Supplementary Methods

Behavioral apparatus: Operant chambers (MedAssociates, St Albans, VT) were equipped with a lever, a drinking tube, and a speaker driven to produce an 8 kHz tone at 72 dBA, using audio equipment either from Tucker-Davis Technologies (Alachua, FL) or manufactured in the Instruments Shop at the Pierce Laboratory. Each chamber contained a house light, which was turned on at the beginning of each session, and a fan, which produced broadband noise at approximately 60 dBA. Behavioral arenas were housed in sound-attenuating chambers (MedAssociates). On correct trials, water was delivered via a pump (MedAssociates) connected to a standard metal drinking tube (AnCare) via Tygon tubing. On error trials, animals experienced a timeout period of 4-8 s during which all experimental stimuli were extinguished. Behavioral devices (houselight, pump, click stimulus) were activated after a delay of 100 ms after lever release. Behavior was monitored during all sessions via closed-circuit video cameras (Allied Electronics, Fort Worth, TX). Operant chambers for neurophysiology were identical to those described above, except that they were mounted on a steel plate within a custom-built Faraday cage, and used a plastic drinking tube that delivered distilled water. Response force was measured using a load cell (part #LCL- 454G {Omega Engineering, Stamford, CT} rated to .454 N, or a thin film load cell, part #S100 {Strain Measurement Devices, Meriden, CT} rated to 1 N), mounted on the lever.

Delayed-response task: Rats were trained in operant chambers (see supplementary methods) according to procedures described in detail elsewhere (Narayanan et al., 2006) to perform a delayed-response task (also called a simple reaction time task with a fixed foreperiod). Trials were initiated when rats pressed a lever. Rats had to maintain the lever press for a 1000 ms delay

period. At the end of this delay period, an auditory trigger stimulus (8000 Hz tone at 72 dBA) was presented (Fig 1). In Experiment 2, trigger stimuli occurred on 50% of trials. *Correct* trials (also called conditioned trials) occurred when rats successfully maintained a lever press for the full delay period and released the lever with short response latencies (within 600 ms of the end of the delay period), and were followed by a liquid reward. Premature error responses occurred when rats did not maintain a lever press for the full delay period. Late responses occurred when rats did not release the lever promptly (>600 ms) from the end of the delay period. Premature and late responses were considered *error* trials and were followed by a timeout period (4-8 s), in which all experimental stimuli were extinguished. Over the course of training, the duration of pump activation was gradually decreased from 3.5 s to 1.0 s (volume per trial: 0.03 mL). Rats learned to make sustained lever presses on at least 60% of trials in 5-15 sessions. Prior to implantation, rats made temporally correct responses on 66±2% of trials.

Rats were motivated by water restriction, while food was available *ad libitum*. Rats consumed 10-15 ml of water during each behavioral session and additional water (5-10 ml) was provided 1-3 hours after each behavioral session in the home cage. Rats were maintained at ~90% of their free-access body weights during the course of these experiments, and received one day of free access to water per week.

Surgery: Aseptic stereotaxic surgery was performed according to methods described elsewhere to implant arrays of microwire electrodes into dmPFC (Experiment 1 and 3) (Fig S1) or motor cortex (Narayanan et al., 2005) (Experiment 2) (Fig S3). Cannulae were implanted into dmPFC according to procedures described elsewhere (Fig S3) (Narayanan et al., 2006). Briefly, anesthesia was initiated with ~4% halothane and intraperitoneal injections of ketamine (100

mg/kg) and diazepam (10 mg/kg). A surgical level of anesthesia was maintained over the course of surgery with supplements (30 mg/kg) of ketamine approximately every 45 mins. Under aseptic conditions, the scalp was retracted, and the skull was leveled between bregma and lambda.

In Experiment 1, a single craniotomy was drilled over the area above dmPFC cortex that spanned both hemispheres. An array of microwire electrodes (3x3x2, 2x8, or 4x4 microwire arrays of 50 μm stainless steel wire, spaced approximately 200 μm apart, impedence range 200-300 ohms, available from NB Labs, Dennison, TX or Neurolinc, New York, NY) was lowered into bilateral dmPFC (Coordinates from bregma: AP: +3.2, ML ± 1.4, DV -3.6 @ 12° in the frontal plane; Fig S1) targeting coordinates of previous inactivation (Narayanan et al., 2006). In Experiment 2, twenty-six gauge guide cannulae (Plastics One, Roanoke, VA) were lowered slowly into bilateral craniotomies targeting the dorsal prelimbic region of dmPFC (Coordinates from bregma: AP: +3.2, ML ± 1.4, DV -3.6 @ 12° in the frontal plane as measured stereotaxically; Fig S3), and a third craniotomy was then drilled and an array of microwire electrodes (identical to those used in Experiment 1) was implanted into rat motor cortex (4x4 or 2x8 configuration; coordinates from bregma: AP: -0.5, ML: ± 2.5-3.5, DV: -1.5 @ -25º in the frontal plane) (Donoghue and Wise, 1982; Laubach et al., 2000; Neafsey et al., 1986) contralateral to the rat's dominant paw (Fig S3). In Experiment 3, separate craniotomies were drilled over dmPFC and motor cortex (coordinates above), and microwire arrays were lowered first into dmPFC, and then into motor cortex.

Craniotomies were sealed with cyanoacrylate ('SloZap', Pacer Technologies, Rancho Cucamonga, CA) accelerated by 'ZipKicker' (Pacer Technologies), and methyl methacrylate (AM Systems, Port Angeles, WA). After implantation of microwire recording arrays and/or

cannulae, animals were allowed to recover for one week, and then acclimatized to recording procedures before testing in the delayed-response task.

Reversible inactivation of dmPFC: Prior to inactivation experiments, fully trained postoperative rats were placed on water restriction and run in the delayed-response task until stable task performance was observed. Next, the animals were acclimatized to recording procedures (i.e., headsets and cables were attached to the implants) for two behavioral sessions. Initially, animals were lightly anesthetized with halothane via a nosecone for 7 min and tested in the delayed-response task 45 min after recovery from anesthesia. On the first day of testing, 0.9% saline (Phoenix Scientific, St. Joseph, MO) was infused into dmPFC (control sessions). On the second and third days of testing, muscimol, a GABA-A agonist (Sigma-Aldrich, St Louis, MO) (Lomber, 1999; Martin and Ghez, 1999; Narayanan et al., 2006), was infused into dmPFC at doses of 0.1 and 1 mg/ml, respectively (inactivation sessions). Animals did not consistently perform enough trials (>50) at the 1 mg / ml dose of muscimol for neurophysiological analysis; therefore, these data were not analyzed. On the fourth day of testing, rats were run without manipulations (recovery sessions).

Infusions of either saline or muscimol were made with a 33-gauge cannula (Plastics One) that protruded 0.2 mm from the tip of the guide cannula. Injectors were inserted into the guide cannula and 0.5 µl of infusion fluid was delivered per site at a rate of 15 µl/hr (0.25 µl/min; Martin and Ghez 1999) via a syringe infusion pump (KDS Scientific, Holliston, MA). Fluid was infused via 0.38 mm diameter polyethylene tubing (Intramedic, New York, NY) joined to the injector on one end and to a 10 µl Hamilton syringe (Hamilton, Reno, NV) on the other end. Injections were confirmed by monitoring movement of fluid in the tubing via a small air bubble.

After injection was complete, the injector was left in place for 2 minutes to allow for diffusion. Rats were tested in the delayed-response task 45 minutes after the start of the infusions. This amount of time was sufficient to allow for full recovery from the halothane anesthesia and for muscimol to reach its maximal effectiveness in inhibiting neural activity (Hikosaka and Wurtz, 1985; Krupa et al., 1999). Fluid spread with these methods has been confirmed previously using fluorescent muscimol (Narayanan et al., 2006).

Electrophysiological recordings: Neuronal ensemble recordings were made using a Many Neuron Acquisition Program (Plexon, Dallas, TX). Putative single neuronal units were identified on-line using an oscilloscope and audio monitor amplified at an average gain of 12000 (range: 1000-22000). The Plexon off-line sorter was used to analyze the signals off-line and to remove artifacts due to cable noise and behavioral devices (pump, click stimulus). Principal component analysis and waveform shape were used for spike sorting. Single units were identified as having 1) consistent waveform shape, 2) separable clusters in PCA space, 3) average amplitude estimated at least three times larger than background activity, and 4) a consistent refractory period of at least 2 ms in interspike interval histograms. Those units identified on-line as potential single units that did not meet these criteria off-line were not included in this analysis (Fig S2).

Analysis of task-related modulations in firing rates: Graphical exploratory analysis of neuronal activity and quantitative analysis of basic firing properties (firing rate, inter-spike intervals, burst rate, surprise entropy) were carried out using Stranger (Biographics, Winston-Salem, NC) and NeuroExplorer (Nex Technologies, Littleton, MA) and subsequently analyzed using custom

routines for MATLAB. Peri-event rasters and average histograms were constructed around lever release, lever press, and tone offset. To compare activity across neurons and animals, average peri-event histograms were normalized to average firing rate in the behavioral epoch of interest (e.g., 2000 ms before to 500 ms after lever release). Task modulated neurons were statistically identified based on Wilcoxon sign tests of epochs of interest (500 ms windows around lever press, during the delay period when animals were waiting to respond, and around lever release) compared with the neuron's spontaneous activity (i.e, firing rates at pseudorandom times; in order to ensure modulation, this test was performed 10 times vs. 1000 random time points).

Finally, all statistical tests consider control sessions and sessions with dmPFC inactivated as two independent sessions, and all statistical analysis comparing control sessions with sessions with dmPFC inactivated were confirmed with balanced numbers of trials (i.e., the same number of trials in each session).

Assessment of paired interactions between dmPFC and MC: In order to investigate whether neurons in dmPFC and motor cortex had functional relationships (Experiment 3), we used cross correlation and joint peristimulus time histograms (JPSTH) (Aertsen et al., 1989). JPSTHs were explored using NeuroExplorer. JPSTH correlation values ([JPSTH-JPSTHpred]/SDPred) were computed for all pairs using MATLAB code provided by MuLab (http://mulab.physiol.upenn.edu/) during the press, delay, and release epochs as described for Experiments 1 and 2. Significant JPSTH interactions were determined by repeating analysis of JPSTH correlations on data in which trial order was shuffled; in this data, all modulations in firing rate are preserved and only correlations are destroyed. For the 1743 trial-shuffled pair wise interactions considered by this analysis (581 interactions for the epochs corresponding to press,

delay, and release), there were 7 JPSTH chance correlations greater than ±0.225. This corresponded to a probability value of 0.004; therefore, we interpret JPSTH correlations with an absolute value greater than 0.225 as significant at $p < 0.005$.

Analysis of multi-unit activity: We separately analyzed multi-unit activity by setting a user-defined threshold for each electrode and capturing all waveforms that passed this threshold. Waveforms typical of cable and behavioral noise were then removed using the Plexon spike sorting software.

Analysis of local field potentials: Raw voltage traces were collected from four wires in each of four animals, analog filtered between 0.5 Hz and 5.9 kHz and sampled at 1 kHz. These signals were subsequently digitally low-pass filtered at 200 Hz.

Predictions of task performance based on correct trials: To predict correct responses, trials with temporally correct responses in the median third of reaction times (between the 33rd and 66th percentile) were selected and perievent histograms were constructed (using 1 ms bins) for the epoch from 250 ms before lever release to 100 ms after release. The histograms were then smoothed, using 5-fold decimation and low-pass filtering, and decomposed into a set of components that were ordered by relative variance. This latter step was carried out using wavelet packet analysis based on the matching pursuit algorithm (Mallet and Zhang, 1993). A 4-point "symmlet" filter was used for the wavelet packet analysis.

Matching pursuit is a sequential decomposition algorithm that finds a series of signal components (up to 10 in this case) that account for decreasing levels of variance in the average

peri-event histograms of each neuron. This procedure is critical for reducing the complexity (i.e., dimensionality) of the data prior to analysis with statistical classifiers. By using matching pursuit, we reduced the dimensionality of the neuronal spike data, from 400 1-ms bins (the number of bins in a peri-stimulus time histogram) to typically 5 or fewer matching pursuit task-related features.

Scores for matching pursuit components defined with the correct responses were also extracted for trials with premature error responses. Scores were calculated as the dot product of the wavelet filter and the measured neuronal firing on each trial. However, the extraction of scores was done in an iterative manner, with the scores for the higher filters defined on the residual neuronal signal after the lower filters were applied.

The scores were then used to train statistical classifiers. Regularized discriminant analysis (RDA; Friedman, 1989) was used with leave-one-out cross-validation. RDA is a non-parametric form of linear discriminant analysis that is a appropriate for data sets with non-Gaussian distributions of the predictor data (i.e., the scores from matching pursuit) and that allows for non-linearities in the discriminant functions (see Hastie et al., 2001 for review). Results obtained from the classifiers were used to construct 'confusion matrices' – i.e., a list of trial outcomes vs. the predictions of trial outcome made by the classifier in the form of a 2x2 table. Importantly, we found that the percent of trials that are classified correctly is a problematic measure of classifier performance for unbalanced data sets, such as in the case of comparing correct and error trials. Randomly guessing the more common of two classes, e.g., in sessions with 75% correct responses, will lead to apparently good classification if the results are expressed in terms of the percent correctly classified. Therefore, we report results obtained with classifiers using two alternative metrics, the area under the curve for the receiver-operating characteristic (which is

near 0.5 for random classification) and the predictive mutual information (which is near 0 for random classification) (Krippendorff, 1986). In particular, mutual information allows comparisons with other studies that apply information theory. These issues are discussed in detail in Narayanan et al., 2005.

Only sessions with more than 20 premature errors and only premature error responses with lever presses longer than 300 ms were included in this analysis. Late trials tended to be rare (<10% of trials) and were excluded from analysis. Finally, all analyses were confirmed with balanced trials (i.e., similar numbers of correct and premature error trials).

Predictions of task performance based on error trials: To predict premature errors, trials with premature errors with lever presses longer than 300 ms were selected and peri-event histograms were constructed as above for the epoch from 250 ms before lever release to 100 ms after release. The method based on matching pursuit algorithm, described above, was then used to identify features for each neuron and to calculate scores for each feature. Scores for matching pursuit components defined with premature errors were also extracted for trials with correct responses. Scores for the set of features defined for each neuron were then evaluated with the statistical classifiers as above.

Software: This analysis was carried out using the WaveLab libraries (http://www-stat.stanford.edu/~wavelab/) for MATLAB and the klaR and verification libraries for R (http://www.r-project.org/), which provided code for RDA and ROC analysis, respectively. For an overview on the use of these methods see Laubach (2004). Code for carrying out these analyses is available on our group's website at http://spikelab.jbpierce.org.

Supplementary Results

Inactivation of dmPFC does not change multiunit activity or motor field potentials

One mechanism by which dmPFC inactivation might alter delay-related firing in motor cortex is via a change in brain networks subserving movement-related potentials, such as the readiness potential or the contingent-negative variation (CNV). Brunia (1999) hypothesized that these brain networks, which include basal ganglia, cerebellum, and thalamic nuclei, might mediate such potentials and may be controlled by dmPFC in anticipation of an impending response. In order to directly test this idea, we compared field potential recordings in control sessions and in sessions with dmPFC inactivated. If these potentials preceding release were influenced by dmPFC, then the motor cortex field potential would be altered by dmPFC inactivation.

We also examined the multiunit records from 80 (of 96; 83%) active wires in six animals. When comparing control sessions to sessions with dmPFC inactivated, no differences were seen in multiunit activity around lever release (2000 ms before to 500 ms after lever release; $T_{(1, 160)} = 0.2$, $p < 0.84$) or while animals were waiting to respond (800 ms before to 300 ms before lever release; $T_{(1, 160)} = 0.17$, $p < 0.86$; Fig S5A).

Comparisons of field potentials in control and inactivation sessions revealed no consistent differences during the entire trial epoch (2000 ms before to 500 ms after lever release; paired $T_{(1, 15)} = 0.66$, $p < 0.52$) or while animals were waiting to respond (800 ms before to 300 ms before lever release; paired $T_{(1, 15)} = 0.48$, $p < 0.63$; Fig S5B). The decrease in delay-related firing by single neurons in motor cortex that was observed with dmPFC inactivated therefore

appears to have resulted from changes in specific synaptic connections within motor cortex and was not due to changes in overall processing as reflected by motor cortex local field potentials.

Supplementary Figures

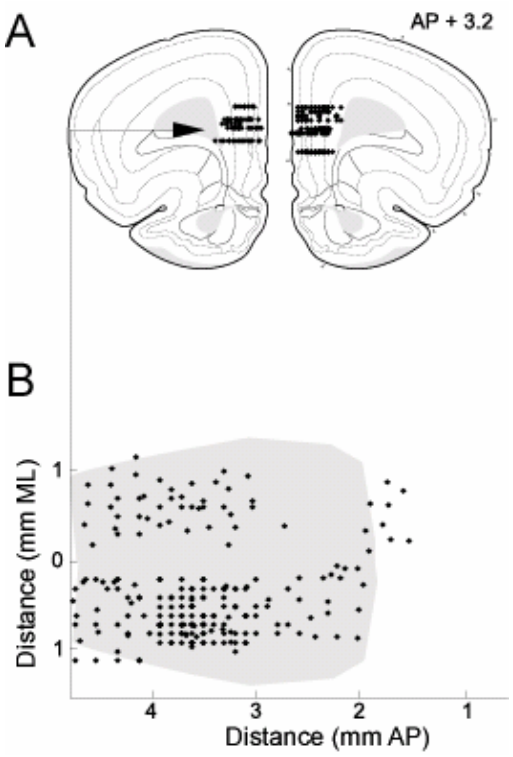

Figure S1: (A) Dorsoventral and (B) anteroposterior location of electrode tips in dmPFC from eleven animals in Experiment 1. Prelimbic cortex (Swanson 1989) is outlined in gray.

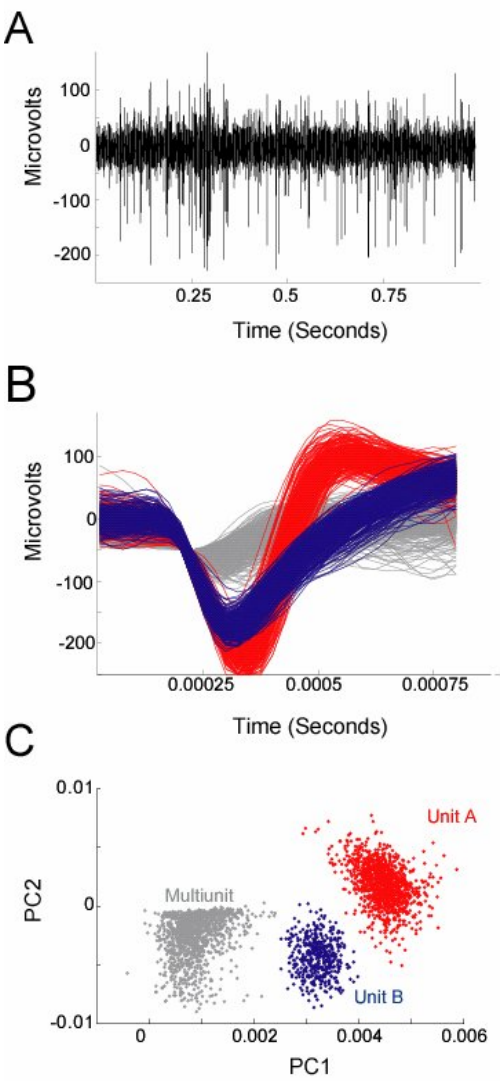

Figure S2: Example of unit isolation from a typical channel (gain = 12000). (A) Raw voltage recorded on a single 50 μm stainless steel wire from the cortex of a rat performing a delayed-response task. (B) Waveforms that crossed a user defined threshold (set at –65 μV). Waveforms were digitized at 40 kHz. (C) Waveforms represented in PCA space. Units were isolated based on consistent waveform shape, separable clusters in PCA space, and average amplitude estimated at least three times larger than background activity. Points in gray represent multiunit data and were not analyzed.

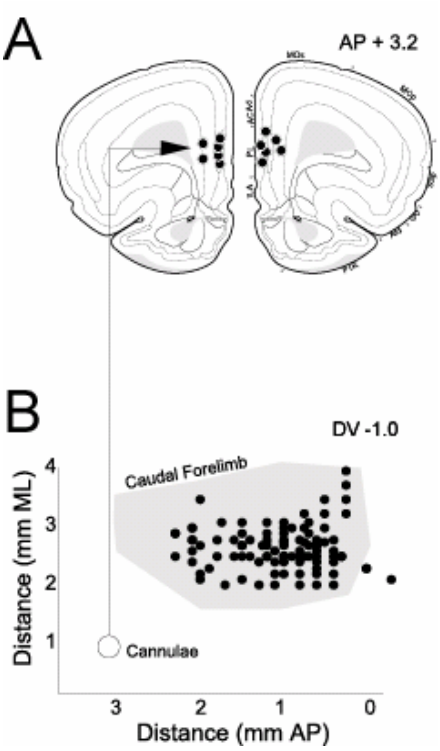

Figure S3: (A) Location of cannulae tips within dmPFC. (B) Location of electrode tips in the caudal forelimb area of the rat motor cortex in relation to cannulae (hollow circle). The caudal forelimb area corresponding to primary motor cortex (Neafsey et al, 1986) is outlined in gray.

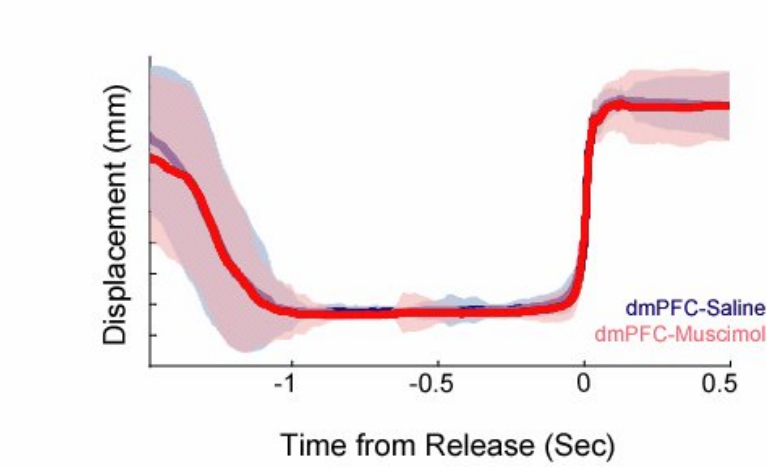

Figure S4: Lever displacement data in control sessions (blue) and in sessions with dmPFC inactivation (red) reveals no change in lever press or release velocity, or lever displacement on conditioned trials (paired t-test: $T_{(1, 276)} = 0.78$, $p < 0.44$).

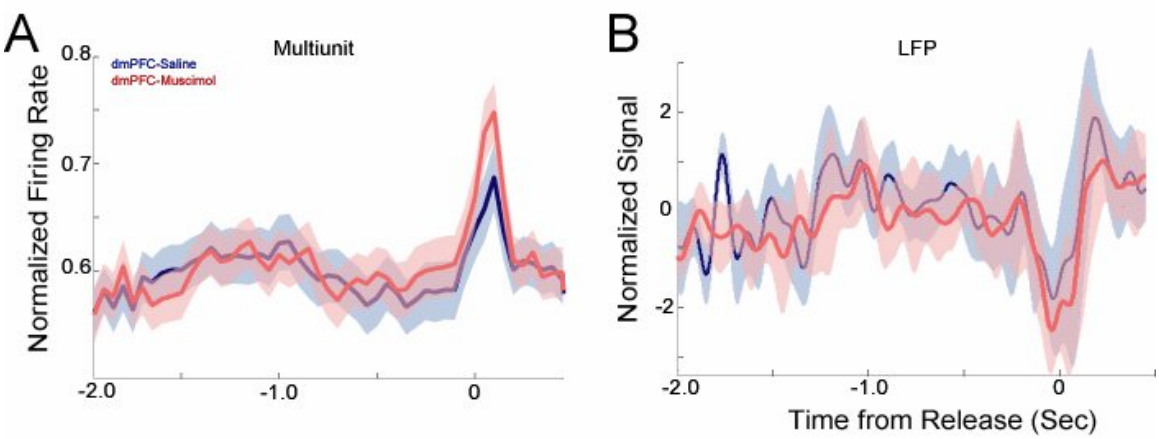

Figure S5: (A) Mean normalized multi-unit activity and (B) Z-score normalized local field potential for conditioned trials in control sessions (blue) and sessions with dmPFC inactivated (red). No differences were apparent in these measures of motor cortical activity in the control and inactivation sessions (paired t-test on MUA: $T_{(1, 160)} = 0.17$, $p < 0.86$; paired t-test on LFP: $T_{(1, 15)} = 0.48$, $p < 0.63$).

Supplementary Videos

Movie KH4_Saline.avi: A conditioned lever press for one animal under control conditions.

Movie KH4_dmPFC_Inactive.avi: A conditioned lever press for the same animal in sessions with dmPFC inactivation.

Supplementary References

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