Stress Impairs Prefrontal Cortical Function via D1 Dopamine Receptor Interactions with HCN Channels

Supplemental Information

Supplementary Methods and Materials

Physiology: Monkey *In Vivo* Recordings and Drug Iontophoresis in Dorsolateral Prefrontal Cortex (DLPFC)

Prior to recording single units from monkeys C and P, a chronic recording chamber was implanted over the right DLPFC based on anatomical coordinates obtained from a magnetic resonance imaging scan. Monkey C's chamber was located at -3 to 2 mm anterior to the caudal end of the principal sulcus, and -4 to 2 mm medial to the principal sulcus. Monkey P's chamber was located 2 to 7 mm anterior to the caudal end of the principal sulcus, and -2 to 3 mm medial to the principal sulcus.

Iontophoretic electrodes were constructed from a seven-barrel, non-filamented capillary glass (Friedrich & Dimmock Inc., Millville, NJ), with a 20 μm pitch carbon fiber (Energy Science Laboratory Inc., San Diego, CA) inserted through the central barrel. The electrode was pulled using the PMP-107 Micropipette Puller (MicroData Instrument, Inc., S. Plainfield, NJ), and the tip was beveled to a diameter of 30 to 40 μm. The outer barrels of the electrode were filled with three charged drug solutions (two consecutive barrels for each drug), and pushed to the tip of the electrode using compressed air. The central recording electrode had a very low impedance of 0.3 to 1.0 MΩ, while the surrounding drug-filled barrels had much higher impedances of over 100 MΩ, depending on the drug. The electrode was then inserted into a 25G stainless steel guide tube, which punctured the dura to facilitate the access of the electrode to the

cortex. The electrode and guide tube were mounted on an MO-95 micromanipulator (Narishige, East Meadow, NY), which controlled the insertion of the electrode into the cortex. Retention currents of 3 to 5 nA with the opposite polarity for each drug were used in a cycled manner (1 s on, 1 s off) when not applying the drugs. As the retention current did not have systematic effects on neuronal activity, the drugs were likely not leaking.

Drugs were ejected from the electrodes with currents of 5-100 nA with the appropriate polarity for each drug, using a Neurophore BH2 iontophoretic system (Medical Systems Corp., Greenvale, NY). The dopamine D1 receptor (D1R) agonist, SKF38393, was dissolved in de-ionized, pH-adjusted distilled water (pH 4.5 to 5.0), and the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel blocker, ZD7288, was dissolved in sterile saline. They were dissolved to a final concentration of 10 mM and stored in 20-40 μL aliquots at -70°C. It was not always possible to obtain the ZD7288 and Recovery conditions, depending on the stability of the neuron and the monkey's motivation. The SKF38393 condition was done after ZD7288+SKF38393, as Vijayraghavan *et al*. (1) found that once suppressed by SKF38393, the firing was often not reversible.

While SKF38393 is a partial D1R agonist, Vijayraghavan *et al.* (1) previously found that SKF38393 ejected at 20-25 nA effectively suppressed delay-related firing in DLPFC. A potential problem was that D1Rs could internalize with excessive stimulation. If neurons became unresponsive, we used a lower dose of SKF38393; this strategy was effective in Vijayraghavan *et al*. (1). ZD7288 is currently the most selective HCN channel blocker available (2, 3), and Wang *et al*. (4) found that ZD7288 ejected at 5-10 nA prevented suppression of firing by the PDE4 inhibitor, etazolate. Higher doses of ZD7288 have been shown to block NMDA and AMPA receptors (5), and also inhibit excitatory transmission in pyramidal neurons (4, 6).

Thus, we aimed to use low doses of ZD7288 that did not significantly affect neuronal activity to prevent these off-target effects, and also to show that the effects of SKF38393 and ZD7288 together were not due to non-specific, additive effects.

Current balancing was not required in this experiment, as drug ejection did not create significant noise in the recorded signals or systematically affect spike characteristics at any ejection current. As discussed in Vijayraghavan *et al.* (1), the extracellular recordings were performed at some distance from neurons, and the drugs were ejected using currents of 5-100 nA – much smaller than currents typically used for microstimulation, which are in the μA range. Sodium ejection with and without current balancing did not show significant differences in effects on neuronal activity. In addition, SKF38393 was dissolved in de-ionized, pH-adjusted distilled water, which had negligible ion content, while ZD7288 was dissolved in sterile saline, which when ejected alone had no significant effects on firing patterns (see Results).

Regularly spiking units that showed delay-related firing were identified using the Spike2 software (Cambridge Electronics Design, Cambridge, UK). Extracellular voltage signals were amplified with a Model 3000 differential amplifier (A-M Systems, Carlsborg, WA), band-pass filtered with a four-pole Butterworth filter (180 to 6000 Hz, 20 dB gain; Krohn-Hite Corporation, Brockton, MA), digitized with a Micro1401 data acquisition unit at 20.83 kHz (Cambridge Electronics Design, Cambridge, UK), and acquired using the Spike2 software (Cambridge Electronics Design). Neural activity was sorted and analyzed using a template-matching algorithm, which allowed us to isolate more than one unit at the same recording site. The Spike2 software measured activity as number of spikes/s, and constructed post-stimulus histograms and rastergrams online to reveal whether a unit showed task-related activity. If so, we continued to record it and applied a sequence of drug conditions.

Physiology: Monkey *In Vivo* Recording Data Analysis

The spike data were re-sorted offline using Spike2, and post-stimulus time histograms were constructed for the eight cue locations in the oculomotor delayed response (ODR) task using 50 ms bins. The data were divided into four task epochs: fixation (0.5 s), cue (0.5 s), delay (2.5 s) and response (1.5 s). The effects of drug conditions on delay firing were first determined at the population level. Population histograms were constructed by taking the average of the number of spikes in each 50 ms bin across all neurons, then smoothed twice by averaging the number of spikes across three consecutive bins. The population-averaged number of spikes in the Control, ZD7288+SKF38393 and SKF38393 conditions was also compared using a 1-way analysis of variance (ANOVA) with repeated measures. User-defined contrasts then compared delay firing between pairs of conditions. Statistical analyses were performed using Systat (Systat Software Inc., Chicago, IL).

Next, the delay firing in individual units was analyzed. Circular regression was used to determine whether the DLPFC delay firing was tuned to a particular cue direction during Control, by testing whether the firing across the eight cue directions deviated significantly from a circle. The number of delay spikes in each trial was regressed to the cue direction using the following model (7, 8):

$$y = b_0 + b_1.\sin\Theta + b_2.\cos\Theta + \varepsilon$$

where y = number of spikes during the delay period; Θ = direction of the cue; b_1, b_2 = regression coefficients.

If b_1 or b_2 was significantly different from 0 ($p < 0.05$) after correcting for multiple comparisons using Bonferroni's correction, the activity of this neuron was deemed spatially tuned during the Control condition. We estimated the preferred direction corresponding to the

cue direction giving rise to the maximum firing rate during the delay period, using the following formula:

$$\Theta = \arctan(b_1/b_2)$$

For the purposes of further analyses, the cue direction closest to Θ was chosen as the neuron's preferred direction. For a neuron without significant spatial tuning, the cue direction with the maximum firing rate during the delay period was used as its preferred direction. For the preferred direction, the spike rate was compared between the delay and fixation periods using paired *t*-tests to determine whether the neuron showed enhanced delay-related firing. If so, the delay firing was compared across drug conditions using *t*-tests.

In neurons that showed reduced delay firing with SKF38393, measures were taken to ensure that this reduction was not caused by other reasons, e.g., the neuron dying or moving away from the electrode. We examined how the number of delay spikes changed across trials before and after the onset of the SKF38393 condition for the direction of interest, i.e., during the ZD7288+SKF38393 and SKF38393 conditions. During these conditions, a correlation coefficient (*r*) was computed and its statistical significance determined between the delay firing rate and time (or trial number). If the number of spikes significantly decreased during ZD7288+SKF38393, i.e., if *r* was significantly negative, then the neurons were removed from further analyses.

Finally, we calculated the value of d-prime (d') as a measure of the strength of tuning during the Control, ZD7288+SKF38393 and SKF38393 conditions, using the following formula:

d' = [mean(preferred) + mean(non-preferred)] / {[SD(preferred)^2 + SD(non-preferred)^2]/2}^(1/2)

D-prime indicated the ability to distinguish preferred vs. non-preferred directions from the delay firing. We used the neuron's preferred direction under the Control condition, and used the opposite direction (i.e., 180° away from the preferred direction) as the non-preferred direction. We compared d' values between conditions using paired *t*-tests.

Physiology: Mouse *In Vitro* Recordings and Drug Application in PFC Slices

Under isoflurane anesthesia, C57BL/6 mice (P22-28) were decapitated, and coronal slices (300 μm) were cut in an ice-cold external solution containing (in mM): 110 CholineCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 10 glucose, 11.6 sodium ascorbate and 3.1 sodium pyruvate, and bubbled with 95% O_2 and 5% CO_2. Slices containing the medial PFC were transferred to artificial cerebrospinal fluid containing (in mM): 127 NaCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2.5 KCl, 1 $MgCl_2$, 2 $CaCl_2$ and 10 glucose, and bubbled with 95% O_2 and 5% CO_2. Slices were incubated for 30 min at 34°C, then maintained at 22–24°C until use. Experiments were conducted at 32–34°C in a submersion-type recording chamber. Whole-cell patch-clamp recordings were obtained from layer V pyramidal neurons identified with video-infrared/differential interference contrast. For current-clamp recordings, glass electrodes (3.2–3.8 MΩ) were filled with internal solution containing (in mM): 135 $KMeSO_3$, 10 HEPES, 4 $MgCl_2$, 4 Na_2ATP, 0.4 NaGTP and 10 Na creatine phosphate (pH 7.3). Recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 4 kHz, and digitized at 10 kHz. Data were acquired using National Instruments (Austin, TX) data acquisition boards and custom software written in MATLAB (Mathworks, Natick, MA).

For all cells, the resting membrane potential was maintained at -65 mV by injecting positive current, and the HCN channel current (I_h) was measured during a 400 ms

hyperpolarizing pulse at -300 pA. I_h was quantified as the ratio of the peak hyperpolarization to the steady-state value during the pulse. To stimulate D1Rs, SKF81297 (10 μM) was bath-applied for 10 min. Data were analyzed offline using custom routines written in IgorPro (WaveMetrics, Portland, OR). I_h values were compared between Control and SKF81297 conditions using paired *t*-tests.

Rat Behavior: Spatial Working Memory Task

Rats were trained individually in a delayed alternation spatial working memory task in a T-shaped maze. In this task, the rats began in the start box behind the start gate at the bottom of the 'T'. For the first trial, the rats were rewarded for entering either arm. The rats were then picked up and returned to the start box for the duration of the delay period. During subsequent trials, rats were rewarded only for entering the arm opposite to the previously entered arm after each delay period. Thus, the rats had to update their spatial working memory for each trial during the delay periods, inhibit distractions, and inhibit the prepotent response to return to the previously rewarded arm. The procedure was repeated for ten trials per day. The choice point was wiped with 75% ethanol solution after each trial to remove any olfactory cues. The delay period ("0" sec to > 1 min) was adjusted for each rat, such that they were performing at a stable baseline of ≥ 70% correct for two consecutive testing days before drug treatment.

Rat Behavior: Implantation of Drug Infusion Cannulae in Rats

Stereotaxic surgery was performed under ketamine (90 mg/kg; Butler Schein, Dublin, OH) + xylazine (4 mg/kg; Akorn, Inc., Decatur, IL) anesthesia using aseptic methods. Guide cannulae (Plastics One, Roanoke, VA), which consisted of 9.0 mm 23G stainless steel, were implanted

such that they extended to DV -1.7 mm. The infusion probes projected 2.5 mm past the tubes to reach DV -4.2 mm for infusions in the prelimbic PFC, and projected 0.3 mm past the tubes to reach DV -2.0 mm for infusions dorsal to PFC (9). Rats were treated with a pre-emptive subcutaneous injection of Metacam (1.0 mg/kg; Boehringer Ingelheim, St. Joseph, MO) to decrease pain. For 48 h following surgery, rats were treated with oral Metacam (1.0 mg/kg), and the wound was treated with a topical antibiotic powder to minimize infection. Rats were monitored on a daily basis for signs of distress or infection. They were allowed to recover for 7 days after surgery, during which they were not tested. Once recovered, the rats were re-trained in the delayed alternation task until they reached a stable baseline performance.

On the day before a treatment, a mock "infusion" was performed by pulling then replacing the stylet, and the treatment was only administered if the rat performed normally with the mock infusion. Similarly, mock intraperitoneal (IP) injections were performed in the stress experiments by inserting a 22G needle into the IP cavity to habituate the rats to this procedure.

Rat Behavior: Preparation of the Pharmacological Stressor, FG7142

FG7142 was made fresh daily by dissolving it in 0.3 mL 100% ethanol and 0.7 mL 2-hydroxylpropylyl-ß-cyclodextrin (HBC) vehicle/kg. The HBC vehicle was made by dissolving 1.5 mg HBC (Sigma-Aldrich, St. Louis, MO) in 1 mL Tween 80 (Sigma-Aldrich) and 7.4 mL sterile saline overnight.

Rat Behavior: Histological Verification of Cannula Positions

Rats were sacrificed via an overdose of inhalation of isoflurane (Butler Schein, Dublin, OH) until the eye blink and toe pinch reflexes were absent, followed by rapid decapitation. Brains were

then removed and fixed in 10% formalin solution. The brains were sectioned coronally at 50 μm using a Leica VT1000 S vibratome (Leica Microsystems Inc., Buffalo Grove, IL); to distinguish between left and right hemispheres when processing floating sections, the right hemisphere was notched. The sections were mounted on gelatin-coated slides and stained with Neutral Red (FD NeuroTechnologies, Inc., Baltimore, MD), preserving left-right orientation. They were then dehydrated in a series of alcohols ending with 100% xylene, and coverslipped with Permount mounting medium (Fisher Scientific, Pittsburgh, PA). The trajectory of gliosis left by the cannulae was observed under the light microscope, and locations of the tips were estimated according to Paxinos & Watson (9) (Figure S1).

Monkey Behavior: Drug Infusions in DLPFC in Monkey Performing the ODR Task

One monkey was trained to perform three versions of the ODR task: 1) 2-target ODR task, 2) 8-target ODR task (Methods), and 3) 2 and 8-target visually guided saccade (VGS) tasks. The 2-target ODR task allowed us to collect numerous trials for each cue to examine the effects of D1R stimulation on ipsi- and contralateral hemifields. The VGS tasks had the same sensory and motor components as the ODR tasks, except that the central target was extinguished when a peripheral cue appeared in the desired saccade location. The animal was simply required to make a saccade to the cue, and thus did not need to use working memory. Schematics of these tasks are shown in Figure S2.

Drug infusions were performed at sites in the right DLPFC that showed spatially tuned, delay-related activity in previous *in vivo* physiology recordings. After a control condition, SKF81297 (10-20 μL) or sterile saline was infused with a 50 μL Hamilton syringe (Hamilton Company, Reno, NV) via two methods. In the first method, the syringe was mounted on a

micro-manipulator (Narishige, East Meadow, NY), and the needle was inserted to 2-3 mm below the dura. The drug was then infused at 2 μL/min. In the second method, a guide tube punctured the dura, and a 25-30G metal tubing within the guide tube was inserted to 2-3 mm below the dura. The syringe was mounted on a PHP 2000 infusion pump (Harvard Apparatus, Holliston, MA), and delivered drugs via polycarbonate tubing at 2-4 μL/min. The numbers of correct and incorrect saccades were measured during and after drug conditions, and compared using the Fisher exact test.

SKF81297 was dissolved to a concentration of 10 mM in sterile saline. SKF81297 infusions were performed 5 times, and the saline infusions were performed twice for the 2-target ODR task; saline infusions were followed by SKF81297 infusions. The experiment was done in only one animal, as multiple infusions ultimately damaged the tissue, and the monkey needed to be sacrificed.

Supplementary Results

D1R Stimulation Reduced Tuning of PFC Delay-Related Firing via Opening of HCN Channels in Monkeys Performing a Working Memory Task

We performed exploratory analyses of changes in spatial tuning across drug conditions using d' as a measure of the strength of tuning. As expected, non-tuned cells started with a smaller value of d' during the Control condition than tuned cells (p = 0.0022 vs. Control for tuned cells). While the value of d' was maintained during the ZD7288+SKF38393 condition (p > 0.05 vs. Control for tuned and non-tuned cells), it significantly decreased with SKF38393 among both tuned cells (p = 0.028 vs. Control; 0.032 vs. ZD7288+SKF38393) (Figure S3A) and non-tuned cells (p = 0.0046 vs. Control) (Figure S3B).

We also hypothesized that ZD7288 would block at least some of the beneficial effects of lower doses of D1R stimulation, whereby D1Rs enhance spatial tuning of delay-related firing (1). We recorded 4 neurons in which d' during the SKF38393 condition was greater than during the Control and ZD7288+SKF38393 conditions, suggesting that ZD7288 blocked the SKF38393-induced spatial tuning (Figure S3C). Of course, these preliminary findings would need to be confirmed in a much larger study in the future.

D1R Stimulation in PFC Impaired Working Memory Performance in a Monkey

Infusions of SKF81297 into the right DLPFC increased the number of inaccurate saccades for cues presented in the visual field contralateral to the infusions in 2- (Figure S4) and 8-target (Figure S5) ODR tasks ($p < 0.05$ vs. Control); saccades to the ipsilateral hemifield were not affected ($p > 0.05$ vs. Control). There were no effects of saline infusions on saccades in the 2-target ODR task (Figure S6) ($p > 0.05$ vs. Control). Finally, there was no effect of SKF81297 on saccades during the 2- (Figure S6) or 8-target (Figure S7) VGS tasks ($p > 0.05$ vs. Control), showing that the deficits observed in the ODR tasks involved working memory. Histological examination confirmed that the infusions were performed in the medial bank of caudal principal sulcus, anterior to the frontal eye fields (data not shown).

Role of General Motor Impairments in Working Memory Performance in Rats

We verified that the changes in working memory performance following D1R stimulation or stress in rats 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. While the response times significantly decreased with SKF81297 ($p = 0.0018$ vs.

vehicle) or FG71242 ($p = 0.0013$ vs. vehicle), they were not correlated with accuracy ($r = 0.29$, $p = 0.36$ for SKF81297; $r = -0.43$, $p = 0.18$ for FG7142). This lack of correlation was consistent with a previous study showing that this compound had no effect on motor performance (10, 11).

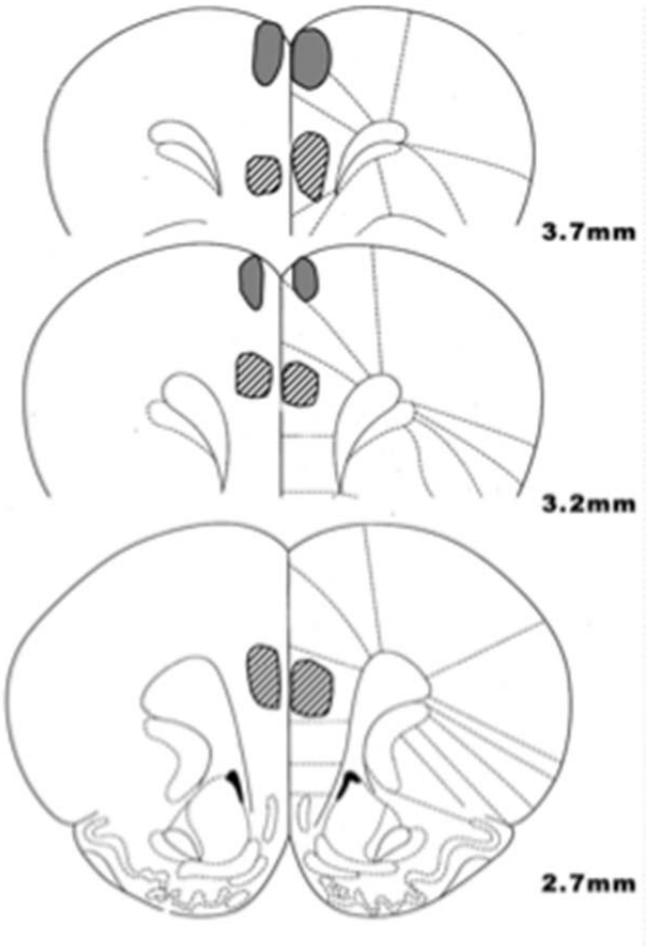

Figure S1. Post-mortem histological data from rats showed that the cannulae were correctly placed in the prelimbic PFC and dorsal to PFC. Lengths (mm) refer to the positions of the sections anterior to Bregma.

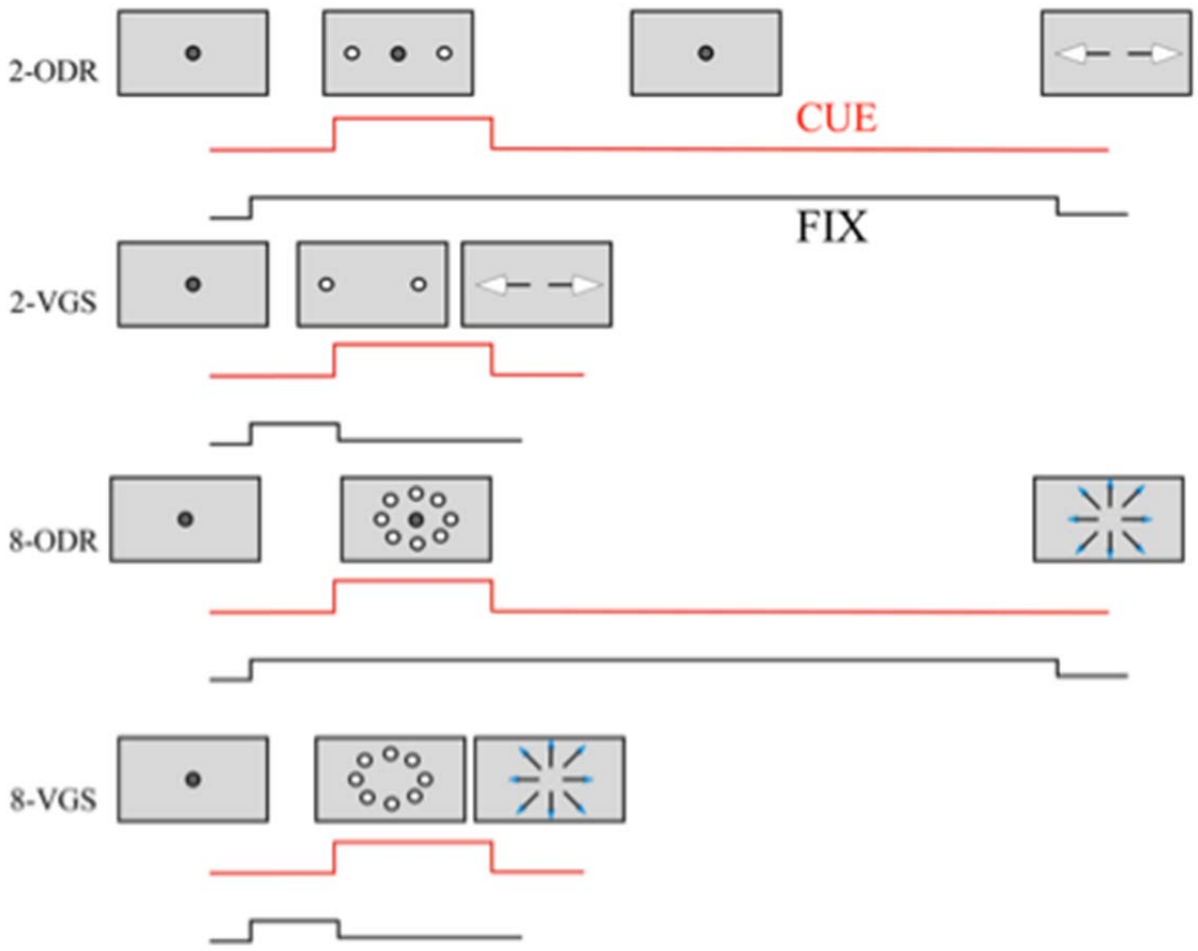

Figure S2. Schematics of 2- and 8-target oculomotor delayed response (2-ODR and 8-ODR, respectively) and visually guided saccade (2-VGS and 8-VGS, respectively) tasks. As for the 8-target ODR task used in the monkey *in vivo* physiology experiments, these tasks started with fixation (0.5 s), followed by cue presentation (0.5 s). In the 2-target tasks, only cues at 0° and 180° were used. For the ODR tasks, the cue was followed by a delay period of up to 3 s, after which the monkey made a saccade to the remembered location of the cue. For the VGS tasks, the monkey made a saccade to the cue after the fixation period, i.e., after 0 s delay. Timing for fixation and cue are indicated in black and red, respectively. Black circles indicate fixation points, white circles indicate cue locations, and arrows indicate putative saccades.

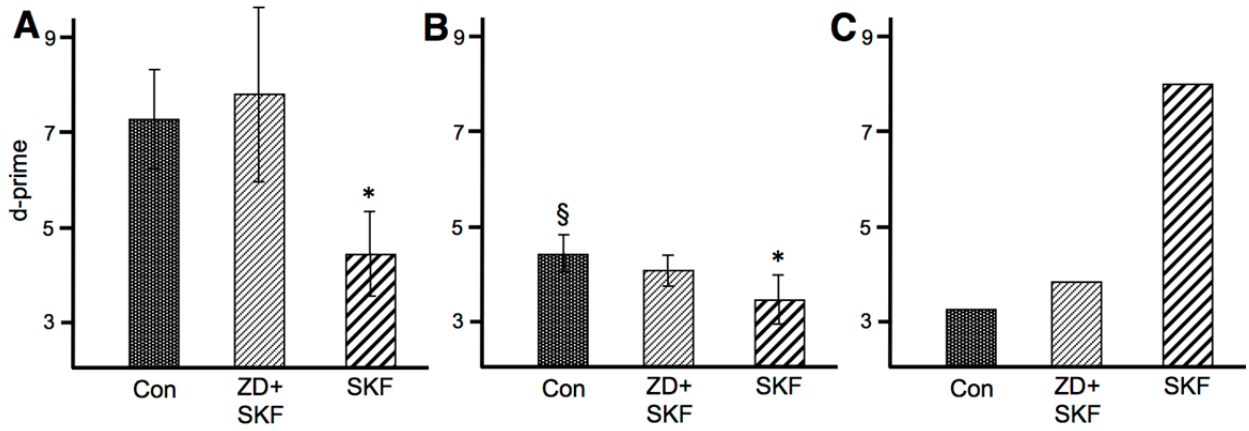

Figure S3. D-prime (d'), a measure of strength of tuning, during the Control, ZD7288 (ZD)+SKF38393 (SKF) and SKF conditions in tuned (**A**) and non-tuned (**B**) cells that showed reduced firing with SKF relative to Control conditions. D-prime significantly decreased with SKF among both tuned (*p = 0.028 vs. Control; 0.032 vs. ZD+SKF) and non-tuned (*p = 0.0046 vs. Control) cells. The non-tuned cells started with a lower value of d' during Control than tuned cells ($^{\S}p$ = 0.022 vs. Control for tuned cells). In addition, 4 neurons showed greater d' during the SKF condition than during the Control and ZD+SKF conditions (**C**), suggesting that ZD blocked the SKF-induced spatial tuning.

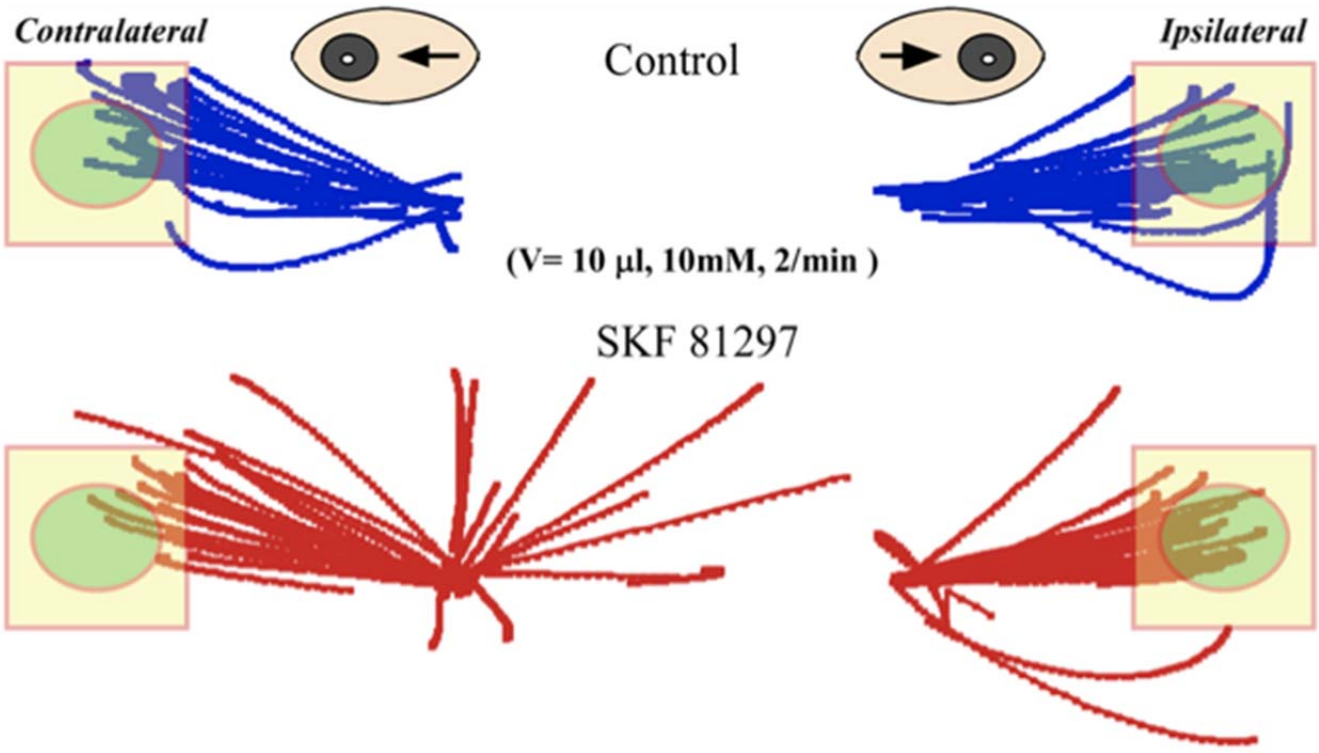

Figure S4. Saccade traces for a monkey performing the 2-target ODR task during Control condition (top) and while SKF81297 (10 µL) was infused into right DLPFC (bottom). Green circles show saccade targets, while surrounding squares show windows within which saccades were considered accurate. SKF81297 increased the number of inaccurate saccades for the contralateral direction (left; $p < 0.05$ vs. Control), but not for the ipsilateral direction (right; $p > 0.05$ vs. Control).

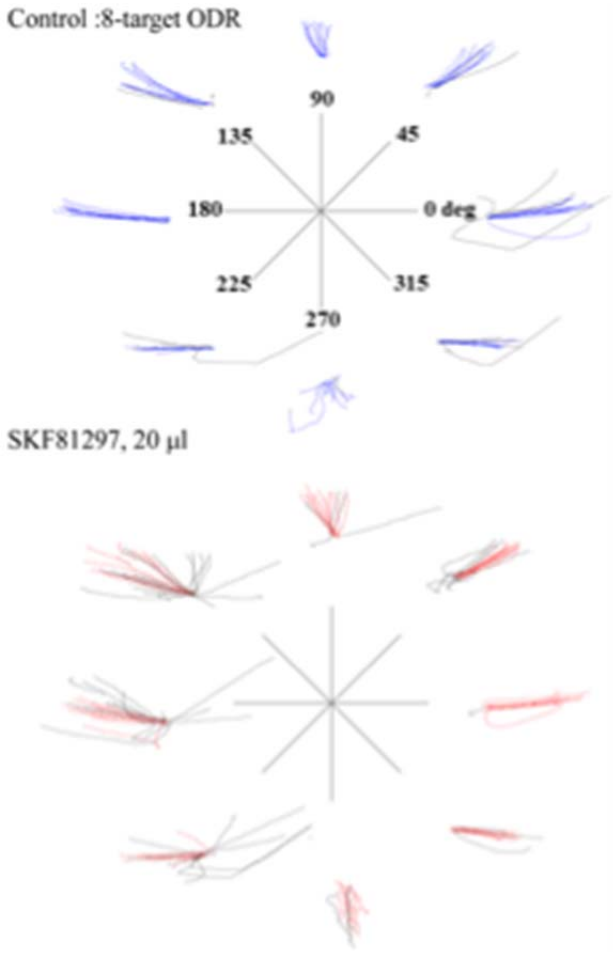

Figure S5. Saccade traces for a monkey performing the 8-target ODR task during the Control condition (top) and while SKF81297 (20 µL) was infused into right DLPFC (bottom). Correct saccades are shown in blue (Control) or red (SKF81297), while incorrect saccades are shown in black. Spokes indicate the eight cue directions. SKF81297 increased the number of inaccurate saccades for the contralateral hemifield (90-225°; $p < 0.05$ vs. Control), but not for the ipsilateral hemifield ($p > 0.05$ vs. Control).

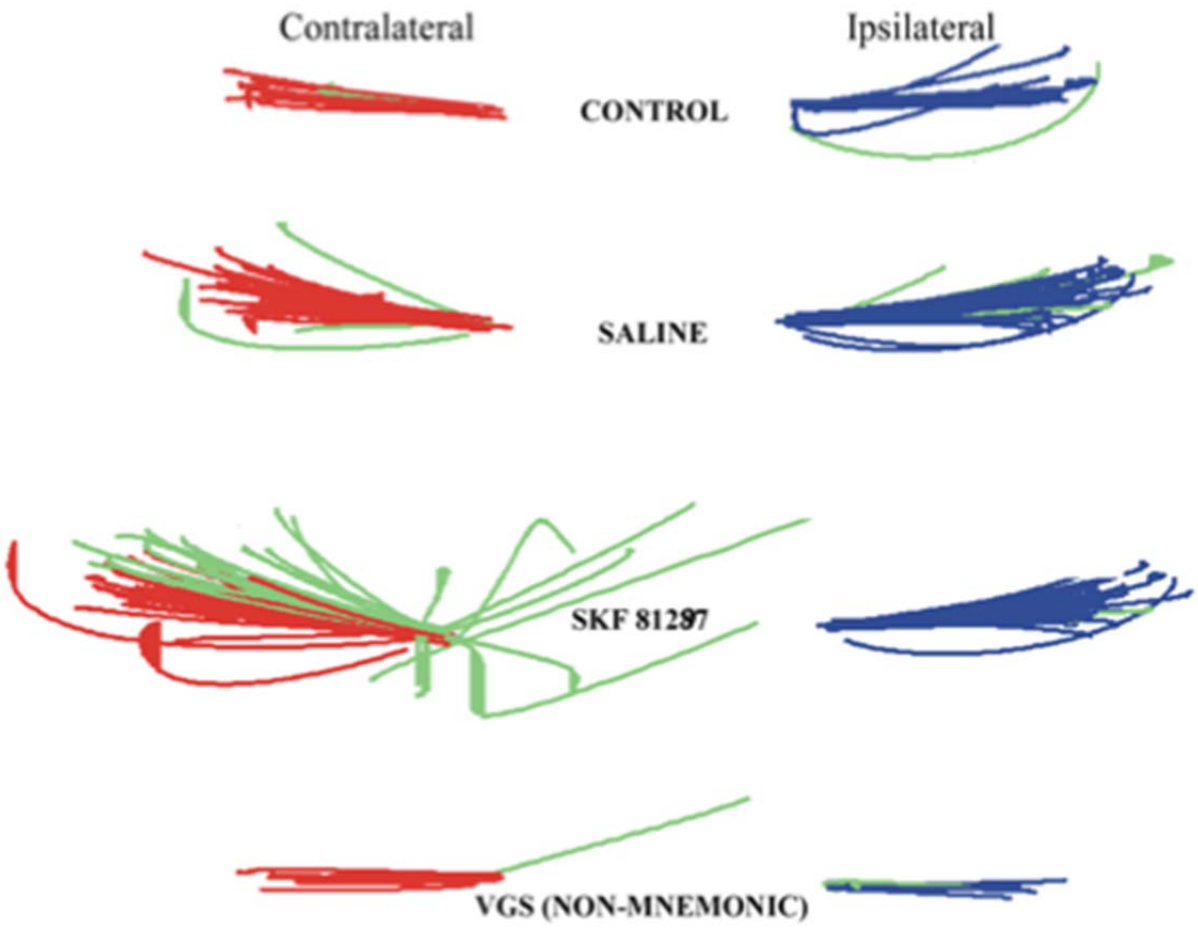

Figure S6. Saccade traces for a monkey performing the 2-target ODR task during Control (top), saline (10 μL; 2nd from top) and SKF81297 (10 μL; 2nd from bottom) conditions, and the VGS task (bottom). The number of inaccurate saccades (green) increased during the SKF81297 condition for the contralateral direction (left; $p < 0.05$ vs. Control), but not for the ipsilateral direction (right; $p > 0.05$ vs. Control). The number of inaccurate saccades also did not increase during the saline condition or VGS task ($p > 0.05$ vs. Control).

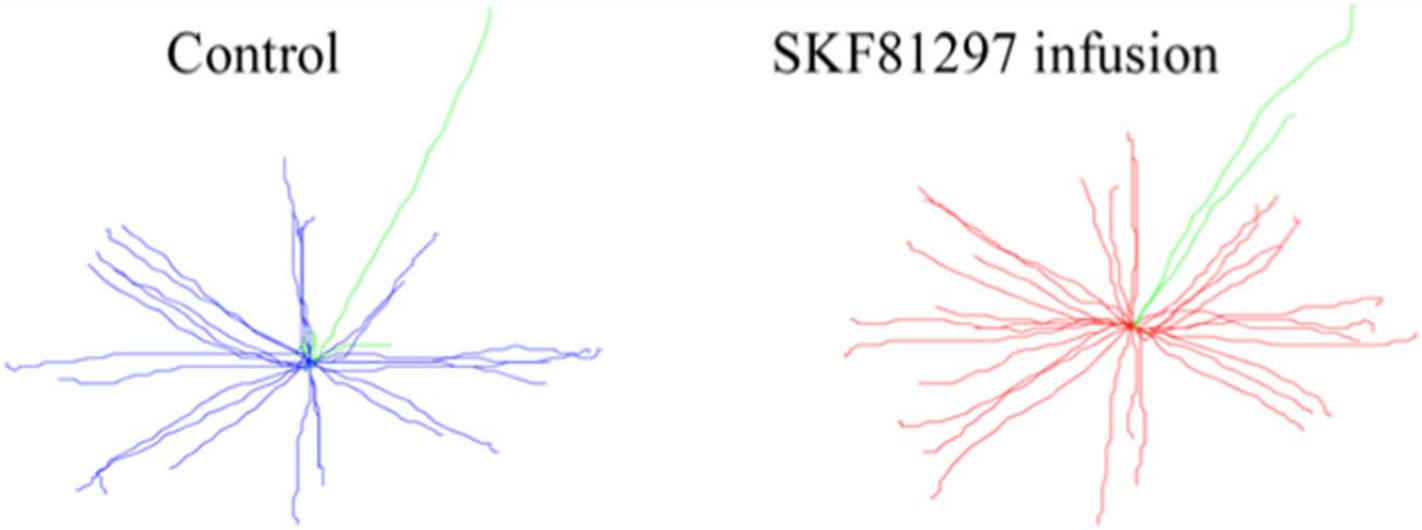

Figure S7. Saccade traces for a monkey performing the 8-target VGS task during Control (left) and SKF81297 (20 μL; right) conditions. The number of inaccurate saccades (green) did not increase during this task ($p > 0.05$ vs. Control).

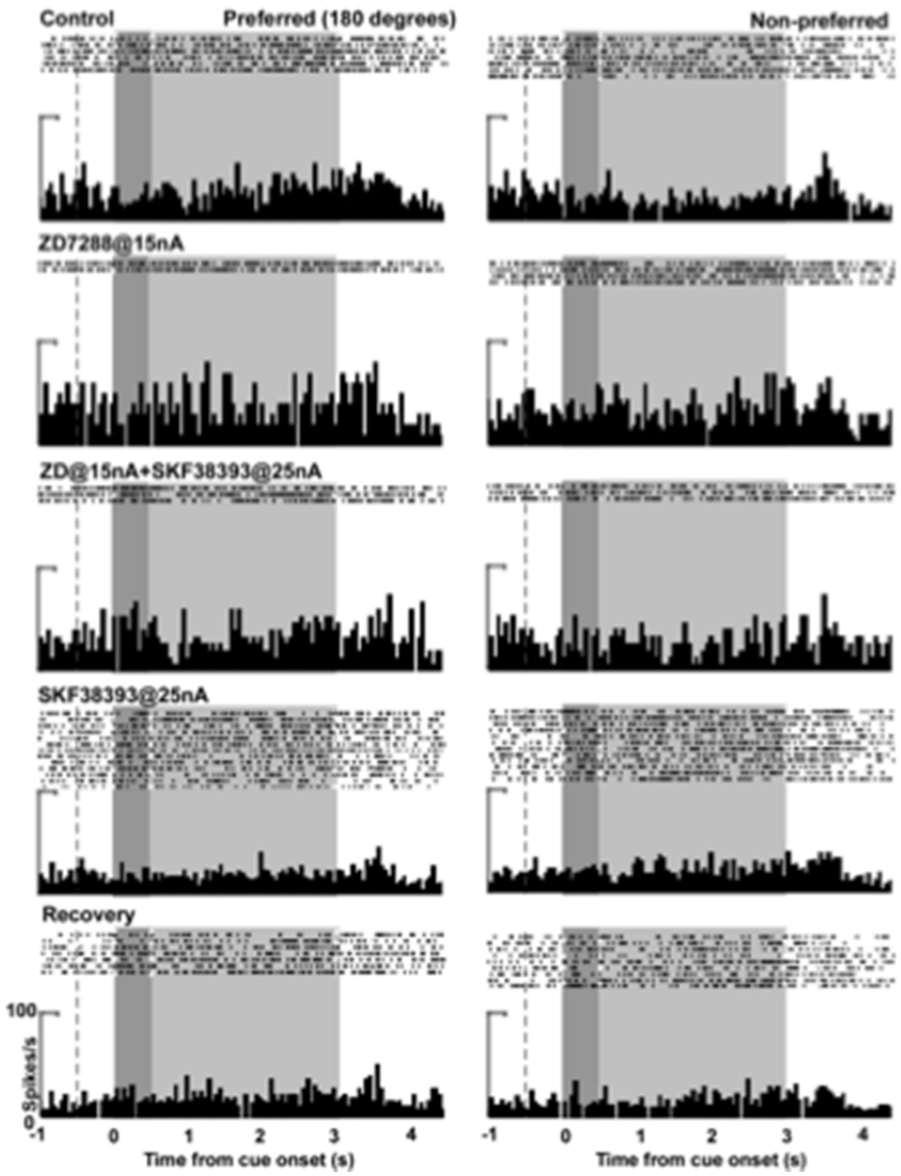

Figure S8. Example of a neuron in DLPFC, in which SKF38393 (SKF) preferentially reduced delay-related firing relative to firing during the fixation period in a monkey performing the ODR task. For the 180° direction, the delay firing remained significantly higher than fixation firing during Control ($p = 0.007$), ZD+SKF ($p < 0.005$), SKF ($p = 0.006$) and Recovery ($p = 0.003$), but not ZD ($p = 0.17$). The delay firing was maintained with ZD and ZD+SKF ($p > 0.05$ vs. Control), but was then reduced with SKF ($p < 0.0014$ vs. Control, ZD+SKF). The delay firing returned during Recovery ($p < 0.016$ vs. Control, ZD, ZD+SKF). 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.

Table S1. Drug conditions applied to single units recorded from DLPFC in two monkeys.

Drug conditions	**Number of neurons**
Control, ZD+SKF, SKF	8
Control, ZD+SKF, SKF, Recovery	2
Control, ZD, ZD+SKF, SKF, Recovery	6

Supplemental References

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