS

Subjects

Male C57BL/6J mice (Jackson Laboratories) aged 2–7 mo at the start of experiments served as subjects. During testing, food was
restrained to maintain mice at ~85% of free-feeding weight. Water was available at all times in the home cages. All experiments were reviewed and approved by the Duke University Institutional Animal Care and Use Committee. For all optogenetic experiments, 129P2-Pvalb\textsuperscript{im}(Cre)\textsubscript{Arb}\textsuperscript{f}1 mice (Jackson Laboratories) were used. This line is characterized by Cre recombinase expression at the locus of PV, a calcium binding protein that is commonly found in interneurons (Bartos et al. 2007; Hippchenmeyer et al. 2005).

Surgery

Excitotoxic lesions. Mice were anesthetized with isoflurane (induction at 3%, maintained at 1% during surgery) and head fixed on a stereotax (David Kopf Instruments). The surface of the skull was exposed, and small holes were drilled into the skull above the targeted sites. Quinolinic acid (60 mM) was infused bilaterally into the target areas with 24-gauge steel cannulas at a rate of 0.1 µl/min and a total volume of 0.4 µl. Five minutes after the infusion, cannulas were removed. Coordinates (in mm relative to bregma) were for mPFC lesions: anteroposterior +2.0, mediolateral ±0.5, dorsoventral (relative to dura) −1.5; for NAc lesions: anteroposterior +1.3, mediolateral ±1.0, dorsoventral −4.0; and for rostral-dorsal hippocampus lesions: anteroposterior −1.8, mediolateral ±1.2, dorsoventral −1.8. Thirty minutes after hippocampal lesions were performed, 0.2 ml of diazepam (Sigma; 2 mg/kg, dissolved in saline) was administered intraperitoneally to prevent seizures. Sham lesions were made with the same procedures except that no drug was infused. Mice were allowed to recover for 7 days after surgery before food restriction began.

Cannula implants in mPFC. After completion of the initial experiments, five sham-lesioned mice and three wild-type mice (n = 8) with the same delayed alternation training were given unrestricted food and then anesthetized as above and implanted with a 24-gauge steel guide cannula (Plastics One). Each cannula had a 5-mm plastic pedestal and was cut 7 mm below the pedestal. Cannulas were implanted through a hole drilled in the skull at anteroposterior +2.0 and mediolateral +0.75 at 22° relative to the dorsoventral axis—to avoid major blood supply—targeting final coordinates anteroposterior +2.0, mediolateral 0, and dorsoventral −1.5. This placement was chosen to target both hemispheres of the mPFC with a single cannula. Cannulas were secured in place with dental acrylic, and a styret was inserted that protruded −0.2 mm beyond the end of the cannula. Mice were allowed to recover for 1 wk after surgery before food restriction began.

Optic fiber implants in mPFC. Mice were anesthetized (as above), and a hole was drilled in the skull just above mPFC at anteroposterior +2.0, mediolateral +0.5, dorsoventral (relative to dura) −1.5 mm relative to bregma. A 24-gauge steel cannula was lowered to the desired depth, and 0.4 µl of either AAV-EF1a-DIO-hChr2(H134R)-eYFP (n = 7) or pAAV-EF1a-DIO-eYFP (n = 5) was infused into the target site at 0.1 µl/min. Five minutes after viral infusion, the cannula was removed and a custom-made, flat-cut fiber-optic implant (5-mm length below ferrule, 105-µm core diameter, 1.25-mm-OD ceramic zirconia ferrule; Precision Fiber Products) was lowered into place 0.2 mm above the site of injection and secured with dental acrylic. All mice were allowed to recover for at least 2 wk after surgery before food deprivation and testing began.

Instrumental Training

Training took place in eight Med Associates (St. Albans, VT) operant chambers (21.6 cm long × 17.8 cm wide × 12.7 cm high) housed within light-resistant and sound-attenuating walls. Each chamber was equipped with a food magazine that received Bio-Serv 14-mg Dustless Precision Pellets from a pellet dispenser, with two retractable levers on either side of the magazine and a 3-W, 24-V house light on the wall opposite the levers and magazine. Computers with the Med-PC-IV program (Med Associates) were used to control the chambers and record behavior. The chamber used for optogenetic stimulation was similar except that it was designed for in vivo electrophysiology, with Plexiglas walls and floor grid, different dimensions (35 cm × 28 cm × 22 cm), and a 2.5-in.-diameter circular opening on the top of the chamber for optical fibers.

One week after surgery, lever-press training began and consisted of five consecutive days of continuous reinforcement (CRF), during which the mice received one pellet for each lever press. At the start of each session, the house light was illuminated and both levers were extended. At the end of the session, the house light was turned off and the levers were retracted. Sessions ended after 60 min or 100 rewards.

The delayed alternation task was adapted from a task described previously (Yin 2010). The task used in the present study included two important differences: 1) both levers retracted after each press, and 2) both left-right and right-left sequences were rewarded instead of only one. Briefly, each testing session began with illumination of the house light and extension of both levers. At the start of each trial, both levers were extended. Pressing either one of them resulted in retraction of both levers for a predetermined delay (2, 8, or 16 s) while the house light remained on. After the delay, both levers were again extended. Pressing the same lever as the initial press resulted in no reward delivery, whereas pressing the opposite lever resulted in delivery of one food pellet (see Fig. 2G). After the second press, regardless of success or failure, the trial ended with retraction of both levers and the house light being turned off for 20 s before the start of the next trial. Each session lasted 90 min. Under these conditions, the mice must alternate their presses to earn rewards. During rule reversal testing, the contingencies of the 2 s condition were reversed such that pressing the same lever twice during one trial resulted in delivery of a reward, whereas alternating between levers was no longer rewarded.

For experiments using local drug infusion in mPFC the task was the same as above, except that the sessions lasted only 60 min. On test days, mice were lightly anesthetized with 1% isoflurane and the styret was removed; 0.4 µl of muscimol (1 µg/µl) or phosphate-buffered saline (PBS, 0.9%) was infused at a rate of 0.2 µl/min on alternating days through a 31-gauge steel cannula (injector), which was inserted through the chronic guide cannula and protruded 0.2 mm beyond the tip of the guide. Two minutes after infusion, the injector was removed, the styret was replaced, and the mice were allowed to recover in their home cages for 15 min before testing began.

Optogenetic Stimulation

For all optogenetic experiments, the Cre-inducible ChR2 with yellow fluorescent protein (YFP)-tagged viral vector pAAV5-EF1a-DIO-hChR2(H134R)-eYFP or the control vector, which lacks the channelrhodopsin sequence, pAAV5-EF1a-DIO-eYFP (~10\textsuperscript{12} infectious units/ml; University of North Carolina Vector Core) from the lab of Karl Deisseroth was used. These viruses provide channelrhodopsin (or eYFP) expression that is specific to cells that express Cre recombinase (Cardin et al. 2010).

Before testing each day, mice were lightly anesthetized with 1% isoflurane and then connected to a 2-m sheathed fiber (62.3-µm core diameter) with a ceramic sleeve (Precision Fiber Products). The fiber was connected to a 473-nm laser (Shanghai Laser & Optics) located outside the operant chamber. The laser was controlled with custom software (MATLAB, MathWorks; LabVIEW, National Instruments). Optogenetic testing lasted 20 min, during which time the laser was active (100 Hz, 5-ns pulse duration) either for the entire 20-min test (tonic) or only during the delay between presses (delay). Before testing each day, the final output of the laser was adjusted, based on the transmittance of each implant, to be 5 mW into the brain.

In Vitro Electrophysiology

For patch-clamp recordings, coronal slices were cut from 1- to 5-mo-old PV-Cre mice (n = 18 males and females) that had been
injected with AAV5-DIO-ChR2-eYFP bilaterally into the mPFC. We waited at least 2 wk after virus injection before starting the slice recording experiments. Brains were removed quickly into ice-cold solution bubbled with 95% O2-5% CO2 containing the following (in mM): 194 sucrose, 30 NaCl, 2.5 KCl, 1 MgCl2, 26 NaHCO3, 1.2 NaH2PO4, and 10 d-glucose. After 5 min brains were blocked and coronal slices were taken at 250 μm. During the recovery period slices were placed at 35°C with oxygenated artificial cerebrospinal fluid (aCSF) solution containing the following (in mM): 124 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 26 NaHCO3, 1.2 NaH2PO4, and 10 d-glucose. All recordings were made under continuous perfusion of aCSF at 29.5°C with a 2–3 ml/min flow rate. Pipettes (2.5–5 MΩ) for voltage-clamp experiments contained the following (in mM): 120 cesium methane sulfonate, 5 NaCl, 10 tetraethylammonium chloride, 10 HEPES, 4 lidocaine N-ethyl bromide, 1.1 EGTA, 4 magnesium ATP, and 0.3 sodium GTP, pH adjusted to 7.2 with CsOH and osmolarity set to 298 mosM with sucrose. The internal solution for current-clamp experiments contained (in mM) 150 potassium gluconate, 2 MgCl2, 1.1 EGTA, 10 HEPES, 3 sodium ATP, and 0.2 sodium GTP, with pH adjusted to 7.2 with KOH and osmolarity set to ~300 mosM with sucrose.

Recordings were made in prelimbic cortex from either visually identified PV interneurons or untransfected pyramidal neurons near ChR2-expressing PV neurons. For recordings of PV and pyramidal neurons, slices were stimulated with 470-nm light generated from an LED assembly (Thor Labs) focused through a ×40 objective (Olympus). During recordings, 5-ms flashes of light were delivered at 1–100 Hz to the entire ×40 field with an LED current driver (Thor Labs). Power density was ~5 mW/mm2.

Histology

After the completion of experiments, lesioned and implanted mice were anesthetized with isoflurane and transcardially perfused with 0.9% saline followed by 10% buffered formalin solution. The brains were sliced into 100-μm coronal sections with a Vibratome 1000 Plus, stained with thionin, and examined under a light microscope to verify the placement of cannula tips and extent of the lesions. For mice used in the optogenetic experiments, the brains were sliced into 35-μm coronal sections with a cryostat, stained with 2% Neurotrace fluorescent Nissl stain (Invitrogen, excitation 530 nm, emission 615 nm), and examined under a fluorescence microscope to verify the extent of ChR2 or YFP expression.

RESULTS

Histological Analysis of Lesions

The brains of all lesioned mice were examined (Fig. 1) after completion of behavioral experiments. Two mice from the NAc group and one from the mPFC group were excluded from all analyses because of inaccurate lesion placement (data not shown). The extent of the lesions is shown in Fig. 1, with the dark shaded region indicating the smallest lesions and the light shaded area indicating the largest lesions. Final group sizes were n = 10 for sham lesion, n = 7 for PFC, n = 6 for NAc, and n = 8 for hippocampus.

PFC Lesions Increased Overall Rate of Pressing

To test the role of the mPFC and areas connected with it in delayed alternation, we assessed the effects of excitotoxic lesions of the dorsal hippocampus, NAc, or mPFC. Mice were first trained to press a lever for food on continuous reinforcement schedules (each press earns a food pellet). Once lever pressing had been successfully acquired, mice were trained to perform the delayed alternation task (Fig. 2G). Data from the first 5 days of training at each of the three delay conditions—2, 8, and 16 s—were analyzed by two-way mixed ANOVAs and Bonferroni post hoc tests to assess the effects of lesion and time on each behavioral measure. In the 2 s condition (Fig. 2A), analysis revealed an interaction between time and lesion [F(12,108) = 4.23, P < 0.0001]. mPFC lesions increased the number of presses per session during the first 5 days of the 2 s condition, whereas sham lesions decreased pressing over the same time period. There was also a main effect of lesion [F(3,108) = 15.39, P < 0.0001] on the number of lever presses such that mPFC-lesioned mice pressed more frequently than sham-lesioned mice on all 5 test days (all P < 0.05). In addition, there was a main effect of time on the number of lever presses at 2 s [F(4,108) = 6.90, P < 0.0001]. In the 8 s condition (Fig. 2B), there was a main effect of lesion [F(3,108) = 7.39, P < 0.001] on the number of lever presses. Bonferroni post hoc tests showed that the mPFC group pressed significantly more than the control group on days 1, 3, 4, and 5 (all P < 0.05) and the hippocampal group pressed less frequently than the control group on day 3 (P < 0.05). There was a main effect of time [F(4,108) = 7.14, P < 0.0001] such that all groups tended to press fewer times on later days, suggesting increased efficiency as they learned the task. In the 16 s condition (Fig. 2C), there was a main effect of lesion [F(3,108) = 5.31, P < 0.01] on the number of lever presses. Post hoc tests showed that on day 2 the mPFC group pressed significantly more than sham-lesioned control animals (P < 0.05) and on day 4 the hippocampus group pressed significantly less than control animals (P <
mPFC is Critical for Performance at Long Delays but Not for Acquisition of Delayed Alternation

Figure 2, D–F, depict the proportion of correct trials for the first 5 days of the 2-, 8-, and 16-s delay conditions, respectively. During the 2 s condition, there was a main effect of time \( F(12,108) = 1.90, P > 0.05 \) and no interaction between the factors \( F(3,108) = 1.27, P > 0.05 \), indicating that, in both the short (2 s)- and moderate (8 s)-delay conditions, all groups of mice learned the delayed alternation task at approximately the same rate (Fig. 2, D and E). mPFC-lesioned mice performed significantly worse than other groups in the 16 s condition (Fig. 2F). There was a main effect of time \( F(12,108) = 4.63, P < 0.01 \), a main effect of group \( F(3,108) = 3.36, P < 0.05 \), and no interaction \( F(12,108) < 1.0, P > 0.05 \), indicating that mPFC is necessary for successful delayed alternation performance when working memory demands are high. Interestingly, hippocampal mice were able to perform this task as well as control mice. This illustrates a dissociable role for this region such that the hippocampus is critical for tasks that require spatial alternation (Dudchenko 2004; Dudchenko et al. 2000) but lesions do not affect performance on tasks, such as the one presented here, that require instrumental delayed alternation.

The mPFC group earned significantly more rewards than the other three groups \( F(3,108) = 8.07, P < 0.001 \); Fig. 3A). Further analysis revealed that the mPFC group received significantly more rewards than all other groups on days 3, 4, and 5 of the 2 s condition (all \( P < 0.05 \)). There was also a significant effect of time on the number of rewards received \( F(12,108) = 62.30, P < 0.0001 \) but no interaction between the factors \( F(12,108) = 1.63, P > 0.05 \). During the 8 s trials (Fig. 3B), there was no main effect of lesion \( F(3,108) = 1.64, P > 0.05 \), a main effect of time \( F(12,108) = 10.47, P < 0.0001 \), and no interaction between the factors \( F(12,108) = 1.06, P > 0.05 \). In the 16-s delay condition (Fig. 3C), there was no main effect of lesion \( F(3,108) = 1.15, P > 0.05 \), a main effect of time \( F(12,108) = 3.13, P < 0.05 \), and no interaction between the factors \( F(12,108) = 0.82, P > 0.05 \) on the number of rewards earned.

Figure 3, D–F, show the average number of magazine entries per test session in the 2, 8, and 16 s conditions, respectively. Under the short-delay condition (Fig. 3D), there was a main effect of lesion \( F(3,108) = 8.70, P < 0.001 \), a main effect of time \( F(12,108) = 42.46, P < 0.0001 \), but no interaction \( F(12,108) < 1.0, P > 0.05 \). Post hoc tests revealed that the mPFC group tended to enter the magazine much more frequently than other groups on days 1, 2, 4, and 5 (\( P < 0.05 \)). At 8-s delay (Fig. 3E), there was no
We therefore compared the median interresponse time (IRT), the time between two actions within each trial, for rewarded and unrewarded trials from each delay condition by two-way ANOVA (Fig. 4). For rewarded trials (Fig. 4A) during the 2-s delay condition, there was a main effect of time \( [F_{(4,108)} = 5.86, P < 0.001] \) but no main effect of lesion \( [F_{(3,108)} < 1.0, P > 0.05] \) and no interaction \( [F_{(12,108)} = 1.20, P > 0.05] \). For rewarded trials in the 8-s condition, there was no main effect of time \( [F_{(4,108)} = 1.52, P > 0.05] \), no main effect of lesion \( [F_{(3,108)} < 1.0, P > 0.05] \), and no significant interaction \( [F_{(12,108)} = 1.56, P > 0.05] \). For rewarded trials during the 16-s delay condition, there was no main effect of time \( [F_{(4,108)} < 1.0, P > 0.05] \), no main effect of lesion \( [F_{(3,108)} = 2.05, P > 0.05] \), and no significant interaction \( [F_{(12,108)} = 1.14, P > 0.05] \). Figure 4B shows the median IRTs for unrewarded trials. At 2-s delay, there was no main effect of time \( [F_{(4,108)} = 1.96, P > 0.05] \) or lesion \( [F_{(3,108)} < 1.0, P > 0.05] \) and no interaction \( [F_{(12,108)} < 1.0, P > 0.05] \). With an 8-s delay, there was no main effect of time \( [F_{(4,108)} < 1.0, P > 0.05] \) or lesion \( [F_{(3,108)} < 1.0, P > 0.05] \) on unrewarded IRT and no interaction between the factors \( [F_{(12,108)} = 1.03, P > 0.05] \). In the 16 s condition, there was again no main effect of time \( [F_{(5,108)} < 1.0, P > 0.05] \) or lesion \( [F_{(3,108)} = 1.48, P > 0.05] \) or interaction \( [F_{(12,108)} = 1.35, P > 0.05] \) on the IRT of unrewarded trials. These results clearly confirm that there was no significant difference between groups in the time between the first and second press during the delayed alternation task, indicating that the actual experienced delays are comparable for all groups.

### Dorsal Hippocampus Lesions Impair Reversal Learning

To test whether selective lesions of dorsal hippocampus, NAc, or mPFC affected cognitive flexibility (the ability to adapt to changing situations and learn new rules), we tested

**Lesions Did Not Affect Interresponse Time**

It is conceivable that poor performance of mPFC-lesioned mice could be due to general motor or attentional deficits (Broersen and Uylings 1999; Muir et al. 1996; Uylings et al. 2003). For example, if the animal is simply distracted and ignores the lever insertion, then the actual experienced delay between the two actions will be significantly longer than the imposed delay. Thus it is critical to find out whether the experienced delay was affected by the lesions.

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**Fig. 3.** Lesions of mPFC temporarily increase activity. A–C: mean number of rewards during 5 days of each delay condition. D–F: mean number of magazine entries during 5 days of each delay condition. At the shortest delay, PFC-lesioned mice received significantly more rewards (A) and entered the magazine significantly more (D) than sham-lesioned control mice. Error bars are SE. ***P < 0.001.

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**Fig. 4.** Lesions did not affect interresponse time (IRT). The median IRT for all 3 delay conditions is plotted for rewarded (A) and unrewarded (B) trials. Two-way ANOVA revealed no group differences for either rewarded or unrewarded trials at 2-, 8-, or 16-s delays, further supporting the conclusion that lesions did not affect overall motor function (all P > 0.05). Error bars are SE.
mice with a reversal paradigm (Fig. 5). Briefly, after completion of the 16-s delay condition, all mice were retrained for 3 days at the 2-s delay condition (data not shown) and then were tested for 3 consecutive days of contingency reversal. During reversal, the instrumental contingency was reversed such that instead of alternating between levers to receive a reward, the mice were instead required to press the same lever twice during one trial in order to earn a reward. Thus the rule was changed from “shift” to “stay.” As shown in Fig. 5, during reversal the hippocampus group performed significantly worse than all other groups (Fig. 5A). Two-way mixed ANOVA on the proportion of correct trials revealed a main effect of time \([F(2,50) = 127.16, P < 0.0001]\), a main effect of lesion \([F(3,50) = 5.69, P < 0.01]\), and no interaction between the factors \([F(6,50) < 1.0, P > 0.05]\). Post hoc tests indicated that the hippocampus group performed significantly worse than all other groups on day 2 of reversal testing (all \(P < 0.05\)). Hippocampal mice also received fewer rewards during reversal testing (Fig. 5B). There was a main effect of time \([F(2,50) = 118.68, P < 0.0001]\), a main effect of lesion \([F(3,50) = 8.62, P < 0.001]\), but no interaction \([F(6,50) < 1.0, P > 0.05]\) on the number of rewards earned during reversal. Post hoc tests revealed that the hippocampus group received significantly fewer rewards than all other groups on days 1 and 2 (all \(P < 0.05\)). The total number of lever presses per day of reversal testing is shown in Fig. 5C. There was a main effect of time \([F(2,50) = 9.16, P < 0.001]\), a main effect of lesion \([F(3,50) = 4.83, P < 0.01]\), but no interaction \([F(6,50) = 2.03, P > 0.05]\). Post hoc tests indicated that on day 1 the hippocampus group pressed significantly fewer times than sham-lesioned control animals (\(P < 0.05\)) and on day 3 the mPFC group pressed significantly more than the hippocampus and sham lesion groups. Dorsal hippocampus lesions significantly impaired mice in learning to reverse the rule of the delayed alternation task, which suggests a role for this area in behavioral flexibility associated with instrumental learning.

Local Inactivation of mPFC Impairs Delayed Alternation Performance

Neuronal microcircuits, involving glutamatergic pyramidal neurons and local inhibitory interneurons within the mouse mPFC, may have essential roles in the encoding and maintenance of information in working memory. Given the behavioral deficits observed after mPFC lesions, we tested whether acute disruption of neuronal microcircuits within mPFC affects delayed alternation performance even after animals are well-trained. The activation of GABAergic interneurons should inhibit the glutamatergic pyramidal neurons, thereby disrupting performance. Therefore, we infused the GABA\(_A\) agonist muscimol (1 \(\mu g/\mu l\)) and PBS locally into mPFC through chronically implanted guide cannulas on alternating days prior to testing in the short (2 s) and long (16 s) delay conditions (Fig. 6, A and B). The effects of muscimol and PBS were analyzed by paired t-tests in which the PBS condition served as a within-subject control. When muscimol was infused into mPFC prior to testing in the 2-s delay condition (Fig. 6, C–F),
the proportion of correct trials was reduced [Fig. 6C; \( t_{(7)} = 4.99, P < 0.01 \)], the number of rewards earned was reduced [Fig. 6D; \( t_{(7)} = 6.50, P < 0.001 \)], and the number of lever presses was reduced [Fig. 6E; \( t_{(7)} = 4.56, P < 0.01 \)]. Muscimol had no effect on the number of magazine entries [Fig. 6F; \( t_{(7)} = 1.79, P > 0.05 \)]. Muscimol infusion into mPFC prior to performance of the 16 s condition (Fig. 6, G–J) also reduced the proportion of correct trials [Fig. 6G; \( t_{(6)} = 4.07, P < 0.01 \)] and rewards earned [Fig. 6H; \( t_{(6)} = 3.29, P < 0.01 \)] and elicited a marginal reduction of lever presses [Fig. 6I; \( t_{(6)} = 2.17, P = 0.07 \)]. There was no change in magazine entries [Fig. 6J; \( t_{(6)} = 0.05, P > 0.05 \)]. Inactivation of mPFC with muscimol significantly impaired delayed alternation performance at both short and long delays. Importantly, motivation and motor activity were not severely affected by temporary inactivation of this area, as illustrated by the fact that the mice showed no differences in magazine entries and were still able to press the levers >10 times after muscimol infusion. These data suggest that acute disruption of microcircuits within mPFC can affect the acquisition and processing of information necessary for delayed alternation performance.

Optogenetic Stimulation of PV Neurons in mPFC Impairs Delayed Alternation Performance

GABAergic interneurons are commonly classified on the basis of morphology (e.g., basket cells, chandelier cells), electrophysiology (e.g., fast spiking, low-threshold spiking), synaptic connectivity (e.g., soma, distal dendrites), and gene expression (e.g., PV, somatostatin) (Markram et al. 2004). Of these, we were particularly interested in the PV-expressing interneurons, which send GABAergic projections to many pyramidal neurons, primarily at the soma and proximal dendrites. PV-expressing interneurons are known to influence the spiking and synchrony of pyramidal neurons by generating the gamma-frequency oscillations that may organize functional neural ensembles (Blatow et al. 2003; Cardin et al. 2009; Sohal et al. 2009). In addition, recent findings demonstrated that targeted disruption of cortical interneurons elicits behavioral deficits (Deans et al. 2001; Lodge et al. 2009).

We first verified that optical stimulation elicits frequency-dependent spiking of ChR2-expressing PV neurons in vitro (Fig. 7). We performed whole cell recordings of visually identified ChR2-expressing neurons in the mPFC. At both low (10 Hz) and high (100 Hz) stimulation frequencies, 5-ms pulses of 470-nm light elicited reliable spiking in PV neurons (Fig. 7, A and B). The magnitude of depolarization was dependent on light intensity in both current-clamp (Fig. 7C) and voltage-clamp (Fig. 7D) recordings. We also recorded from neighboring uninfected pyramidal neurons while stimulating ChR2-expressing PV interneurons with blue light (Fig. 8). Both low- and high-frequency stimulation hyperpolarized pyramidal neurons (Fig. 8). Stimulating PV interneurons generated a significant hyperpolarization in current-clamp mode (\(-4.0 \pm 0.75 \text{ mV, } n = 10 \)) in neighboring pyramidal neurons. This is in agreement with previous reports that have used AAV5-DIO-ChR2 in the same line of PV-Cre transgenic mice to hyperpolarize pyramidal neurons (Sohal et al. 2009).

To test the role of PV interneurons during delayed alternation, we exploited Cre-loxP techniques and light-activated ChR2. Briefly, we expressed a double-floxed inverted open-reading frame (DIO) adeno-associated virus (AAV) encoding ChR2 in PV-Cre transgenic mice (PV::ChR2), in which expression of ChR2 and the eYFP tag is restricted to GABAergic PV neurons. As a control, we expressed a DIO-AAV5 encoding only eYFP, which lacks the channelrhodopsin sequence (PV::eYFP). Figure 9B depicts a coronal brain slice from a PV::Cre mouse injected in mPFC with Cre-dependent DIO-ChR2-eYFP virus (green) and stained with fluorescent Nissl stain (red) to illustrate the location of the injection and stimulation within mPFC. All viral injection sites were confirmed to be in the prelimbic region of mPFC. After viral infection, mice were trained on the delayed alternation task and then stimulated on alternating days with three stimulation conditions: no stimulation, delay stimulation, in which the laser was on for the entire trial, and tonic stimulation, in which the laser was on for the entire session (Fig. 9A). We compared stimulation at low (10 Hz) and high (100 Hz) frequency.

Two-way repeated-measures ANOVAs and Bonferroni post hoc tests were used unless otherwise noted to compare the effects of stimulation (none, delay and tonic stimulation) between PV::...
ChR2 and PV::eYFP groups for both 10 and 100 Hz conditions for all behavioral measures. In the 2-s delay condition, 10-Hz stimulation elicited no significant main effect of stimulation on the proportion of correct trials [Fig. 9C; $F_{(2,8)} = 3.91, P > 0.05$], a main effect of group [$F_{(1,8)} = 23.54, P < 0.01$], and an interaction between stimulation and group [$F_{(2,8)} = 5.26, P < 0.05$]. Post hoc tests revealed that both delay and tonic stimulation significantly reduced the performance of PV::ChR2 mice ($P < 0.01$). During the 2-s delay condition, there was also a main effect of 100-Hz stimulation on the proportion of correct trials [$F_{(2,20)} = 5.89, P < 0.01$] and a main effect of group [$F_{(1,20)} = 3.03, P < 0.05$] but no interaction [$F_{(2,20)} = 1.80, P > 0.05$] (Fig. 9D). Bonferroni post hoc test revealed that the performance of the PV::ChR2 group was significantly impaired compared with the PV::eYFP group in the tonic stimulation condition ($P < 0.05$) but not the other conditions. At 16 s, 10-Hz stimulation yielded a main effect of stimulation [$F_{(2,12)} = 5.52, P < 0.05$], a main effect of group [$F_{(1,12)} = 24.41, P < 0.01$], and a marginally significant interaction [$F_{(2,12)} = 3.42, P = 0.07$] (Fig. 9E). Post hoc tests showed that the performance of PV::ChR2 mice was reduced by both delay and tonic stimulation ($P < 0.01$). There was a main effect of 100-Hz stimulation on proportion correct at 16 s [Fig. 9F; $F_{(2,20)} = 13.89, P = 0.0001$], no main effect of group [$F_{(1,20)} = 1.24, P > 0.05$], and an interaction [$F_{(2,20)} = 5.96, P < 0.01$]. Tonic stimulation, but not delay stimulation, reduced delayed alternation performance of PV::ChR2 mice ($P < 0.05$). These data confirm that both high- and low-frequency stimulation of PV interneurons impair performance on the delayed alternation task and the deficit observed was a result of neither viral infection nor nonspecific effects of laser stimulation on brain tissue.

We next analyzed the behavior of PV::ChR2 and PV::eYFP groups as they performed the delayed alternation task to confirm that laser stimulation per se did not cause any adverse motor or motivational effects (Fig. 10). In the 2-s delay condition, 10-Hz stimulation yielded a main effect of stimulation [$F_{(2,8)} = 12.08, P < 0.01$], a main effect of group [$F_{(1,8)} = 47.85, P < 0.01$], and an interaction between the factors [$F_{(2,8)} = 10.20, P < 0.01$] on the number of rewards earned (Fig. 10A). Post hoc tests showed that both delay and tonic stimulation reduced the number of rewards ($P < 0.001$). During 10-Hz stimulation there was a main effect of stimulation [$F_{(2,8)} = 15.16, P < 0.01$] and group [$F_{(1,8)} = 16.77, P < 0.05$] and an interaction [$F_{(2,8)} = 8.57, P < 0.05$] on the number of lever presses. Both stimulation during the delay ($P < 0.05$) and tonic stimulation ($P < 0.001$) reduced the number of presses. Using a 10-Hz frequency did not alter the number of magazine entries [$F_{(2,8)} < 1.0, P > 0.05$]. Using a frequency of 100 Hz yielded a main effect of stimulation [$F_{(2,20)} = 17.76, P < 0.0001$] but no group effect [$F_{(1,20)} < 1.0, P > 0.05$] and no interaction [$F_{(2,20)} = 2.70, P > 0.05$] on the number of rewards earned (Fig. 10B). There was a main effect of stimulation on the number of lever presses [$F_{(2,20)} = 9.66, P < 0.01$] but no group effect [$F_{(1,20)} < 1.0, P > 0.05$] and no interaction [$F_{(2,20)} > 1.0, P > 0.05$]. At the 2-s delay there was a main effect of stimulation on the number of magazine entries [$F_{(2,20)} = 4.00, P < 0.05$] but no group effect [$F_{(1,20)} < 1.0, P > 0.05$] and no interaction between the factors [$F_{(2,20)} = 3.28, P > 0.05$].

In the 16-s delay condition, there was a main effect of 10-Hz stimulation on the number of rewards earned [$F_{(2,12)} = 10.73, P < 0.01$] but no group effect [$F_{(1,12)} = 3.73, P > 0.05$] and no interaction [$F_{(2,12)} = 1.84, P > 0.05$] (Fig. 10C). There was a...
marginal significance effect of 10-Hz stimulation \( F_{(2,12)} = 3.38, \ P = 0.07 \) but no group effect and no interaction \( F < 1.0, P > 0.05 \) on the total number of lever presses. As with the 2-s delay condition, there were no effects of 10-Hz stimulation or group and no interaction \( P > 0.05 \) on the number of magazine entries. There was a main effect of 100-Hz stimulation \( F_{(1,20)} = 4.95, P < 0.05 \) on the number of rewards earned during the 16-s delay condition (Fig. 10D). A t-test revealed that tonic 100-Hz stimulation, but not delay stimulation, reduced the number of rewards for the PV::ChR2 group \( t_{(10)} = 2.53, P < 0.05 \). There was a main effect of 100-Hz stimulation on the number of lever presses \( F_{(2,20)} = 5.60, P < 0.05 \) and no effect of group or interaction \( F < 1.0, P > 0.05 \). There was no effect of 100-Hz stimulation or group or interaction \( P > 0.05 \) on the number of magazine entries in the 16-s condition. In agreement with the local muscimol inactivation data, these data show that tonic optical stimulation—both high and low frequency—of PV interneurons in the mPFC significantly reduces delayed alternation performance at both short and long delays through increased inhibitory tone within PFC circuits.

DISCUSSION

Natural behaviors often involve delays between two different elements in a sequence. To know what to do next, one must recall what was just completed. In this study we attempted to examine the neural substrates underlying this cognitive function in mice. Using a combination of excitotoxic lesions, reversible inactivation, whole cell patch-clamp recording, and optogenetic stimulation techniques, we showed that the mPFC is critical in the performance of delayed alternation. The role of the mPFC was revealed when we made the task more difficult by increasing the duration of the delay period between the two actions. As shown in Fig. 4, mice performed the second action as soon as the delay ended, so the scheduled delay roughly approximated the actual experienced delay. With longer delays, not only is there a stronger demand on working memory for the recently completed action, but the additional waiting period also provides more opportunities to become distracted.

Previous studies have suggested that the frontostriatal circuit is critical for instrumental delayed alternation in rats (Dunnett et al. 1999, 2005; Izaki et al. 2001; Seamans et al. 1995). Similarly, connections between hippocampus and PFC are thought to be necessary for delayed alternation performance (Czerniawski et al. 2009; Floresco et al. 1997). However, these studies had several limitations: 1) they did not use excitotoxic lesions that spare fibers of passage; 2) they did not measure the detailed behavioral parameters to rule out deficits in general sensory-motor functions; and 3) they did not compare irreversible excitotoxic lesions with reversible online manipulations of neural activity such as muscimol inactivation and PV-expressing interneuron stimulation in order to assess the relative contributions of the PFC to acquisition and performance.

Our results not only confirm previous work on the functional contribution of the PFC, but also provide additional insight into the circuit and cellular mechanisms underlying proper functioning of the mPFC. Experiment 1 (excitotoxic lesions) shows that the mPFC (specifically, the prelimbic and infralimbic areas that are heavily connected with mPFC) reveals a role of rostral-dorsal hippocampus in cognitive flexibility during instrumental reversal learning. Experiment 2 (muscimol inactivation) shows that mPFC involvement is not limited to the acquisition of the delayed alternation rule but is necessary for online performance, even when the task is well-learned. Finally, experiment 3 (optogenetic stimulation) shows that the activation of PV interneurons, which increased GABA-ergic inhibition in mPFC, also impairs delayed alternation performance without affecting general motor activity or motivation.

Excitotoxic Lesions

The rodent mPFC projects extensively to the NAc and receives projections from many cortical and subcortical areas including the hippocampal formation (Almada et al. 2009; Hoover and Vertes 2007; Tanji and Hoshi 2008; Tzschentke and Schmidt 2000). We therefore assessed delayed alternation performance after mPFC, dorsal hippocampus, and NAc lesions. Using excitotoxic lesions, which limit the damage to cell bodies but spare fibers of passage, we showed that only mPFC lesions produced a significant impairment at long (16 s) but not short (2 s) delays. Neither the ventral striatum nor the dorsal hippocampus appears to be required for successful performance of this task, although these areas are extensively connected with the mPFC. These findings confirm a role of mPFC
in delayed alternation performance and extend the results of previous studies to include operant mouse models (Deacon et al. 2003; Dunnett et al. 2005).

Interestingly, a novel dissociation was found between mPFC and the dorsal hippocampus. Hippocampus lesions did not affect acquisition of delayed alternation but impaired performance when the rule was reversed from “shift” to “stay” (i.e., instead of pressing a different lever for the second action, press the same lever again; Fig. 5). Previous studies have shown intact spatial reversal learning in hippocampus-lesioned rats (Marston et al. 1993; Whishaw and Tomie 1997), but the reversal deficit observed here requires more than simply spatial reversal; rather, it is a rule reversal—a change in the instrumental contingency. This result is in accord with previous lesion studies of the dorsal hippocampus (Winocur and Olds 1978). Our results suggest a more general role for the hippocampus in cognitive flexibility.

It may be surprising that only mPFC-lesioned mice showed deficits in delayed alternation. Maze tasks, with higher demands in spatial navigation, may require proper hippocampal functioning. This could also explain why such tasks often take longer for mice to learn. In contrast, most mice in our experiments showed >80% success rate after three to five training sessions. Such rapid acquisition and reliability are promising for the development of a high-throughput screening method to identify behavioral deficits associated with impaired prefrontal functioning.

Muscimol Inactivation

Muscimol, an agonist of GABA<sub>A</sub> receptors, was used to reversibly inactivate the mPFC. Using a within-subject design, we compared the performance of the same animal after muscimol injection to performance after PBS injection, which served as the control condition. It is important to note that the mice were already fully trained on the task when they received the injections, so the data revealed that a functioning mPFC is critical for successful performance on the delayed alternation task, even when the animal has already acquired the task. Local inactivation of mPFC did not significantly increase the press rate; however, excitotoxic lesions of the same area resulted in increased pressing in the initial stages following surgery. This may be explained by compensatory changes in the neural circuits involved that are caused by permanent lesions, which do not occur when the mPFC is only temporarily inactivated by local infusion of muscimol.

Optogenetic Stimulation of PV-Positive Interneurons

The mPFC can only perform its computational role in the circuit by transmitting signals from its pyramidal projection neurons to other areas in the brain. The effects of muscimol injection suggest that inhibiting the pyramidal neurons by activating GABA<sub>A</sub> receptors can disrupt prefrontal function.

The main source of GABAergic inhibition to the pyramidal neurons is cortical interneurons. To further elucidate the role of the local cortical circuitry in delay alternation performance, we took advantage of optogenetic tools, which were used successfully to study the function of PV-expressing GABAergic interneurons in the brain (Cardin et al. 2009; Sohal et al. 2009; Yizhar et al. 2011). These interneurons play a key role in GABAergic inhibition of the pyramidal projection neurons. Forming an extensive network that can be synchronized through electrical coupling (Connors and Long 2004), they are thought to be involved in the generation of fast oscillations in the gamma range (30–80 Hz) (Wang 2010).

Here we report the first results on the role of mPFC PV-positive interneurons in operant behavior. The present results support a critical role for prefrontal interneurons in the coordination of rule-based and goal-directed behavior. High- and low-frequency stimulation (10 and 100 Hz) of GABAergic interneurons can directly inhibit pyramidal neurons, the source of glutamatergic efferents from mPFC. Our in vitro and in vivo optogenetic stimulation results corroborate the results from local injection of muscimol. Optical stimulation of PV interneurons can mimic the effects of chronic lesion and temporary inactivation in a spatially and temporally precise way. A recent study also using manipulations of PV interneurons (Yizhar et al. 2011) has suggested that excessive cortical excitation could result in severe social deficits found in psychiatric disorders, while elevation of inhibition can rescue such deficits. The present results, on the other hand, reveal that excessive inhibition can also result in strong behavioral deficits, suggesting the importance of the proper balance of cortical excitation and inhibition.

Conclusions

We have performed the first systematic examination of the effects of prefrontal cortical manipulations on delayed alternation performance in mice. Our results have significant implications for understanding of the mPFC. To perform successfully on the delayed alternation task, the mouse must remember which action it completed recently, as well as the rule that a different lever must be pressed on the next action. We showed that local GABAergic inhibition by PV-expressing interneurons can interfere with the prefrontal output to downstream structures such as the basal ganglia, thus impairing performance on the delayed alternation task.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES

Almada RC, Borelli KG, Albrecht-Souza L, Brandao ML. Serotonergic mechanisms of the median raphe nucleus-dorsal hippocampus in condi-


Yin HH. The role of the murine motor cortex in action duration and order. *Front Integr Neurosci* 3: 23, 2009.

