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Stress Impairs Prefrontal Cortical Function via D_1 Dopamine Receptor Interactions With Hyperpolarization-Activated Cyclic Nucleotide-Gated Channels

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ABSTRACT

BACKGROUND: Psychiatric disorders such as schizophrenia are worsened by stress, and working memory deficits are often a central feature of illness. Working memory is mediated by the persistent firing of prefrontal cortical (PFC) pyramidal neurons. Stress impairs working memory via high levels of dopamine D_1 receptor (D_1R) activation of cyclic adenosine monophosphate signaling, which reduces PFC neuronal firing. The current study examined whether D_1R-cyclic adenosine monophosphate signaling reduces neuronal firing and impairs working memory by increasing the open state of hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels, which are concentrated on dendritic spines where PFC pyramidal neurons interconnect.

METHODS: A variety of methods were employed to test this hypothesis: dual immunoelectron microscopy localized D_1R and HCN channels, in vitro recordings tested for D_1R actions on HCN channel current, while recordings in monkeys performing a working memory task tested for D_1R-HCN channel interactions in vivo. Finally, cognitive assessments following intra-PFC infusions of drugs examined D_1R-HCN channel interactions on working memory performance.

RESULTS: Immunoelectron microscopy confirmed D_1R colocalization with HCN channels near excitatory-like synapses on dendritic spines in primate PFC. Mouse PFC slice recordings demonstrated that D_1R stimulation increased HCN channel current, while local HCN channel blockade in primate PFC protected task-related firing from D_1R-mediated suppression. D_1R stimulation in rat or monkey PFC impaired working memory performance, while HCN channel blockade in PFC prevented this impairment in rats exposed to either stress or D_1R stimulation.

CONCLUSIONS: These findings suggest that D_1R stimulation or stress weakens PFC function via opening of HCN channels at network synapses.

Keywords: cAMP, D_1 dopamine receptor, HCN channel, Prefrontal cortex, Stress, Working memory

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Dysfunction of the prefrontal cortex (PFC) is central to cognitive deficits observed in many psychiatric disorders (1). The PFC uses representational knowledge to provide top-down guidance of behavior, thought, and affect (2). The working memory functions of PFC are rapidly and markedly impaired by exposure to even mild, uncontrollable stress (3). Dopamine (DA) D_1 receptor (D_1R) signaling via cyclic adenosine monophosphate (cAMP) plays a critical role in PFC function, producing an inverted U-shaped dose-response curve whereby moderate levels improve PFC function, while higher levels impair function, e.g., taking PFC offline during stress (4,5). While the PFC expands and differentiates greatly from rodents to primates, there are some effects that bridge across species, e.g., the D_1R inverted U-shaped dose-response curve with impairment at high levels of DA stimulation has been observed in mice (6), rats (4), monkeys (7), and humans (8,9). The detrimental effects of high levels of D_1R stimulation during stress are particularly important to understand, as D_1Rs are altered in disorders such as schizophrenia (10,11), and the symptoms of such disorders are often precipitated or exacerbated by stress (3,12).

Much of the research on the molecular mechanisms of PFC function has focused on spatial working memory in animal models. In primates, spatial working memory is mediated by recurrent excitation among networks of pyramidal neurons in layer III and likely layer V that sustain firing over a delay period in dorsolateral PFC (DLPFC), considered to be the electrophysiological correlate of representational knowledge (2,13,14). Maintenance of persistent firing depends upon effective contacts between pyramidal neurons via *N*-methyl-D-aspartate (NMDA)

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receptor synapses at dendritic spines (2,15). These networks can be spatially tuned by lateral inhibition from gamma-aminobutyric acidergic interneurons (2,16,17) and are dynamically altered by neuromodulators via intracellular signaling at the spine, referred to as dynamic network connectivity (18). Moderate levels of D_1R stimulation enhance spatial tuning by sculpting away noise, reducing neuronal firing for nonpreferred directions, while higher levels of D_1R stimulation erode all task-related firing (5).

Exposure to stress produces high levels of DA release in the rodent PFC, and D_1R-mediated generation of cAMP reduces PFC network firing and impairs working memory (4,5,19). Similarly, direct application of D_1R agonists onto DLPFC neurons reduces delay-related firing via increased cAMP in monkeys performing a working memory task (5), and high doses of D_1R agonists impair spatial working memory in mice (6), rats (4), and monkeys (7). However, it is not understood how D_1R stimulation reduces PFC neuronal firing, as D_1Rs can have multiple actions in PFC, e.g., in layer V neurons of ferrets and rodents, D_1R stimulation can reduce glutamate release from axon terminals (20) or alter opening of calcium channels (21). D_1Rs may also reduce firing by increasing the open probability of hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels via cAMP. Previous research has shown that cAMP reduces PFC neuronal firing by increasing the open state of HCN channels on dendritic spines (22). In cerebral cortex, HCN1 and HCN2 subunits form heterotetramers that are particularly responsive to cAMP (23–25) and associate with cAMP-regulating proteins in spines of monkey layer III DLPFC (22,26). In monkeys performing a working memory task, low doses of an HCN channel blocker, such as ZD7288, enhance task-related DLPFC neuronal firing, whereas treatments that increase the HCN channel current (I_h) reduce firing (22).

The current study investigated whether high levels of D_1R stimulation, as occur during stress exposure, reduce PFC neuronal firing and impair working memory through cAMP interactions with HCN channels. Understanding the molecular basis of cognitive function requires molecular, cellular, and behavioral approaches. Thus, we employed a cross-species approach, integrating monkey anatomy and in vivo physiology, mouse in vitro physiology, and rat and monkey behavior.

METHODS AND MATERIALS

All procedures were approved by the Yale Institutional Animal Care and Use Committee.

Immunoelectron Microscopy

Brains of two adult, male rhesus macaques (*Macaca mulatta*) were fixed via transcardial perfusion and blocked coronally. Sections of DLPFC were used for dual immunolabeling using peroxidase and/or gold immunoprobes for D_1Rs and the HCN1 subunit of HCN channels. Tissue processing and all immunoprocedures, including antibody characterization, are described in detail in Paspalas *et al.* (26).

Monkey In Vivo Recordings

Subjects. Two adult, male rhesus macaques, C (13 years old) and P (7 years old), were housed individually under standard laboratory conditions. A highly palatable juice reward was used to minimize need for dietary regulation. Water was provided ad libitum, and animals were fed monkey chow (Purina Mills, Gray Summit, Missouri) and fruit immediately following testing. Care was taken to habituate them to all procedures.

Spatial Working Memory Task, Physiological Recording, and Iontophoresis in Monkey DLPFC. Monkeys were trained on the oculomotor delayed response (ODR) task, a test of spatial working memory as outlined in Figure 1A. Single-unit recordings were performed in the principal sulcus region of DLPFC (Figure 1B), the brain region typically associated with performance of the ODR task (13). Many regularly spiking neurons (presumed pyramidal neurons) in DLPFC exhibit persistent firing that is maintained throughout the delay period (delay cells). Spatial tuning is observed in a large subset of delay cells, whereby they increase firing during the delay period following a cue in their preferred direction but show smaller increases or even inhibition of firing for nonpreferred directions (2) (Figure 1C). The microcircuitry underlying spatially tuned delay-related firing is shown in Figure 1D.

Once delay cell firing was stabilized, drugs were applied via iontophoresis in minute amounts that were insufficient to alter behavior (Supplementary Methods and Materials in Supplement 1). Drugs were applied in the following sequence for at least six trials for each of the eight cue locations: 1) control; 2) ZD7288 (5–10 nA; Tocris, Ellisville, Missouri); 3) ZD7288 + SKF38393 (20–25 nA; Tocris); 4) SKF38393; and 5) recovery (Table S1 in Supplement 1 lists the drug conditions applied to each neuron). Data were analyzed using analysis of variance and circular regression using Stata (StataCorp LP, College Station, Texas) (Supplementary Methods and Materials in Supplement 1). A working model of molecular mechanisms that weaken PFC network connectivity at dendritic spines is illustrated in Figure 1E.

Mouse In Vitro Recordings

Whole-cell current-clamp recordings were obtained from layer V pyramidal neurons in mouse (postnatal days 22–28) PFC slices; very young animals are needed to enhance slice viability. HCN channels are prominent along the dendrites of layer V pyramidal neurons in both monkeys and rodents (26,27). In electrophysiological recordings, these cells exhibit a prominent sag current in response to hyperpolarizing voltage steps, corresponding to the slow activation kinetics of I_h (27,28).

To measure I_h, a 400-ms hyperpolarizing current (−300 pA) was injected via the patch pipette. I_h was quantified as the ratio of the peak hyperpolarization to the steady state value during the pulse. SKF81297 (10 μmol/L; Tocris) was bath-applied, and the response in membrane potential was measured. I_h values were compared between control and SKF81297 conditions using paired *t* tests.

Rat Behavioral Studies

Subjects. Young adult male Sprague Dawley rats (Taconic, Germantown, New York) were housed individually under standard laboratory conditions. They were kept on a 12-hour light/dark cycle, and behavioral experiments were conducted during the light phase. Highly palatable rewards (chocolate

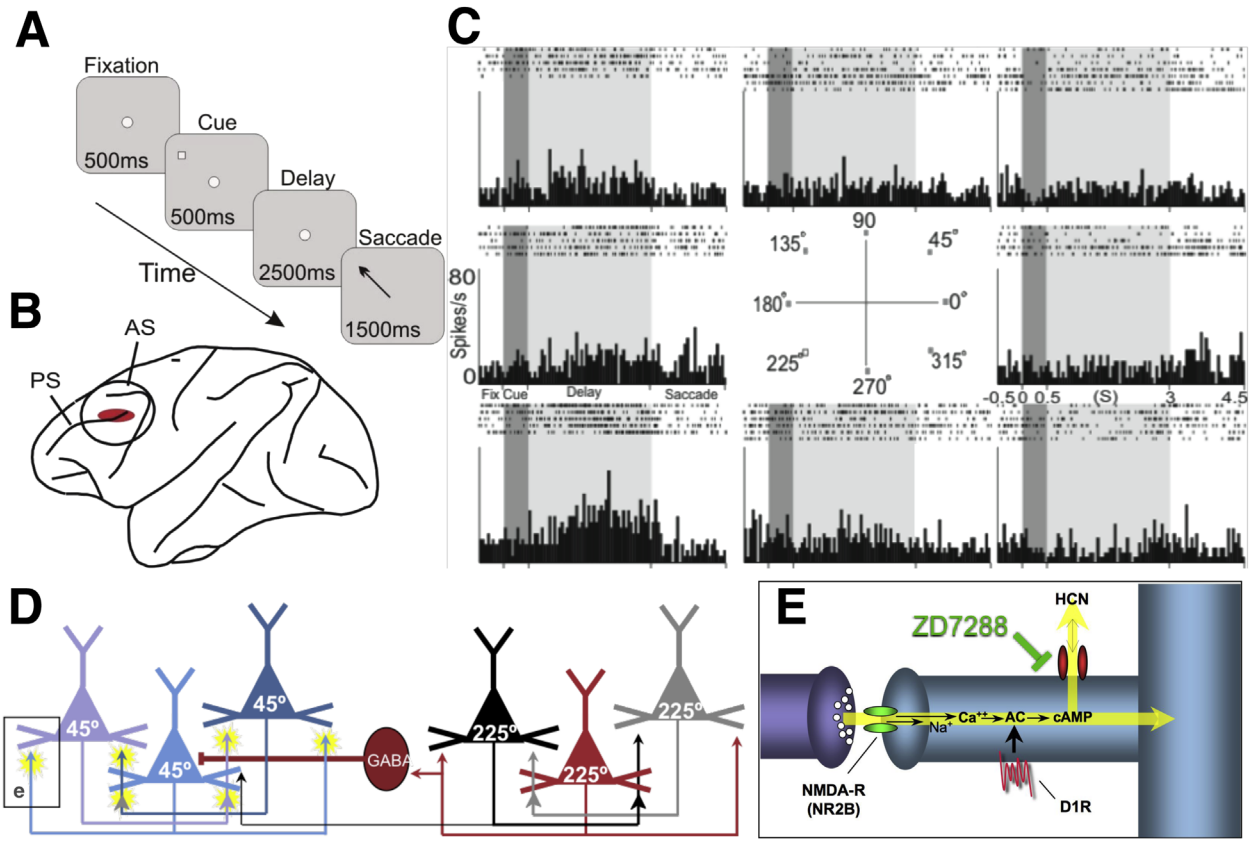

Figure 1. Single-unit recording from monkey dorsolateral prefrontal cortex (DLPFC) was combined with drug iontophoresis in monkeys performing the oculomotor delayed response task. **(A)** The oculomotor delayed response task, a test of spatial working memory. The monkey was seated in front of a screen, and the trial began when he fixated on a central target on the screen for .5 second (fixation period). Next, a cue appeared briefly (.5 second) in one of eight peripheral locations on the screen (cue period), followed by a 2.5-second delay period, during which the monkey continued to maintain fixation. At the end of the delay period, the fixation target was extinguished, and the monkey was required to make a memory-guided saccade to the remembered location of the cue (response period); monkeys were rewarded with a drop of juice for each correct response. Each test session consisted of hundreds of trials across which the cued location randomly changed, thus requiring the monkey to update his working memory. The TEMPO Experiment Control System (Reflective Computing, St. Louis, Missouri) generated the task, while the ISCAN Eye Movement Monitoring System (ISCAN Inc., Woburn, Massachusetts) monitored eye position. **(B)** Single-unit recording was performed in DLPFC. **(C)** Firing patterns of a sample neuron from the current study. Under optimal conditions, neurons show delay-related firing for a preferred direction (e.g., 225°) but suppress firing for nonpreferred directions (2). The dark gray background indicates the cue period, and the light gray background indicates the delay period. **(D)** Circuit basis for spatial working memory (2). Spatial working memory is maintained in DLPFC by recurrent excitation among networks of *N*-methyl-D-aspartate receptor (NMDA-R) glutamatergic pyramidal neurons with shared stimulus inputs (e.g., 225°). Spatial tuning is enhanced by lateral inhibition of nonpreferred inputs (e.g., 45°) from gamma-aminobutyric acid (GABA)ergic interneurons (16). **(E)** Working model of molecular mechanisms that weaken prefrontal cortex network connectivity. Cyclic adenosine monophosphate (cAMP) directly increases the open probability of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, while ZD7288 blocks them. Dynamic network connectivity signaling proteins are often found in long, thin spines with narrow spine necks at NR2B NMDA-R synapses in layer III monkey DLPFC (2). AC, adenylyl cyclase; AS, arcuate sulcus; Ca^{++}, calcium; D_1R, D_1 receptor; Na^+, sodium; PS, principal sulcus.

chips) were used during the experiments to minimize need for dietary regulation. Water was provided ad libitum, and animals were fed 12 g to 16 g of autoclaved rat chow (Purina Mills) immediately following testing. They were weighed weekly, and weights were maintained at 400 g to 450 g. The rats were habituated to all procedures and tested by a single experimenter, who was blind to drug treatment conditions.

Spatial Working Memory Task and Drug Infusions Into Rat Prelimbic PFC. Rats were trained individually in a delayed alternation spatial working memory task in a T-shaped maze (Supplementary Methods and Materials in Supplement 1).

The medial PFC, including prelimbic PFC, is required for delayed response tasks and behavioral flexibility in rats (29–35). Rats were implanted with chronic infusion cannulae directed above prelimbic PFC (anterior-posterior +3.2 mm; medial-lateral ±.75 mm; dorsal-ventral −4.2 mm) (36) or in a control region above the dorsal anterior cingulate cortex (ACd) (anterior-posterior +3.2 mm; medial-lateral ±.75 mm; dorsal-ventral −2.0 mm), a region that is not needed for the delayed alternation task (37,38) (Figure 2). Drug was slowly infused at .25 μL/min. Mock treatments adapted rats to all procedures (Supplementary Methods and Materials in Supplement 1). Experiments used a within-subjects design, with at least a 1-week washout period between treatments.

In the first experiment, we tested whether blocking HCN channels with ZD7288 would prevent the impairing effects of

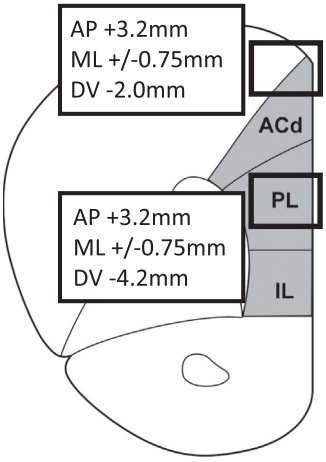

Figure 2. Drug infusions into rat prelimbic prefrontal cortex. Rats were implanted with chronic infusion cannulae directed above prelimbic prefrontal cortex (PL) (anterior-posterior [AP] +3.2 mm; medial-lateral [ML] ±.75 mm; dorsal-ventral [DV] −4.2 mm) or dorsal anterior cingulate cortex (ACd) (AP +3.2 mm; ML ±.75 mm; DV −2.0 mm), as indicated by boxes. IL, infralimbic cortex.

D_1R agonist infusion into prelimbic PFC. Each rat (n = 6) received the following four treatments in pseudorandom order: 1) ZD7288 + SKF81297; 2) ZD7288 + vehicle; 3) vehicle + SKF81297; and 4) vehicle + vehicle. ZD7288 (.001 μg/.5 μL) was infused into PFC 30 minutes before testing, as described in Wang *et al.* (22), as this dose had no effect on performance on its own in pilot studies. Thus, any effect of the drug on working memory performance would be due to an interaction between ZD7288 and SKF81297, rather than to nonspecific additive effects of the two treatments. Pilot studies determined a dose of SKF81297 (.001 μg to .5 μg/.5 μL) for each rat that impaired accuracy. Previous research showed that SKF81297 must be infused into prelimbic PFC to be effective (4). SKF81297 and ZD7288 were made daily by dissolving them in sterile saline to the appropriate concentrations.

In the second experiment, we examined whether ZD7288 would also block working memory impairments induced by a pharmacologic stressor, FG7142. FG7142 is a benzodiazepine partial inverse agonist that mimics the stress (39). This pharmacologic stressor was used instead of physical stressors (e.g., restraint stress), as it minimizes habituation that occurs with repeated physical stressors (40). Pilot studies determined an FG7142 dose (3.0 mg to 7.5 mg/kg; Tocris) for each rat that impaired accuracy but allowed task performance. Each rat (n = 5) received the following four treatments in pseudorandom order: 1) ZD7288 + FG7142; 2) ZD7288 + vehicle; 3) vehicle + FG7142; and 4) vehicle + vehicle. FG7142 was suspended in solution (Supplementary Methods and Materials in Supplement 1) and injected intraperitoneally 30 minutes before testing, as described in Birnbaum *et al.* (41). ZD7288 (.001 μg/.5 μL) was infused into PFC 30 minutes before testing, as described above.

Finally, an anatomical control experiment was performed to verify that ZD7288 improved performance via actions in prelimbic PFC, rather than via drug diffusion up the cannulae. This study followed the above methods, except that ZD7288 was infused in ACd (n = 7).

Histologic Verification of Cannula Positions

Following completion of the experiments, the locations of the cannulae were verified by histologic examination (Supplementary Methods and Materials in Supplement 1). Only data from rats with correctly placed cannulae were analyzed (Figure S1 in Supplement 1).

Behavioral Data Analysis

Data were analyzed using two-way analysis of variance with repeated measures with within-subjects factors of HCN channel blockade (ZD7288 vs. vehicle) and D_1R stimulation (SKF81297 vs. vehicle) or stress (FG7142 vs. vehicle). User-defined contrasts then compared performance between pairs of drug conditions. Statistical analyses were performed using Systat.

Monkey Behavior

Parallel behavioral experiments were performed in a monkey performing the ODR task with unilateral infusions of vehicle (10 μL or 20 μL) or SKF81297 (10 mmol/L; 10 μL or 20 μL) into DLPFC (Supplementary Methods and Materials in Supplement 1). Previous research showed that unilateral lesions of DLPFC impaired working memory for cues in the visual field contralateral to the lesion site (42).

RESULTS

HCN Channels and D_1Rs Colocalize in Dendritic Spines of Monkey DLPFC

Dual immunoelectron microscopy revealed coexpression of D_1Rs and HCN channels in pyramidal neurons, as well as spatial interaction on spine membranes in layer III monkey DLPFC. Both proteins were localized in the synthetic machinery in the soma (Figure 3A) and colocalized at the plane of the plasma membrane of dendritic spines (Figure 3B–E). Although HCN channels are prominent along the distal pyramidal dendrites, D_1Rs are not present at this location (26). Thus, colocalization was found in the spine head near the synapse (Figure 3B,C,E) and in the spine neck (Figure 3D), where D_1Rs could generate cAMP next to HCN channels and regulate their open state. These findings built on previous work revealing HCN channel interaction with key cAMP-regulating molecules (26).

D_1R Stimulation Suppressed PFC Delay-Related Firing via Opening of HCN Channels in Monkeys Performing a Working Memory Task

This experiment examined whether the HCN channel blocker, ZD7288, could block the impairing effect of the D_1R agonist, SKF38393, on delay-related firing in DLPFC. Two monkeys (C and P) were trained to perform an ODR spatial working memory task (Figure 1A), while 42 delay-related cells (25 from monkey C; 17 from monkey P) with varying degrees of spatial tuning were recorded in DLPFC. For tuned neurons, the preferred direction was determined by circular regression, whereas for neurons without significant tuning, the direction with the highest mean firing during the delay period was selected as the preferred direction for further analyses. Neurons were only included in the analyses if their activity during the ZD7288 + SKF38393 condition did not change significantly with time or trial number. As a result, one neuron

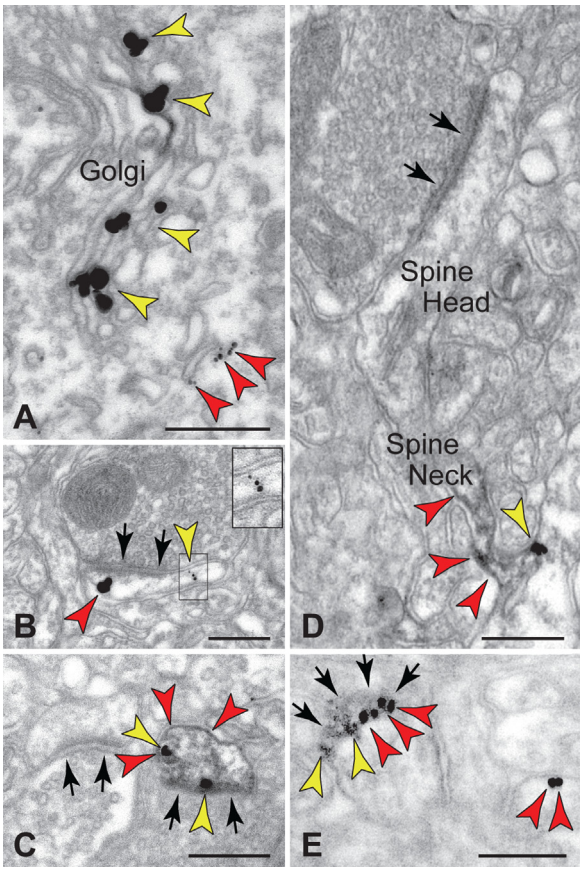

Figure 3. Dual immunoelectron microscopy for hyperpolarization-activated cyclic nucleotide-gated channel subunit 1 (HCN1) and dopamine D_1 receptors (D_1Rs) in monkey dorsolateral prefrontal cortex. **(A)** HCN1 channels (red arrows) and D_1Rs (yellow arrows) were coexpressed in layer III pyramidal neurons in the Golgi and reticular endomembranes, showing that D_1Rs are manufactured by layer III pyramidal cells. **(B–E)** In the neuropil, HCN1 channels and D_1Rs were colocalized in dendritic spines, as shown by gold-gold **(B)** or peroxidase-gold **(C–E)** labeling. HCN1 channels were localized at the elongated spine neck with D_1Rs positioned at the base of the emerging spine **(D)**. An oblique section through a synapse (the synaptic disk is indicated by multiple arrows) demonstrated perisynaptic localization for both proteins **(E)**; lead-contrasting was omitted from **(E)** to facilitate visualization of D_1R-immunoperoxidase. Black arrows point to axospinous synapses. Scale bars: 200 nm.

from monkey C ($r = -.90$, $p = .014$ vs. time; $r = -.81$, $p = .052$ vs. trial number) and one neuron from monkey P ($r = -.86$, $p = .0003$ vs. time; $r = -.87$, $p = .0002$ vs. trial number) were excluded. As Vijayraghavan *et al.* (5) already established that D_1R stimulation suppressed PFC firing, the remaining 16 cells (8 from monkey C; 8 from monkey P) that showed reduced delay-related firing with SKF38393 were analyzed. Of these, 8 cells (4 from monkey C; 4 from monkey P) showed spatial tuning during the control condition, as determined by circular regression.

As shown in Figure 4A, drug application significantly altered neuronal firing relative to the control condition ($F_{2,30} = 30.671$, $p < .0005$; $n = 16$ neurons). User-defined contrasts revealed that iontophoresis of SKF38393 alone significantly reduced firing relative to the control condition ($F_{1,15} = 84.151$, $p < .0005$). However, co-application of ZD7288 and SKF38393 led to significantly higher firing than with SKF38393 alone ($F_{1,15} = 26.364$, $p < .0005$) and was similar to the control condition ($F_{1,15} = 2.365$, $p = .145$). Thus, blockade of HCN channels prevented the suppression of firing caused by D_1R stimulation. Figure 4B shows the drug effects on population firing across all task epochs.

A subset of neurons was sufficiently stable over long testing sessions to assess ZD7288 alone, in addition to the other conditions. These neurons showed a significant main effect of drug treatment ($F_{4,20} = 6.017$, $p = .002$; $n = 6$) (Figure 5A). User-defined contrasts showed that there was no significant effect of ZD7288 alone relative to the control condition ($F_{1,5} = .621$, $p = .467$) and that the control condition levels of firing were maintained following co-application of ZD7288 and SKF38393 ($F_{1,5} = 1.351$, $p = .298$). However, removal of ZD7288 while continuing iontophoresis of SKF38393 significantly reduced firing relative to the control condition ($F_{1,5} = 193.632$, $p < .0005$) and to ZD7288 + SKF38393 ($F_{1,5} = 16.607$, $p = .010$). Figure 5B shows the drug effects on population firing across all task epochs. Figure 6 shows sample data from an individual neuron.

D_1R Stimulation Opened HCN Channels in Mouse PFC Slices

Whole-cell recordings from layer V pyramidal neurons in mouse PFC slices were performed to verify that D_1R stimulation increased I_h. The D_1R agonist, SKF81297, significantly enhanced the hyperpolarization-induced sag in membrane potential corresponding to I_h ($p = .038$ vs. the control condition; $n = 7$; Figure 7).

D_1R Stimulation in PFC or Stress Impaired Working Memory Performance via Opening of HCN Channels in Rats

Effects of D_1R Stimulation and HCN Channel Blockade on Working Memory. Rats were trained in a T-maze spatial working memory task to examine the behavioral effects of D_1R-HCN channel interactions. Infusions of ZD7288 into rat medial PFC (Figure 2) significantly reversed the impairing effects of SKF81297 on working memory (Figure 8A). There was a significant main effect of ZD7288 ($F_{1,5} = 26.03$, $p = .004$) and trends toward a main effect of SKF81297 ($F_{1,5} = 4.33$, $p = .092$) and interaction between ZD7288 and SKF81297 ($F_{1,5} = 6.231$, $p = .055$). User-defined contrasts revealed that SKF81297 significantly impaired performance relative to vehicle ($F_{1,5} = 15.92$, $p = .010$), in agreement with previous findings (4,5). Coinfusion of ZD7288 reversed this impairment (ZD7288 + SKF81297 vs. SKF81297: $F_{1,5} = 40.00$, $p = .001$) to performance levels similar to vehicle ($F_{1,5} = .12$, $p = .74$). ZD7288 alone did not significantly affect performance relative to vehicle ($F_{1,5} = .36$, $p = .57$), which verified that the effects observed with ZD7288 + SKF81297 were not due to nonspecific additive effects.

Effects of Pharmacologic Stress and HCN Channel Blockade on Working Memory. Infusions of ZD7288 into rat medial PFC significantly reversed the impairing effects of the pharmacologic stressor, FG7142, on working memory (Figure 8B). There was a significant main effect of ZD7288

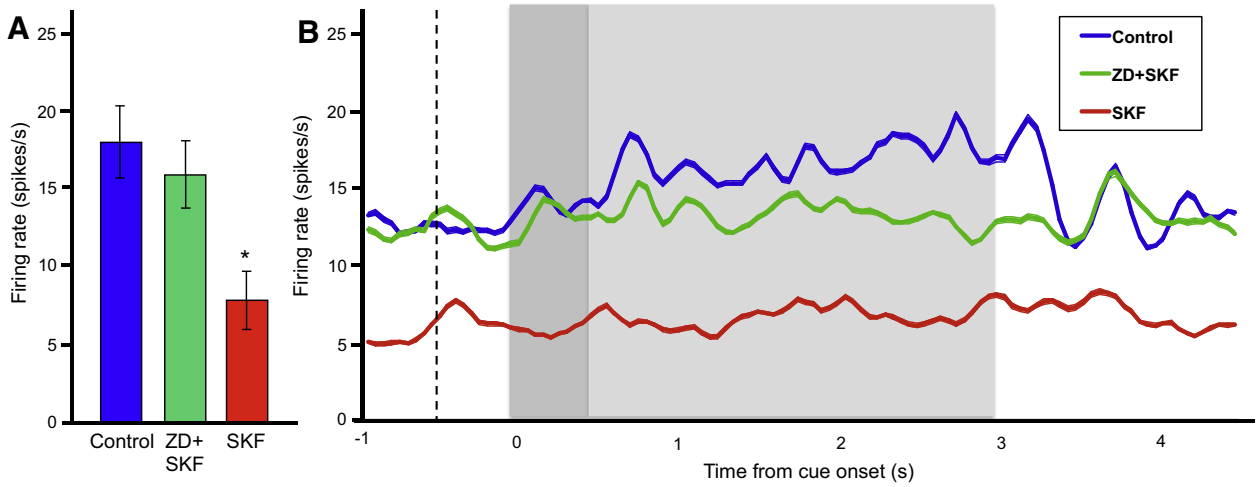

Figure 4. The D_1 receptor agonist, SKF38393 (SKF), suppressed delay-related firing, and this suppression was blocked by the hyperpolarization-activated cyclic nucleotide-gated channel blocker, ZD7288 (ZD), in monkeys performing the oculomotor delayed response task (n = 16 neurons). **(A)** Drug effects on the mean firing rate during the delay period. Error bars represent SEM. *$p < .0005$ versus control, ZD + SKF. **(B)** Drug effects on population firing across all task epochs. The dotted line indicates onset of the fixation period, the dark gray background indicates the cue period, and the light gray background indicates the delay period.

($F_{1,4}$ = 10.39, p = .032), a nonsignificant main effect of FG7142 ($F_{1,4}$ = 3.55, p = .133), and a significant interaction between ZD7288 and FG7142 ($F_{1,4}$ = 25.76, p = .007). User-defined contrasts revealed that FG7142 + vehicle significantly impaired performance relative to vehicle ($F_{1,4}$ = 35.71, p = .004), in agreement with previous findings (43). Infusion of ZD7288 reversed this impairment (ZD7288 + FG7142 vs. FG7142: $F_{1,4}$ = 28.24, p = .006), returning performance to vehicle levels ($F_{1,4}$ = .074, p = .80). ZD7288 alone did not significantly affect performance relative to vehicle ($F_{1,4}$ = 2.25, p = .21), which verified that the effects observed with ZD7288 + FG7142 were not due to nonspecific additive effects.

Anatomical control experiments verified that ZD7288 improved working memory via actions in prelimbic PFC, rather than via drug diffusion up the cannulae (Figure 8C). When ZD7288 was infused into ACd (Figure 2), there was no significant main effect of ZD7288 ($F_{1,6}$ = 1.23, p = .309), a significant main effect of FG7142 ($F_{1,6}$ = 45.14, p = .001), and no significant interaction between ZD7288 and FG7142 ($F_{1,6}$ = 2.87, p = .14). User-defined contrasts revealed that FG7142 significantly impaired performance relative to vehicle ($F_{1,6}$ = 28.90, p = .002). Coinfusion of ZD7288 did not reverse this impairment (ZD7288 + FG7142 vs. FG7142: $F_{1,6}$ = 2.21, p = .19), and performance following ZD7288 + FG7142 was still significantly impaired relative to vehicle ($F_{1,6}$ = 15.28, p = .008). Again, ZD7288 alone did not significantly affect performance relative to vehicle ($F_{1,6}$ = .092, p = .772).

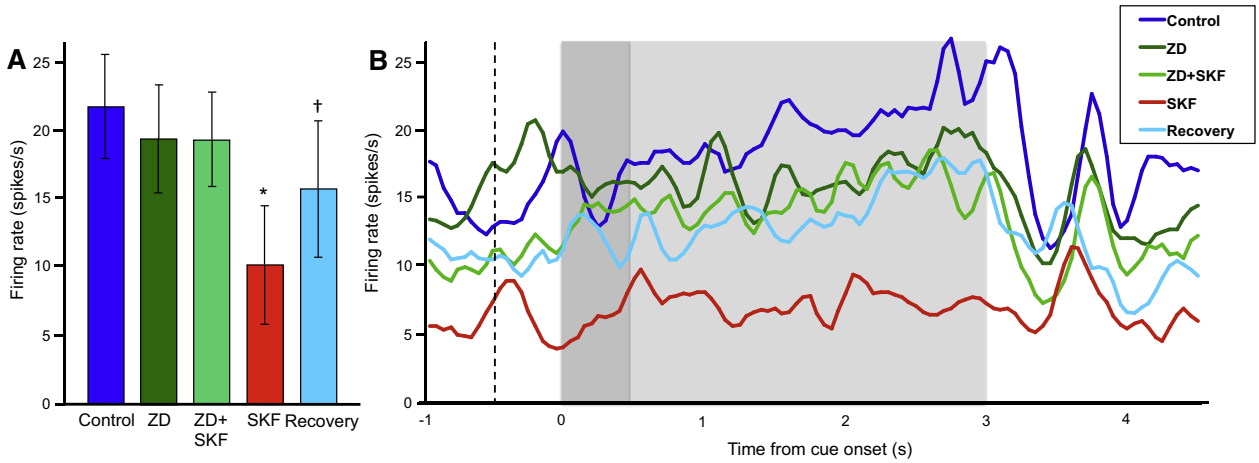

Figure 5. In the subset of neurons in which we assessed ZD7288 (ZD) alone and recovery, SKF38393 (SKF) suppressed delay-related firing, and this suppression was blocked by a nonimproving dose of ZD in monkeys performing the oculomotor delayed response task (n = 6). Firing increased during recovery, following the removal of SKF. **(A)** Drug effects on the mean firing rate during the delay period. Error bars represent SEM. *$p < .0005$ versus control, ZD + SKF; †p = .030 versus SKF. **(B)** Drug effects on population firing across all task epochs. The dotted line indicates onset of the fixation period, the dark gray background indicates the cue period, and the light gray background indicates the delay period.

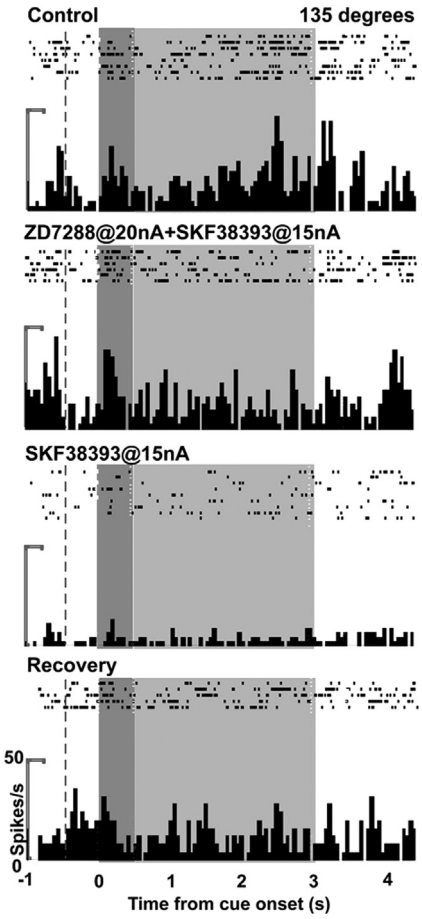

Figure 6. The effects of SKF38393 (SKF) and ZD7288 (ZD) on neuronal firing in an individual neuron recorded in dorsolateral prefrontal cortex while a monkey performed the oculomotor delayed response task. For the 135° direction, the neuron showed enhanced delay-related firing (p = .0020 vs. fixation) during control. The delay firing was maintained with ZD + SKF (p > .05 vs. control) but was then reduced with SKF (p = .00017 vs. control; .0015 vs. ZD + SKF). The delay firing returned during recovery (p = .00013 vs. SKF). The ZD condition was not performed in this neuron. The dotted line indicates onset of the fixation period, the dark gray background indicates the cue period, and the light gray background indicates the delay period.

Finally, we verified that the changes in performance following D_1R stimulation or stress were due to working memory impairments and not to general motor impairments, by analyzing the rats' response times for each trial during vehicle and SKF81297 or FG7142 conditions (Supplementary Results in Supplement 1).

D_1R Stimulation in PFC Impaired Working Memory Performance in a Monkey

Unilateral infusions of SKF81297 into monkey DLPFC impaired working memory performance in the ODR task for spatial cues contralateral to the infusion sites (p < .05 vs. the control condition; Figures S4–S6 in Supplement 1). Infusions of vehicle had no effect (p > .05 vs. the control condition; Figure S6 in Supplement 1). In addition, infusions of SKF81297 did not impair performance under visually guided control conditions when working memory was not needed (p > .05 vs. the control condition; Figures S6 and S7 in Supplement 1). Further details are provided in the Supplementary Results in Supplement 1.

DISCUSSION

The current study showed that the impairment in PFC function during stress involves D_1R opening of HCN channels to weaken PFC network connections. Combining anatomical, physiological, and behavioral evidence, we found that 1) D_1Rs and HCN channels colocalize and spatially interact at dendritic spines in monkey layer III DLPFC; 2) stimulation of D_1Rs increases I_h in mouse PFC slices; 3) suppression of neuronal firing by D_1R-cAMP signaling can be prevented by blocking HCN channels in monkey DLPFC; and 4) working memory impairment induced by D_1R stimulation or pharmacologic stress can be prevented by blocking HCN channels in rat PFC.

While cAMP is known to be involved in a multitude of intracellular pathways [e.g., (44–46)], the current study demonstrated that suppression of DLPFC function by D_1Rs involves cAMP-mediated increase in I_h, thus revealing an ionic component to this important physiological response. It is of interest that D_1R stimulation suppressed firing throughout all epochs of task-related firing, replicating earlier findings with high doses of D_1R stimulation (5). As the firing of delay cells is thought to be driven by recurrent excitation from neurons with shared network properties (2), D_1R weakening of these network inputs may alter all phases of task-related firing.

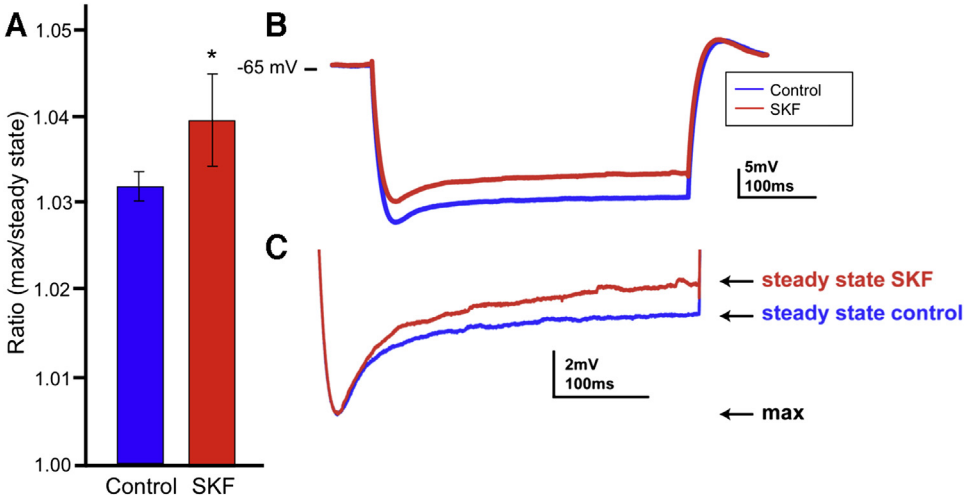

Figure 7. The effects of the D_1 receptor agonist, SKF81297 (SKF), on the hyperpolarization-activated cyclic nucleotide-gated channel current in layer V pyramidal neurons in mouse prefrontal cortex slices. **(A)** SKF increased the ratio of the maximum change in membrane potential to the subsequent steady state potential during the hyperpolarizing pulse (*p = .038 vs. control; n = 7). Error bars represent SEM. **(B)** A sample trace showing that SKF induced a sag in the membrane potential relative to control conditions, which indicated an increase in hyperpolarization-activated cyclic nucleotide-gated channel current. **(C)** The trace in **(B)** scaled to highlight the difference in the membrane potential response between SKF and control conditions.

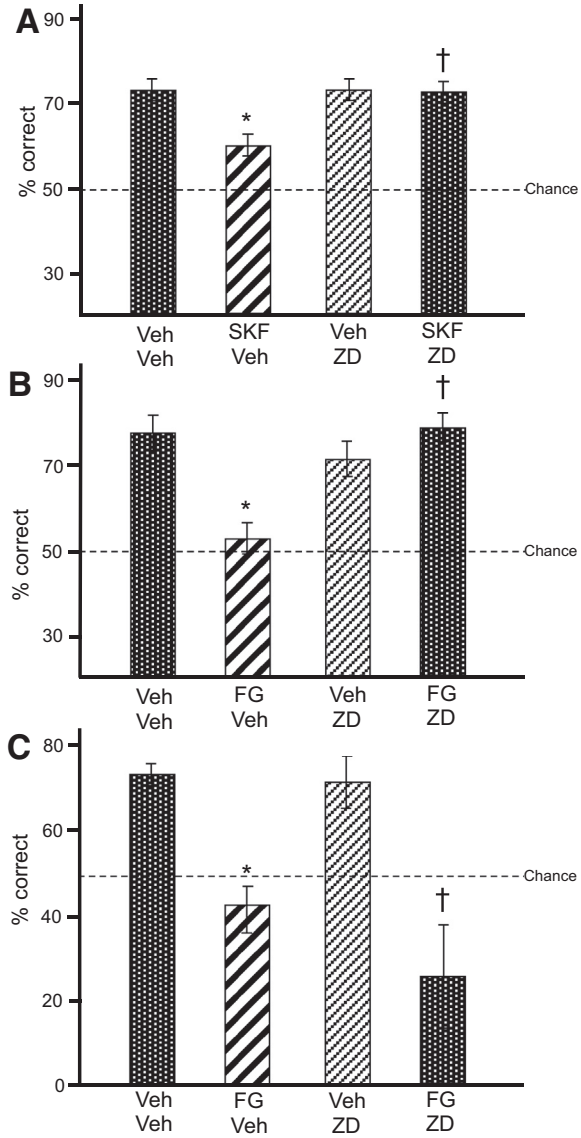

Figure 8. The effects of SKF81297 (SKF) and stress on spatial working memory performance in rats. **(A)** SKF impaired spatial working memory performance in rats (n = 6) relative to vehicle (Veh) (*p = .010 vs. Veh + Veh), and this impairment was blocked by prefrontal cortex (PFC) infusions of ZD7288 (ZD) (†p = .001 vs. SKF + Veh). Results represent % correct out of 10 trials. Error bars represent SEM. **(B)** The pharmacologic stressor, FG7142 (FG), impaired spatial working memory performance in rats (n = 5) relative to Veh (*p = .004 vs. Veh + Veh), and this impairment was blocked by PFC infusions of ZD (†p = .006 vs. FG + Veh). Results represent % correct out of 10 trials. Error bars represent SEM. **(C)** FG impaired spatial working memory performance in rats (n = 7) relative to Veh (*p = .002 vs. Veh + Veh). However, this impairment was not blocked by ZD infusions dorsal to PFC (†p = .008 vs. Veh + Veh). Results represent % correct out of 10 trials. Error bars represent SEM.

Lower levels of D_1R stimulation likely engage several mechanisms that would also enhance spatially tuned persistent firing (5), some of which would not involve HCN channels, e.g., increasing NMDA receptor insertion into the postsynaptic density (47,48) and enhancing gamma-aminobutyric acidergic lateral inhibition (49–51). However, HCN channel opening likely contributes as well (Figures S3C and S8 in Supplement 1).

Rodent Versus Monkey PFC Homology

Although there are many differences between the rodent medial PFC and the monkey DLPFC (29,31), almost two decades of research suggest that some mechanisms do extend across species. For example, DA depletion in these regions impairs working memory in both rodents (52) and monkeys (52–54), while excessive D_1R stimulation in PFC impairs cognitive function in rats and reduces neuronal firing in monkeys via increased cAMP signaling (5). Our in vitro recordings in rodent PFC were made from layer V pyramidal neurons, which have properties of both layer III and layer V pyramidal neurons in primates (55), e.g., they respond to both D_1R and D_2R agonists. These neurons are often used for intracellular recordings and have been essential for direct examination of ionic mechanisms. The current data indicate that D_1R impairment in PFC working memory function involves HCN channel opening in both rodents and monkeys. However, the precise contributions of these channels to neuronal physiology may differ across species, particularly as HCN channels play a variety of roles depending upon their ultrastructural localization and molecular interactions.

Excitatory Versus Inhibitory Nature of D_1R-HCN Channel Signaling

D_1Rs have been shown to enhance excitability of rodent PFC pyramidal neurons in vitro, both via cAMP (21,56–58) and independently of cAMP (59,60), through increase in sodium and reduction in potassium currents (21,59,61,62) and possibly NMDA receptors (63–65). These basic, excitatory influences may be saturated in a behaving animal, with additional inhibitory effects being observed with higher levels of stimulation (5), or the inhibitory effects in vivo may override these excitatory mechanisms (3,66).

HCN channels also show both excitatory and inhibitory influences on membrane potential, likely depending on the laminar position of the neuron and whether the recording is from a highly active neuron in vivo versus a hyperpolarized neuron in a PFC slice. It should be noted that I_h does not necessarily require hyperpolarization to open, as HCN channels have a tonically active leak current component (67–71) that is blocked by ZD7288 (67,72). Furthermore, HCN channels and D_1Rs are found near a constellation of cAMP signaling proteins at dendritic spines, whereas HCN channels on dendrites have few cAMP signaling proteins nearby (26). While speculative, these findings suggest that HCN channels at spines may open primarily in response to cAMP and reduce firing by shunting network inputs and/or reducing temporal summation [e.g., (73–75)]. Finally, HCN channels may also interact with other potassium channels to alter dendritic excitability, e.g., KCNQ (Kv7) channels (76) and Kir2.2/2.3 and potassium-selective leak (K_{leak}) channels (77).

While the current study and previous work (26) indicate that HCN channels on spines can colocalize with D_1Rs, a future, dual quantitative analysis of HCN1 and D_1Rs in the PFC neuropil will be necessary to determine the extent of this co-expression. Low doses of ZD7288 may be especially

potent in blocking HCN channels on spines due to D_1R-mediated phosphorylation of channels keeping them open (78–81) and/or because channel blockade may be more efficacious in a thin spine, given its very small volume compared with that of a large dendrite.

Relevance to Psychiatric Disorders

These mechanisms are likely relevant to a range of psychiatric disorders associated with dysregulated DA signaling, in which patients often show precipitation or exacerbation of symptoms with stress (3,12). For example, D_1Rs are upregulated in DLPFC of patients with schizophrenia (82–84), especially in young, drug-naïve patients (11), and this increase correlates with poor working memory (82,83). The current data suggest that some of this impairment may arise from D_1R-HCN channel weakening of PFC network firing.

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