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# Selective Delay Activity in the Medial Prefrontal Cortex of the Rat: Contribution of Sensorimotor Information and Contingency

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**Cowen SL, McNaughton BL.** Selective delay activity in the medial prefrontal cortex of the rat: contribution of sensorimotor information and contingency. *J Neurophysiol* 98: 303–316, 2007. First published May 16, 2007; doi:10.1152/jn.00150.2007. The medial prefrontal cortex (mPFC) plays a critical role in the organization of goal-directed behaviors and in the learning of reinforcement contingencies. Given these observations, it was hypothesized that mPFC neurons may store associations between sequentially paired stimuli when both stimuli contribute to the prediction of reward. To test this hypothesis, neural-ensemble spiking activity was recorded as rats performed a paired-associate discrimination task. Rats were trained to associate sequentially presented stimuli with probabilistic reward. In one condition, both elements of the stimulus sequence provided information about reward delivery. In another condition, only the first stimulus contributed to the prediction. As hypothesized, stimulus-selective, prospective delay activity was observed during sequences in which both elements contributed to the prediction of reward. Unexpectedly, selective delay responses were associated with slight variations in head position and thus not necessarily generated by intrinsic mnemonic processes. Interestingly, the sensitivity of neurons to head position was greatest during intervals when reward delivery was certain. These results suggest that a significant portion of delay activity in the rat mPFC reflects task-relevant sensorimotor activity, possibly related to enhancing stimulus detection, rather than stimulus–stimulus associations. These observations agree with recent evidence that suggests that prefrontal neurons are particularly responsive during the performance of action sequences related to the acquisition of reward. These results also indicate that considerable attention must be given to the monitoring and analysis of sensorimotor variables during delay tasks because slight changes in position can produce activity in the mPFC that erroneously appears to be driven by intrinsic mechanisms.

## INTRODUCTION

The medial prefrontal cortex (mPFC) rests at the top of a complex cortical and subcortical hierarchy that includes sensory, motor, emotional, and neuromodulatory centers (Conde et al. 1995; Groenewegen et al. 1997). The mPFC is therefore situated in a unique position to integrate sensory and emotional information for the organization of adaptive and goal-directed behavior. Central to such behavior is the capacity to learn associations and contingencies among stimuli, actions, and outcomes. Evidence from lesion studies suggests that the mPFC may be critical for such learning (Balleine and Dickinson 1998; Cardinal et al. 2003; Parkinson et al. 2000). Furthermore, this learning may be facilitated by the strong input the region receives from various neuromodulatory centers such as the nucleus basalis, raphe nucleus, ventral tegmental area

(VTA), and the locus coeruleus (reviewed in Robbins 2000). Neurons in these regions respond preferentially to stimuli that consistently predict reward (e.g., Aston-Jones et al. 1997; Bouret and Sara 2004; Schultz 1998; Wilson and Rolls 1990). Furthermore, the neuromodulators associated with these regions are all capable of facilitating associative neural plasticity (e.g., Baskerville et al. 1997; Hotte et al. 2005; Izumi and Zorumski 1999; Jay et al. 2004; Matsuda et al. 2006). It was hypothesized that the action of such neuromodulatory signals will facilitate the formation of associative connections between neurons that respond to sequentially presented stimuli that reliably predict reward. In contrast, associative connections should not form between stimuli that are consistently paired but do not reliably predict reward. This follows from the observation that neurons in most neuromodulatory systems do not respond to frequently presented stimuli that are not associated with reinforcement. It was further hypothesized that the strength of association would be indicated by the degree to which the presentation of the first stimulus of the sequence triggered the representation of the paired associate (e.g., pattern completion). Therefore an appropriate measure of the strength of the association would be the degree to which the delay activity between the two stimuli was selective for the anticipated stimulus. Selective delay activity in the prefrontal cortex has been frequently reported in the primate (Fuster and Alexander 1971; Kubota and Niki 1971) and, more recently, in the rat (Baeg et al. 2003; Batuev et al. 1990; Narayanan and Laubach 2006; Sakurai and Sugimoto 1986).

To test this hypothesis, activity from multiple, simultaneously recorded single neurons in the rat mPFC was recorded as animals performed a discrimination task in which sequentially presented stimuli and rewards were delivered according to a probabilistic schedule. The task involved three conditions. In the “predictive” condition, the first stimulus of a two-stimulus sequence predicted the delivery of the second stimulus with a probability of 0.5, whereas the second stimulus predicted the delivery of reward with a probability of 1.0. In this condition, the absence of the second stimulus indicated that no reward would be presented. In the “nonpredictive” condition, the first stimulus also predicted the second stimulus with a probability of 0.5; however, the presence or absence of the second stimulus was entirely random with respect to reward delivery ( $P = 0.5$ ). In the “never-rewarded” condition the stimulus–stimulus contingencies were identical to the previous two conditions, but no rewards were ever delivered. It was hypothesized that activity during the delay interval that separated the first and second stimuli would be more selective for the anticipated CS2 stimulus in the predictive condition relative to the nonpredictive condition.

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This hypothesis was evaluated by using a go/no-go protocol with discrete stimuli and delay intervals. Furthermore, animals were required to maintain their nose within a small nosepoke hole for the duration of a trial. This requirement was adopted to minimize the potential contribution of sensorimotor effects to delay activity. The potential contribution of such effects to delay activity was identified in some of the earliest studies of the primate prefrontal cortex (Kubota and Niki 1971). The difficulty of disambiguating sensorimotor responses from memory-guided activity has been a persistent challenge to the study of delay activity (reviewed in Wang 2005) and it is a challenge that has not necessarily been completely resolved for any experimental paradigm.

The results of this investigation suggest that responses during delay periods were indeed selective to predictive stimulus sequences; however, these responses were often associated with the sensory or motor consequences of slight variations in head position. This result was surprising, considering the restrictions imposed on the animal's position. The variations in head position were also selective to specific stimulus sequences and thus may have been the result of a behavioral strategy used by the animal to solve the task. Such a strategy might obviate the need for an intrinsically sustained memory, at least in such highly overtrained contexts. The observed sensitivity of mPFC neurons to slight changes in head position suggests that, in the absence of precise measurement and analysis, the contribution of sensorimotor information to putative memory-driven delay activity may be seriously underestimated. Although it is conceivable that the observed behaviors served no adaptive function, it is also possible that the behaviors formed a component of an "embodied" strategy used to solve short-interval delay tasks.

## METHODS

### *Animals and surgical procedures*

Neurophysiological studies were conducted on three Brown Norway/Fisher 344 hybrid male rats between 14 and 20 mo old. The rats were housed individually and maintained on a 12:12 light–dark cycle. Recordings took place during the dark phase of the cycle. Surgery was conducted according to National Institutes of Health guidelines for rodents and approved IACUC protocols. Before surgery, the rats were administered bicillin (30,000 units intramuscularly in each hind limb). The rats were implanted, under isoflurane anesthesia, with an array of 14 separately movable microdrives ("Hyperdrive"). This device, general implantation methods, and the parallel recording technique have been described in detail elsewhere (Gothard et al. 1996). Briefly, each microdrive consisted of a drive screw coupled by a nut to a guide cannula. Twelve guide cannulae contained tetrodes (McNaughton et al. 1983; Recce and O'Keefe 1989; Wilson and McNaughton 1993). Each tetrode consisted of four polyimide-coated nichrome wires 14- $\mu$ m diameter, electroplated with gold to a final impedance of 300–1,000 k $\Omega$  (Kanthal Palm Coast, Palm Coast, FL). Two additional tetrodes with their individual wires shorted together served as an indifferent reference and an EEG recording probe.

Two craniotomies were drilled over the right and left mPFCs ( $\pm 1.3$  mm mediolateral, +3.2 mm anteroposterior) and 1.5-mm craniotomies were drilled over the left and right hippocampi ( $\pm 2.0$  mm mediolateral,  $-3.0$  mm anteroposterior to bregma). A hyperdrive was cemented in place over either the right or the left prefrontal craniotomy. The drive was implanted at a 9° angle toward the midline (see Fig. 1). The unused craniotomy was sealed with KwikSil (World Precision Instruments, www.wpiinc.com) and covered with dental acrylic. EEG probes (coated diameter of 0.0045 in., 300-k $\Omega$  imped-

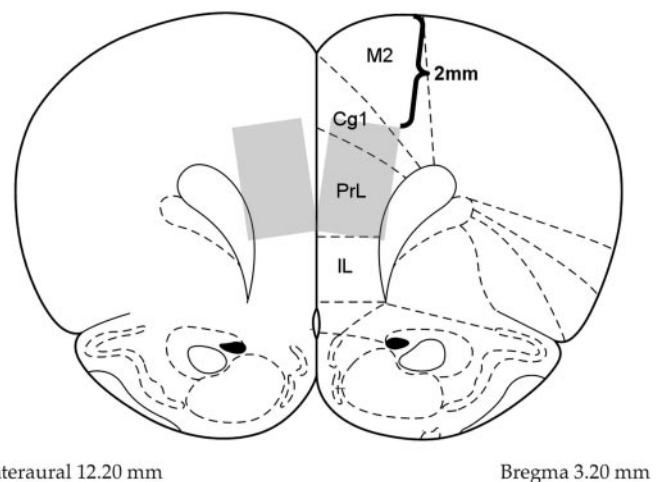


FIG. 1. Location of electrodes. Target for the recording array was the anterior cingulate (Cg1) and prelimbic (PrL) cortex. Shading indicates the region in which neural activity was recorded. In 2 animals, recording arrays were implanted at different times over both hemispheres. M2, secondary motor cortex; Cg1, anterior cingulate dorsal; PrL, prelimbic cortex; IL, infralimbic cortex. (Adapted from Paxinos and Watson 1998.)

ance; 316SS3T; twisted pair Teflon-coated stainless steel wire; Medwire, Mt. Vernon, NY) were lowered into the hippocampal craniotomy contralateral to the hyperdrive implant. The EEG electrode was lowered into the hippocampal fissure [2.7 mm dorsoventral (DV)]. Data from the hippocampal EEG were not analyzed for this study. An EMG electrode was also implanted into the neck muscle (coated diameter of 0.0045 in.; 316SS3T; Teflon-coated stainless steel wire; Medwire). The implant was cemented in place with dental acrylic anchored by dental screws. After surgery, rats were given ibuprofen (1 ml/1 kg of children's Motrin, McNeil PPC). They also received oral ampicillin (Bicillin, Wyeth Laboratories, Madison, NJ) on a 10-days-on/10-days-off regimen for the duration of the experiment. In two animals, after 3 mo of successful recording, the unused craniotomy was opened and implanted with a new hyperdrive.

### *Neurophysiological recordings*

Tetrodes were lowered after surgery into a region just above the anterior cingulate cortex ( $\sim 1.3$  mm DV), allowed to stabilize for 1 mo, and then gradually advanced. Analysis was restricted to cells located between 2 and 4 mm DV. The neutral reference electrode was located in the superficial layers of the cortex (0.5 mm from brain surface). The four channels of each tetrode were each attached to a separate channel of a 50-channel unity-gain headstage (Neuralynx, Tucson, AZ). A multiwire cable connected the headstage to digitally programmable amplifiers (Neuralynx). The spike signals were amplified by a factor of 1,000–5,000, band-pass-filtered between 600 Hz and 6 kHz, and transmitted to the Cheetah Data Acquisition system (Neuralynx). Signals were digitized at 30 kHz and events that reached a predetermined threshold were recorded for a duration of 1 ms. Spikes were sorted off-line on the basis of the amplitude and principal components from the four tetrode channels by means of a semiautomatic clustering algorithm (KlustaKwik, authored by K. D. Harris, Rutgers–Newark). The resulting classification was corrected and refined manually with custom-written software (MClust, authored by A. D. Redish, University of Minnesota; Waveform Cutter, authored by S. L. Cowen, University of Arizona), resulting in a spike-train time series for each of the well-isolated cells (0 to 16 cells per tetrode). No attempt was made to match cells from one daily session to the next and therefore the numbers of recorded cells reported do not take into account possible recordings from the same cells on consecutive days. EEG signals were band-pass filtered between 1 and 300 Hz and

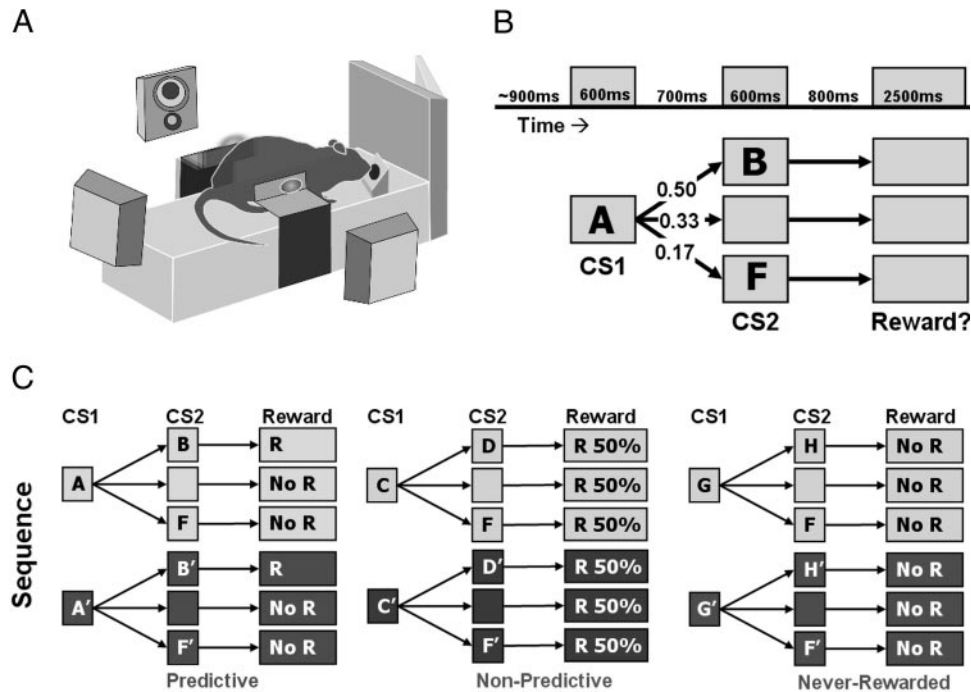


FIG. 2. Apparatus and stimulus and reward contingencies for the sequence-discrimination task. *A*: illustration of the experimental apparatus. Apparatus consisted of a narrow platform surrounded by 4 speakers. Two cell-phone vibration motors, mounted on flexible aluminum plates, rested on either side of the animal. Trials were initiated when the animal placed its nose in a nosepoke hole located to the front and right of the animal. An automatically controlled aluminum door was located in front of the animal. Liquefied rat mash food reward was delivered by an automatically controlled dipper located behind the door. *B*: time course of a trial and stimulus contingencies. Timeline at the top of the figure indicates the time course of events for a single trial. Trials began with a variable fixation period (~900 ms) followed by an auditory stimulus (CS1). If the animal maintained the nosepoke throughout the subsequent 700-ms delay interval, a second stimulus was presented according to the contingency relationship presented in the box and arrow diagram below the timeline. As the diagram indicates, the paired associate stimulus (B) was presented after the CS1 stimulus on 50% of trials, omitted (blank box) on 32.5% of trials, and a foil stimulus (F) was presented on 17.5% of trials. A second delay also followed the presentation of the CS2 stimulus. Delay was followed with reward according to the reinforcement contingency relationships presented in the bottom panel. Note that, in a given session, 80% of presented sequences were in the predictive and nonpredictive conditions and only 20% were in the never-rewarded condition. *C*: reinforcement contingencies. This panel summarizes the reinforcement contingencies for all 6 stimulus sequences used in the task. Two diagrams in the left column indicate the 2 unique sequences of stimuli in the predictive condition. In this condition, the presence of the paired associate CS2 (B or B') was always followed by reward and its absence was never followed by reward. In the nonpredictive condition (middle), the presence or absence of the paired CS2 (C or C') had no predictive value because the reward was delivered with a probability of 0.5 regardless of CS2 presentation. In the never-rewarded condition (right), reward never followed the presentation of either stimulus sequence.

sampled at 2.4 kHz. The EEG signals were amplified on the headstage with unity gain and then again with variable-gain amplifiers ( $\leq 5$  K).

*Apparatus*

All behavior was performed in a custom-designed operant chamber (see Fig. 2*A*). The chamber consisted of a narrow platform surrounded by four speakers. Two cell-phone vibration motors, mounted on flexible aluminum plates, rested on either side of the animal. Trials were initiated when the animal placed its nose in a nosepoke hole (2-cm diameter) located to the front and right of the animal. Nosepoke entry and withdrawal were identified by an infrared emitter-detector mounted within the hole. An automatically controlled aluminum door was located in front of the animal. Liquefied rat mash food reward was delivered by an automatically controlled dipper located behind the door. The apparatus was controlled by a microcontroller (BasicX-35, NetMedia) using custom software written in the BasicX programming language (BasicX, NetMedia).

The position of the animal was tracked from a video camera mounted 1 m above the operant chamber. Position was tracked automatically through video hardware and software that monitored the light from light-emitting diodes (LEDs) that were mounted on the headstage. The proximity of the camera to the headstage enabled precise tracking (~1-mm resolution). Tracking information was collected at 60 frames/s.

*Behavioral protocol*

To examine how reinforcement contingencies can influence associative plasticity, it was important to develop a task that required animals to distinguish among a variety of stimuli and reinforcement schedules. A diagram of the overall organization of the task and the various reinforcement and stimulus delivery schedules is presented in Fig. 2. In general, the task was a go/no-go discrimination task that required animals to distinguish between stimuli presented sequentially as the animals maintained their noses within the nosepoke hole (see Supplementary Table 1 for a summary of the specific stimuli used in each sequence).<sup>1</sup> Reward was delivered at the end of the stimulus sequence according to the schedule described in Fig. 2 (bottom). There were three experimental conditions in the task and two unique stimulus sequences were assigned to each condition so that stimulus selectivity might be assessed within condition. In the “predictive” condition, reward was always delivered if the second stimulus was presented and the animal’s nose remained within the nosepoke hole until the “go” signal. In the “nonpredictive” condition, reward was delivered randomly with respect to the delivery of the second stimulus. In the “never-rewarded” condition, reward was never delivered. Performance was measured as the percentage of trials in which the animal withdrew from the nosepoke hole (aborting the trial) during

<sup>1</sup> The online version of this article contains supplemental data.

conditions in which reinforcement was unlikely and remained within the nosepoke hole in conditions when reinforcement was likely.

Trials began with a variable delay interval (random between 500 and 1,000 ms), termed the "fixation" interval, during which the animal had to remain with its nose within the nosepoke hole ("nose fixation") to receive the first stimulus of the sequence (CS1). The CS1 stimuli consisted of six unique sounds (mixed frequency). These sounds were pulsed in triplets during the CS1 interval (133-ms pulse duration, 100-ms interpulse interval) and presented from the front speaker. The termination of CS1 was followed by a 700-ms delay interval (D1). This interval was followed by either the presentation of the paired second stimulus (CS2), the delivery of a foil stimulus, or the omission of any stimuli (see Fig. 2). The second stimulus was either a pulsating white noise sound presented from the left, right, or rear speaker or a pulsating vibration stimulus delivered from either the left, right, or simultaneously from both vibration motors that rested on either side of the animal (Supplementary Table 1). Stimulus duration was typically 600 ms, during which the stimulus was pulsed three times (133-ms pulse duration, 100-ms interpulse interval). The paired second stimulus was delivered on about 50% of trials and the foil stimulus was delivered on about 17% of trials according to a pseudorandom schedule. No second stimuli were presented on about 33% of trials. Foil stimuli were not unique to any particular stimulus sequence (i.e., they were not paired associates). The second stimulus interval was followed by a second delay interval (D2), which lasted 800 ms. This delay was followed by either the opening of the door that blocked entry into the reward chamber or by a 1.8-s pulsating tone (and no reward) that was delivered 1.2 s after the typical time the door would have opened, had the trial been a rewarded trial. A trial could be aborted at any time if the animal withdrew from the nosepoke hole. Withdrawal before the completion of a trial was not followed by any error signal.

As briefly discussed, the contingency between the delivery of reward and the presentation of the first (CS1) and second (CS2) stimuli was varied in three different conditions (predictive, nonpredictive, and never-rewarded). Reward was delivered in the predictive condition if the paired second stimulus was presented, but reward was omitted if the foil stimulus was delivered or if no stimuli were presented. In the nonpredictive condition, the omission or presentation of any stimuli during the CS2 interval did not influence the probability of reward delivery on a given trial ( $P = 0.5$ ). In the never-rewarded condition, animals were never rewarded regardless of the stimuli presented in the CS1 or CS2 intervals. Stimulus sequences from the predictive and nonpredictive conditions constituted 80% of all trials, whereas stimulus sequences from the never-rewarded condition constituted 20% of trials. Animals typically completed 90 trials in each predictive or nonpredictive stimulus sequence on a given behavioral session (~180 trials per condition). Sequences from the predictive or nonpredictive conditions were presented in blocks of eight trials. The order of the blocks during an experimental session was random. The use of blocks was originally implemented to facilitate training and the original design called for the elimination of blocked trials once behavior improved. From a practical perspective, however, it was observed that animals would not sample trials in the predictive and nonpredictive conditions equally without blocked trials. An analysis of neural activity and animal behavior during trials within each block is presented in Supplementary Fig. 5.

Animals were considered to have learned the task when they successfully performed the following three behaviors: First, they maintained nose fixation during the predictive sequences when the paired-associate CS2 was presented and withdrew from the hole during the presentation of the foil or during the CS2-omission trials. Second, they maintained nose fixation for the duration of the trial during the nonpredictive conditions. Third, they withdrew from the hole during the never-rewarded conditions.

### Pretraining

All animals used in this study required a minimum of 5 mo of daily training (~2 h/day, 6 day/wk) to reach criterion. Performance was evaluated after each training session by visually inspecting plots similar to the summary plot presented in Fig. 4. Criterion was reached when several conditions were met. First, animals were required to withdraw from the nosepoke hole during 70% of trials in the never-rewarded condition, but maintain nose fixation during 70% of trials in all other conditions. Second, animals were required to maintain nose fixation for the duration of 70% of nonpredictive trials if the paired CS2 stimulus was delivered but to withdraw during the majority (>70%) of trials if the CS2 stimulus was absent (foil or omission). Criterion was achieved only if animals could accurately perform the task with delay intervals (D1 and D2) of at least 700 ms. A total of five animals reached criterion; however, two animals were eliminated because one died from liver cancer and another was omitted because of problems with the microdrive implant. Performance at the time of recording was more accurate than the level set by the criteria described earlier, as a result of the extra training the animals received during the postsurgery recovery period (~1 mo).

Training proceeded according to the following stages. The early stages required animals to learn to discriminate between pairs of unique auditory stimuli while keeping their noses within the nosepoke hole. Success at this stage (~1 mo) was followed by the introduction of a second stimulus that followed the auditory cue. The contingency relationship between the second stimulus and reward delivery was also introduced at this stage. Delay intervals and foil stimuli were introduced only after animals could successfully discriminate between stimulus sequences in the predictive, nonpredictive, and never-rewarded conditions (~2 mo). Successful discrimination between the stimulus sequences resulted in the introduction of the foil stimuli and delay intervals. This final stage of training typically lasted 2 mo. Surgery was performed once the animals could discriminate between the CS2, omit, and foil stimuli in the predictive condition.

### Analysis

MEASURES OF SELECTIVITY TO HEAD POSITION AND STIMULUS SEQUENCE. The analysis of behavior revealed the presence of small but sequence-selective variations of head position (see RESULTS). As a result, it was important to determine whether sequence-selective delay activity was a result of the sensorimotor consequences of such postural shifts. To address this issue, two methods were adopted to assess the degree of sensorimotor contribution to selective delay activity. The first approach measured sensitivity to head position as the  $R^2$  value of the regression between trial-by-trial values of head position and firing rate during the first delay interval. Only correct trials were included in this analysis. This approach is described in more detail in RESULTS.

The second approach was developed under the assumption that if head position influences stimulus-specific delay activity, then the difference in firing-rate activity between stimulus sequences should be greatest during trials in which the head position was most divergent. This analysis was accomplished by separating trials into groups that depended on the degree to which head position overlapped or diverged between stimulus sequences within either the predictive or the nonpredictive conditions. The measure of selective delay activity was subsequently and independently calculated for each of these two groups.

This method was implemented by segmenting trials into two groups of equal size according to the position of the animal's head during the delay interval. The "diverge" group contained trials in which head position was to the outside of the median of the distributions of head position, whereas trials in the "overlap" group were those trials on the inside of the medians of the distributions (see Fig. 3 for an illustration). Segmentation was performed separately for the two sequences in either the predictive or the nonpredictive conditions. The strength

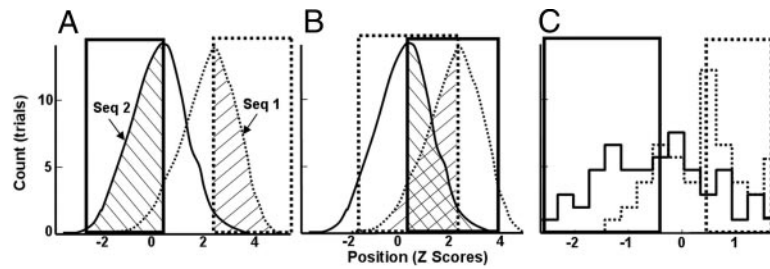


FIG. 3. *A* and *B*: schematic illustration (artificial data) of the method used for segmenting trials into 2 groups based on head position. Solid and dashed lines indicate the 2 different stimulus sequences in either the predictive or nonpredictive trial conditions. Trials in the “diverge” group were those trials in which the position was maximally divergent from other trials. Divergent trials were operationally defined as the outside halves (split by the median) of the distributions of position (dashed boxes in plot *A*), whereas the overlap group consisted of those trials on the inner halves of the 2 distributions (dashed boxes in plot *B*). Once trials were segmented, the firing rates during the delay intervals in the diverge and overlap groups were compared. *C*: example from one recording session (animal 7885) highlighting (boxes) the halves of the 2 distributions for the “diverge” condition.

and significance of delay activity within the diverge and overlap groups were compared by using a two-factor ANOVA with stimulus sequence being the first factor (“sequence 1,” “sequence 2”) and head position (“diverge” or “overlap”) being the second. Neurons were considered to be modulated by sensorimotor information if the effect of delay activity was significantly enhanced or reduced respectively as a function of membership to the diverge or overlap group. It should be

mentioned that the animal’s go/no-go choice performance during the trials in the two groups was identical. As a result, it can reasonably be assumed that a significant difference in delay activity between diverge and overlap groups was not a result of differences in factors such as recall or attention.

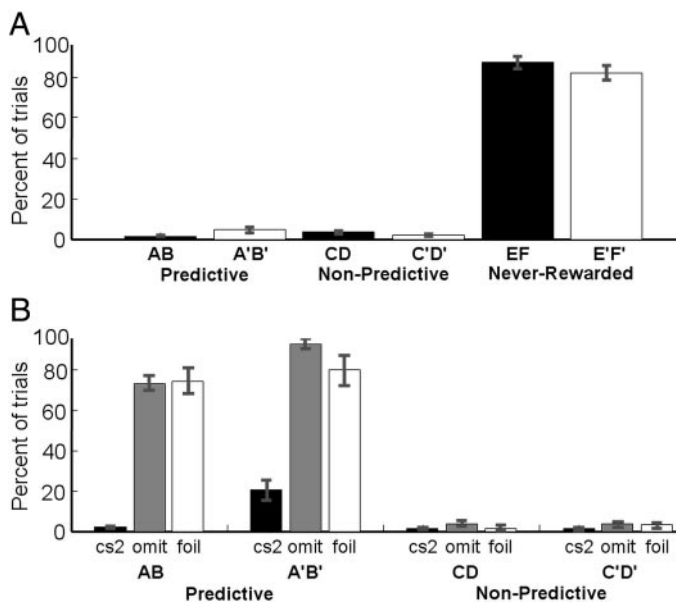


FIG. 4. Behavioral performance during recording sessions. Each bar in the figure presents the mean percentage of trials that were aborted (by withdrawing from the nosepoke hole) during various stimulus intervals. A withdrawal was counted in the CS1 interval if it occurred between 50 ms after CS1 onset and the onset of CS2. A withdrawal was counted in the CS2 interval if it occurred between 50 ms after CS2 onset and before the time when the door would open. Error bars indicate the SE ( $n = 90$  sessions). *A*: withdrawal during the CS1 interval. A separate bar is presented for each unique stimulus sequence. Animals remained in the hole for the majority of the predictive and nonpredictive trials. Animals most frequently withdrew from the hole during the never-rewarded sequences, indicating that they had learned to discriminate between the CS1 stimuli. Difference between the never-rewarded and the predictive and nonpredictive groups was significant ( $P < 0.001$ , ANOVA). *B*: withdrawal during the CS2 interval. During nonpredictive trials, animals remained in the hole during the CS2 interval regardless of the second stimulus; however, in the predictive condition, animals withdrew from the hole during the presentation of the foil stimulus or during omission trials. Difference between the CS2 and the omit and foil withdrawal probabilities was significant in the predictive condition but not in the nonpredictive condition ( $P < 0.001$ , ANOVA). Accurate choice behavior was consistent across sessions and animals (see Supplementary Fig. 2).

CLASSIFICATION OF NEURONS BY SELECTIVE DELAY ACTIVITY. A two-factor ANOVA was used to categorize cells into groups based on the sensitivity of the firing rate to the stimulus sequence (factor 1) or to head position (factor 2). Firing rate was determined by counting spikes on each trial within a 400-ms window during the delay interval (D1) that began 150 ms after the offset of the CS1 interval and ended 150 ms before the onset of the CS2 interval. The trial-by-trial firing rates were evaluated by ANOVA ( $P < 0.05$ ) and the results of that ANOVA were used to assign cells to one of the following four groups.

1) No effect of stimulus sequence (*Nonselective*). This group includes those cells that did not exhibit any effect of sequence; those that exhibited an effect of position, but not sequence (purely sensorimotor); and those that exhibited an interaction between sequence and position, but no effect of sequence. Because our analysis was focused on sequence-specific delay activity, these subgroups were not analyzed individually.

2) Effect of stimulus sequence, no effect of position, no interaction (*Sequence*). There was an effect of sequence, but no significant effect of head position (overlapping head positions vs. divergent head position).

3) Independent effects of stimulus sequence and position, no interaction (*Sequence/Position*). There was an effect of sequence and position, but there was no interaction between the two groups. In other words, significant differences in position did not appear to influence the strength of the sequence effect.

4) Effect of stimulus sequence and position and an interaction (*Interaction*). There was a significant interaction between sequence and position, suggesting that the strength of the sequence effect was influenced by head position or, alternatively, that the strength of the position effect was influenced by the sequence.

The *Sequence* and *Sequence/Position* groups could be considered to be the categories most often associated with intrinsically generated (e.g., memory-related) delay activity. In these categories, head position did not significantly influence the selectivity of the delay activity. Membership in the *Interaction* category, however, would suggest that position does influence selective delay activity for that cell.

MEASURES OF NEURAL SELECTIVITY TO STIMULI, SEQUENCE, AND POSITION. The degree of stimulus selectivity during the delay interval was quantified as the absolute value of the difference between the mean firing rate activity in the two within-condition sequences (e.g., predictive or nonpredictive). An advantage of this measure was its simplicity and ease of interpretation. A disadvantage was the fact that it does not take into account the variance of the two distributions and

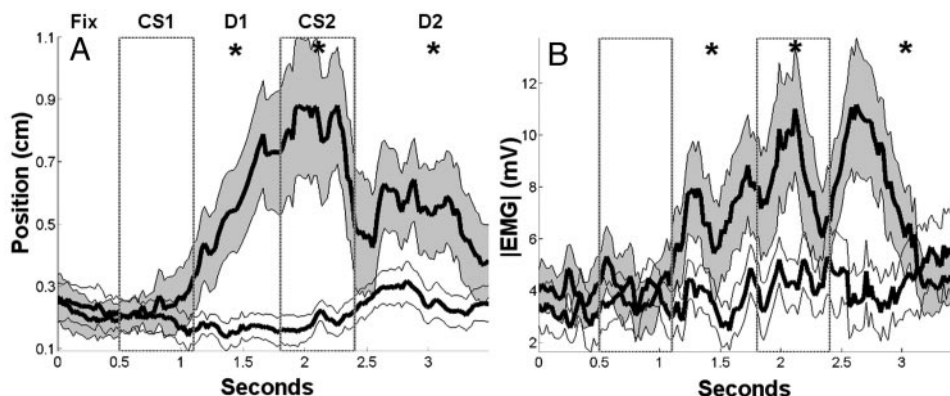


FIG. 5. Deviation in head position and EMG amplitude between sequences. *A*: head position varied as a function of sequence and condition (predictive or nonpredictive). During a given session, the mean head position during each of the sequences in the predictive and nonpredictive conditions was determined. For the 2 sequences within a given condition, the difference between these means was calculated. Absolute value of this difference was used as a measure of the between-sequence deviation in head position. These values were averaged across sessions ( $n = 90$ ) and the average response is presented; y-axis indicates the median deviation of head position (cm) between the 2 sequences within the predictive (gray error bars) or the nonpredictive (clear error bars) conditions. Error bars indicated the 95% notch confidence intervals (Chambers et al. 1983). No significant difference was observed during the fixation (Fix) or CS1 epochs ( $P < 0.36$ , paired sign test); however, the differences in the D1, CS2, and D2 epochs were significant ( $*P < 10^{-8}$ , paired sign test). Durations of the first (D1) and second (D2) delays were constant across animals; however, the duration of the second stimulus (CS2) was longer on 15% of recording sessions. For those data sets, only the first and last 300 ms of the CS2 interval were used. *B*: analysis that was identical to the analysis performed in *A* was applied to the EMG data ( $n = 80$ ). Results were quite similar, with significantly greater variation being observed during the D1, CS2, and D2 intervals ( $*P < 10^{-5}$ , paired sign test).

thus the degree of confidence that the difference was significant. For most analyses, this was not a major concern because neurons were screened for significant selective activity before the analysis of effect size.

Selectivity in the population of simultaneously recorded cells was measured using linear discriminant classification (classify.m in Matlab, The MathWorks). The classifier was trained to discriminate between the two within-condition sequences given the trial-by-cell matrix of spike counts (bin size = 400 ms) associated with a given stimulus epoch (e.g., CS1, D1, CS2, or D2). All population vectors (rows in the matrix) were normalized to have a vector length of one to emphasize relative change in firing activity in the population of cells and eliminate the influence of global changes in rate on the quality of the discrimination. Leave-one-out cross-validation (Bishop 1995) was used to determine a percentage-correct score of the classification. The degree to which these scores deviated from chance ( $P = 0.5$ ) was interpreted as the strength of selectivity. The significance of the difference was determined by evaluating the table of correct and incorrect classifications (the “confusion matrix”) with a chi-square test ( $P < 0.05$ ). Experimental sessions with fewer than ten simultaneously recorded cells (30% of sessions) were eliminated from the analysis. The degree of prospective and retrospective activity was identified through a similar procedure; however, in this procedure the classifier was trained on either the CS1 or CS2 intervals but tested on the population vectors associated with the delay (D1) interval. Significantly higher percentage-correct performance of the classifier trained on the CS1 interval relative to the CS2 interval was interpreted as suggesting retrospective coding, whereas enhanced performance for the CS2 classifier was interpreted as suggesting prospective coding.

## RESULTS

### Behavior during the sequence-discrimination task

Accuracy in the sequence-discrimination task was extremely high; average performance across all sessions and animals is summarized in Fig. 4. All animals reliably withdrew from the nosepoke hole during the presentation of the never-rewarded CS1 stimuli, but maintained nose fixation during all other CS1 stimuli. Furthermore, animals maintained nose fixation during

the presentation of the paired stimulus in the predictive condition but withdrew during foil and omission trials. In contrast, animals continued to nosepoke during omission, foil, and CS trials in the nonpredictive condition. This behavior was also clearly identifiable in the distribution of withdrawal latencies for each condition (Supplementary Fig. 1) and was consistent across sessions and animals (Supplementary Fig. 2). These results are in clear accord with the reinforcement contingencies adopted in this task (see METHODS, Fig. 2). The differential response to the foil stimuli in the predictive and nonpredictive conditions is particularly interesting. In the predictive condition, the foil stimulus was 100% predictive of the absence of future reward; however, in the nonpredictive condition, the same stimulus had no predictive value. The differential response to the identical foil stimuli in the two conditions suggests that the animals were able to discriminate between the different sequence types and conditions based in predictions developed during the presentation of CS1. Alternatively, the presence of the foil stimulus makes the task context driven in the sense that the identical stimulus had different meanings depending on the preceding stimulus.

In addition to evaluating performance accuracy, head movement and EMG activity were also analyzed. This analysis revealed that the animals expressed patterns of head movement and EMG activity that were associated with specific stimulus sequences. This observation was surprising given the restrictions placed on the physical movement of the animal (e.g., nosepoke-maintenance requirement, narrow platform). Analysis also revealed that these patterns of behavior were most selective for individual sequences in the predictive condition (Fig. 5), suggesting that, on average, the LEDs on the animal’s headstage deviated 1 cm ( $\sim 10^\circ$  of head rotation) between the two sequences.

### Selective responses to stimuli: effect of contingency

Evaluation of the perievent time histograms (PETHs) for individual neurons identified numerous clear examples of cells

with selective activity during the various stimulus and delay intervals as predicted by the hypothesis motivating this study. Almost all of the selective responses were observed during the two predictive sequences. Examples of individual neurons with selective responses are presented in Fig. 6.

Neurons with selective activity were identified as those neurons with significantly different distributions of firing rates (across trials) between the two sequences in either the predictive or the nonpredictive conditions (ANOVA,  $P < 0.05$ ). The number of cells with selective activity during each experimental epoch is presented in Fig. 7. Significantly more cells were selective during the predictive sequences during all experimental epochs ( $P < 0.05$ , chi-square test).

A similar pattern was observed following the evaluation of ensemble activity during each experimental session (Fig. 7C). Selectivity in the population was measured as the percentage-correct performance of a classifier trained to discriminate between the two sequences (see METHODS) given the population vectors of simultaneously recorded cells. Selectivity was considerably higher during all epochs in the predictive condition, although the degree of selectivity was significantly lower during the presentation of the CS1 stimulus ( $P < 0.05$ , paired ANOVA; factor: epoch; Tukey–Kramer test for multiple comparisons).

*Delay responses*

EFFECT OF CONTINGENCY. The majority of the analyses that follow evaluate the neural activity that occurred during the delay interval between the presentation of the CS1 and CS2 stimuli. It was initially hypothesized that strong, stimulus-selective, and prospective activity would be observed during this interval, in which both stimuli in the sequence contribute to the prediction of reward (predictive condition). Examples of

cells with selectivity to the stimulus sequence and to the reinforcement contingency relationship are presented in Fig. 6. In these examples, delay responses were selective to sequences in the predictive condition but not the nonpredictive condition. This pattern of response was also observed in the population.

The presence of selective delay activity in the population of recorded cells was evaluated by examining the binned, trial-by-trial firing rates during the various trial conditions. Activity was binned during a 400-ms window in the center of the 700-ms delay period (D1). Comparisons were made between activity during the two paired stimulus sequences, to produce a measure of selectivity. It should be noted that this measure of selectivity is agnostic with regard to whether the activity during the delay interval is prospective (CS2), retrospective (CS1), or some combination thereof. For this reason, selective activity is termed sequence-selective instead of stimulus-selective.

Of the 1,310 neurons included in this study, a total of 248 (19%) exhibited activity during the delay that was selective for specific sequences in the predictive condition. A total of 122 (9%) neurons were selective in the nonpredictive condition (Fig. 7A). The mean effect size, quantified as the absolute value of the difference in firing rate, was also largest in the predictive group [Fig. 7B, Predictive:  $1.4 \pm 0.25$  Hz (mean  $\pm$  SE), Nonpredictive:  $0.7 \pm 0.12$  Hz,  $P < 0.05$ , ANOVA]. Similar results were obtained following the analysis of the population of simultaneously recorded cells (Fig. 7C). The ensemble expressed significant selectivity for individual sequences during D1 and D2 and the degree of selectivity was notably stronger in the predictive condition [ $P < 0.05$ , paired ANOVA (factor: epoch) with Tukey–Kramer test for multiple comparisons]. Results from the analysis of single units and the population are in agreement with previous reports that suggest that delay activity in the prefrontal cortex is selective for task-relevant stimuli (Freedman et al. 2001; Rainer et al. 1998);

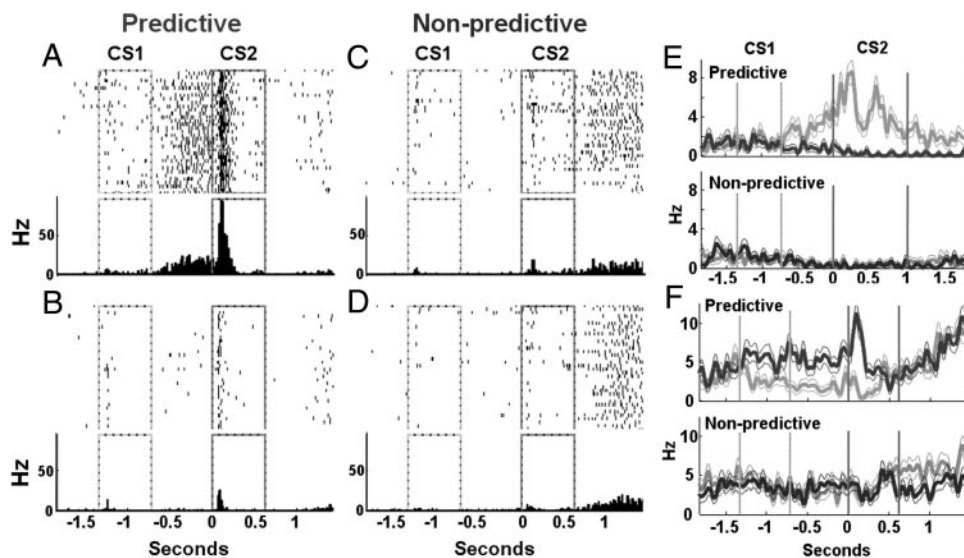


FIG. 6. Responses of neurons with selective delay activity. A–D: spike rastergrams and perievent time histograms (PETHs) for one neuron with selective delay activity. Rasters were aligned on the presentation of the second stimulus in each of the 4 unique stimulus sequences. Plots A and B present the rastergrams for trials associated with the 2 sequences in the predictive conditions and plots C and D present the responses to the 2 sequences in the nonpredictive condition. Onset and offset of the first and second stimuli are indicated by vertical lines. A clear selective response was observed during the first sequence in the predictive condition (plot A). E and F: average PETH responses for 2 cells with selective delay activity aligned on the time of presentation of the paired associate CS2 stimulus. Green lines in the top row of each panel indicate the average response during the 2 sequences in the predictive condition. Blue lines in the 2nd row of each panel indicate the average response in the nonpredictive condition. Lines indicate the mean and SE. Spike trains were smoothed with a 100-ms Hanning window before averaging.

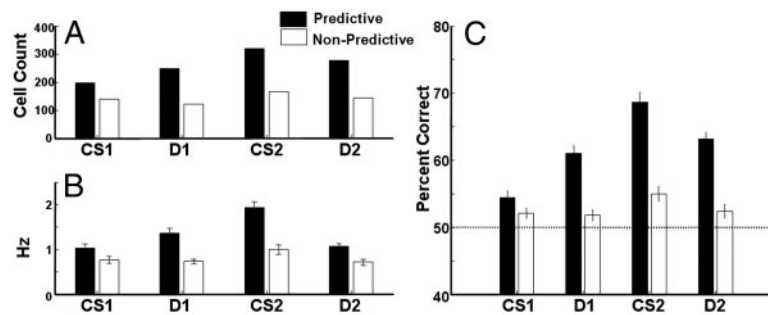


FIG. 7. Neural activity was most selective during delay intervals in the predictive condition. *A*: more neurons were identified with selective activity in the predictive condition during all stimulus-presentation and delay intervals; y-axis indicates the number of cells (out of a total of 1,301 neurons) identified as having selective responses to the 2 paired sequences in either the predictive (black) or nonpredictive (white) conditions. Differences in the counts in the predictive and nonpredictive counts were significant during all epochs (chi-square,  $P < 0.001$ ). *B*: effect size (means  $\pm$  SE) in the predictive (black) and nonpredictive (white) sequences; y-axis indicates the effect size, measured as the absolute difference between the delay activities (Hz) for the 2 paired sequences. Difference between the predictive and nonpredictive conditions was significant in the D1, CS2, and D2 epochs but not in the CS1 epoch ( $P < 0.05$ , ANOVA). *C*: selectivity of the ensemble of recorded cells during stimulus and delay intervals. Degree of selectivity was determined by evaluating the performance of a linear classifier trained on the population of simultaneously recorded neurons. Measure of discriminability was the percentage of correct classifications (means  $\pm$  SE, y-axis); x-axis presents the epoch. Difference between the percentage correct in the predictive and nonpredictive conditions was significantly lower during CS1 than during all other epochs [ $P < 0.05$ , paired ANOVA (factor: epoch) with Tukey–Kramer test for multiple comparisons].

however, as will be discussed, this activity was strongly influenced by sensorimotor information.

**PROSPECTIVE AND RETROSPECTIVE CODING.** To determine whether the activity during the delay intervals was prospective or retrospective, it was necessary to quantify the degree to which the activity was selective for either the previously presented stimulus or the anticipated stimulus. Simply comparing the delay activity to the activity in the CS1 or CS2 intervals within a given sequence did not address this issue because such a comparison does not account for the selectivity of the response relative to responses to stimuli in other sequences. To address this issue, linear discriminant classification was used to quantify the degree to which delay activity could discriminate between the two within-condition sequences. This analysis was performed separately for the CS1 and CS2 intervals (see METHODS). Higher percentage-correct performance of the classifier trained on the CS1 interval relative to the CS2 interval was interpreted as evidence for retrospective coding, whereas higher values for the CS2 classifier were interpreted as evidence for prospective coding. Results of this analysis are consistent with the presence of prospective coding (Fig. 8) because the percentage correct was significantly higher in the predictive condition ( $P < 0.001$ , paired  $t$ -test). This result was

consistent for both single units and for the population of recorded cells. No significant difference ( $P > 0.05$ , paired  $t$ -test) was observed during the nonpredictive condition. Put more concisely, the results suggest that in the predictive condition, but not in the nonpredictive condition, the delay activity is significantly more similar to the CS2 activity than to the CS1 activity.

**CONTRIBUTION OF SENSORIMOTOR INFORMATION.** Although sequence-selective delay activity was observed in 19% of neurons, it was unclear whether this activity was sustained by memory-driven processes that were intrinsic to the medial prefrontal cortex (or anywhere in the CNS) or whether the activity had an extrinsic source such as sensorimotor feedback. This was of particular concern given the observation of patterns of head movement that were specific to the stimulus sequences in the predictive condition (Fig. 5). This observation posed a serious problem for the interpretation and analysis of selective delay activity. Because position covaried with sequence, it becomes difficult to determine whether the observed sequence-selective delay activity was intrinsically sustained through memory-guided processes or whether it was driven by sensorimotor information. Two approaches were taken to address this issue. Results of these two approaches are described in the following sections.

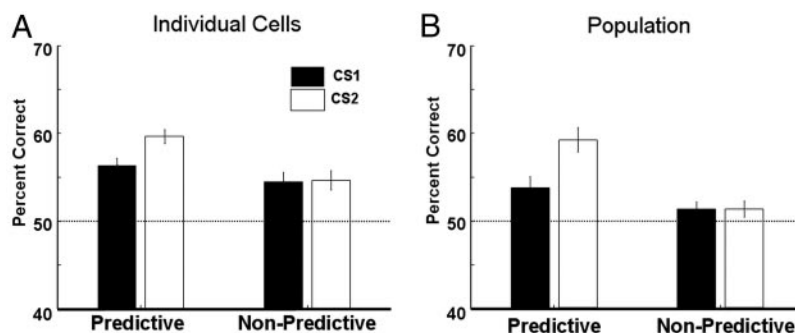


FIG. 8. “Prospective” activity during predictive sequences. Degree of prospective and retrospective activity in single units and in the population of simultaneously recorded cells was measured as the capacity of a classifier to discriminate between trials in the 2 paired sequences based on firing rate activity in either the CS1 or the CS2 intervals. Classifier was trained on trials in the CS1 or CS2 intervals but tested on activity during the delay interval (leave-one-out cross-validation). Performance of the classifier (percentage correct) is indicated on the y-axis. Dashed line indicates the level of performance expected by chance. Difference in performance between the CS1 and CS2 conditions was significantly different in the predictive condition for individual cells (plot A:  $P < 0.001$ , paired  $t$ -test) and for the population of simultaneously recorded cells (plot B:  $P < 0.001$ , paired  $t$ -test), whereas no significant difference ( $P > 0.05$ ) was observed in the nonpredictive condition.

*Sensitivity to position variation: regression of position and spike rate.* The essential difficulty with the previously described covariation between head position and sequence was the ambiguity surrounding whether neural activity was driven by memory-guided processes or by sensorimotor information. One strategy to address this issue is to restrict the analysis of neural activity and head position to trials associated with an individual stimulus sequence. This approach is advantageous because both the stimuli and the reinforcement contingencies are held constant within a stimulus sequence. As a result, this approach avoids any selective effect that may arise from either memory-guided activity or differences in motivation/expectation. It should be noted that this analysis presents a conservative lower-bound estimate of position sensitivity because it is restricted to a relatively small portion of the total head variation (variation within a sequence).

This analysis was accomplished by performing a multivariate regression on the trial-by-trial firing rate (dependent variable) as a function of position (independent variable). Head position was binned into four 200-ms bins during the 800 ms that preceded the onset of the CS2 stimulus (four variables for the regression). Significance of the full regression model and the  $R^2$  value of the regression were determined for each neuron and compared with values obtained from a shuffled control where the trial order of firing rates, but not position, was randomly resorted. The analysis was restricted to only those trials in which the second stimulus was presented and the animal maintained nose fixation for the duration of the trial (e.g., A → B → Reward; see Fig. 2).

Of the 248 neurons that exhibited sequence-selective delay activity, 105 (42%) expressed significant covariation with position during one of the two sequences ( $P < 0.05$ ). Only 11 (4%) neurons exhibited such covariation in the shuffled control condition. The degree of sensitivity of neurons to head position was quite high during all delay intervals, including the pre-stimulus fixation interval (Fig. 9A). Interestingly, the strength of position sensitivity was greatest during the second delay interval of the predictive condition (ANOVA,  $P < 0.05$ , Tukey–Kramer test for multiple comparisons) even though the average firing rates of neurons during the predictive and

nonpredictive conditions were not different ( $P > 0.05$ , ANOVA). In this condition, the animal was presumably certain about future reward delivery because the presence of the second stimulus always indicated that the reward would be delivered. This analysis was also repeated with the rectified EMG data. In contrast to head position, EMG activity was a poor predictor of trial-by-trial variations in firing rate (mean  $R^2 = 0.036$  during D1 for EMG compared with  $R^2 = 0.125$  for the position data).

To explore further the relationship between sensitivity to sensorimotor variation and delay activity, a regression was performed on the measure of position sensitivity (the  $R^2$  value of the within-sequence regression with position) and the measure of sequence selectivity during the various delay intervals (Fig. 9B). This regression was significant ( $P < 0.0001$ ,  $R^2 = 0.21$ ), further supporting the presence of a relationship between sensitivity to head-position variation and selective delay activity. In sum, cells exhibiting strong sequence-selective delay activity were likely to be highly sensitive to position.

The relationship between sequence selectivity and the measure of delay activity was further investigated by evaluating the activities of cells with or without a significant correlation with position ( $P < 0.05$  or  $P > 0.15$  for the full regression model between rate and position). The firing rates of cells in these two groups are presented in Fig. 10A. Position-modulated cells fired at significantly higher rates relative to position-insensitive cells ( $P < 0.001$ , ANOVA; factor: position sensitivity). The degree of selectivity, measured as the absolute value of the firing rate differences in the two sequences, was significantly larger in the position-sensitive group ( $P < 0.0001$ , ANOVA).

*Sensitivity to position variation: trial segmentation by position variability.* Results of the regression analysis described earlier suggest that delay activity was significantly affected by sensorimotor information. To confirm this, a second approach was applied that involved the segmentation of trials into groups according to head position and sequence. It was hypothesized that if delay activity was significantly influenced by head-position variation, the strength of delay activity should be weakest during trials in which head position overlapped the most between sequences and strongest when head position was

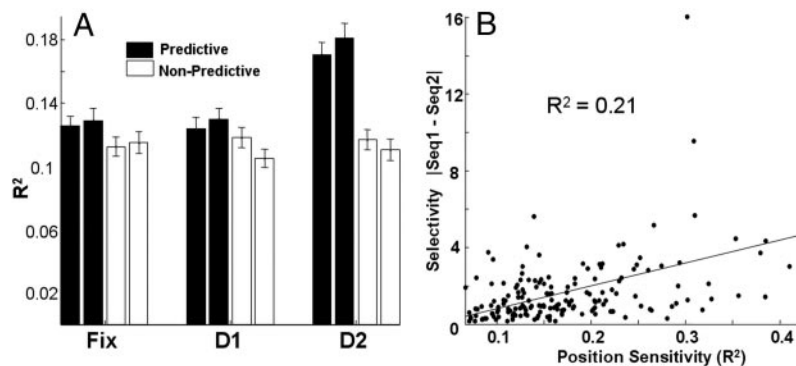


FIG. 9. Sensitivity to position and sequences. *A*: firing activity was correlated with variations in head position during delay intervals; y-axis indicates the explained variance ( $R^2$ ) between trial-by-trial variations in firing rate and head position after subtracting the  $R^2$  value obtained from the shuffled control. All values in the predictive and nonpredictive conditions were significantly greater than zero (ANOVA,  $P < 0.05$ , Tukey–Kramer test for multiple comparisons). Values were significantly larger in the predictive condition relative to the nonpredictive condition during the D2 intervals, but not during the other intervals (ANOVA,  $P < 0.05$ , Tukey–Kramer test for multiple comparisons). *B*: degree of sensitivity to head position ( $R^2$ ) was correlated with the degree of selectivity to sequences during the first delay interval. A regression was performed between the measure of sensitivity of each neuron to position ( $R^2$  of within-sequence regression to prediction) and the measure of selective delay activity (absolute difference in mean firing rates between sequences). Regression was significant ( $P < 0.0001$ ,  $R^2 = 0.21$ ), indicating that the independent measure of position sensitivity was correlated with the between-sequence measure of delay activity.

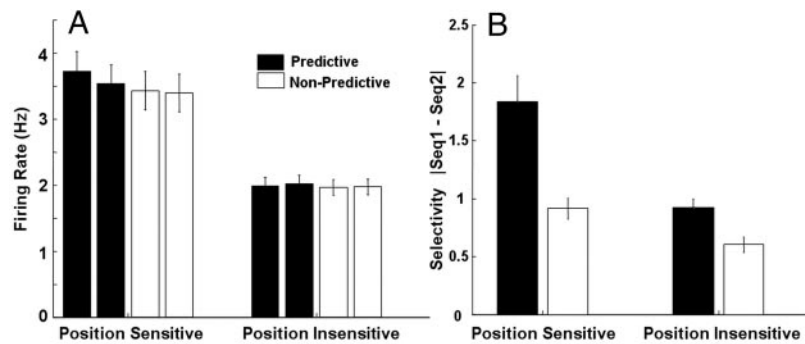


FIG. 10. Position-sensitive neurons had higher within-trial firing rates and were more selective during delay intervals. *A*: average firing rate of neurons during the first delay interval (D1) associated with each predictive and nonpredictive stimulus sequence. Neurons were divided into 2 groups: those that were sensitive to head position and those that were relatively insensitive to head position ( $P < 0.05$  or  $P > 0.15$  for the full regression model). Each bar presents the mean firing rate during the 4 stimulus sequences (black, predictive sequences; white, nonpredictive sequences). Only neurons with selective delay activity were considered. Position-sensitive neurons fired at higher rates relative to insensitive neurons ( $P < 0.001$ , ANOVA; factor: position sensitivity). No difference was observed between the firing rates of predictive and nonpredictive neurons. *B*: strength of the selective activity and position sensitivity. Selectivity was evaluated independently for neurons with selective delay activity during the predictive sequences (black) or nonpredictive sequences (white). In both conditions, the selectivity of neurons in the position-sensitive group was larger than the selectivity in the position-insensitive group ( $P < 0.001$ , ANOVA).

maximally divergent. Trials were therefore separated into “overlap” and “diverge” groups according to the distributions of head position, and sequence-selective delay activity was evaluated for each of these two groups (two-factor ANOVA,  $P < 0.05$ ; factors: sequence/head position; see METHODS). All analyses of delay activity were restricted to those trials in which the CS2 paired-associate stimulus was presented and the animal maintained nose fixation until the typical time of reinforcement delivery. Error trials (e.g., pulling out after CS2 presentation in the predictive condition) were not evaluated because animals typically committed fewer than five errors on

a given session. Therefore such an analysis would lack sufficient statistical power. Restricting the analysis to correct trials also minimized the potential contribution of errors of recall as a potential factor in variations in firing rate or head position.

For a given neuron, there are three potential outcomes of the analysis. First, selective delay activity may not be affected by segmenting trials into the overlap and diverge groups, suggesting that head position may not influence activity in the cell (e.g., Fig. 11A). Second, delay activity may be nearly abolished in the overlap group, suggesting that delay was almost entirely a function of sensorimotor information (e.g., Fig. 11B). Finally,

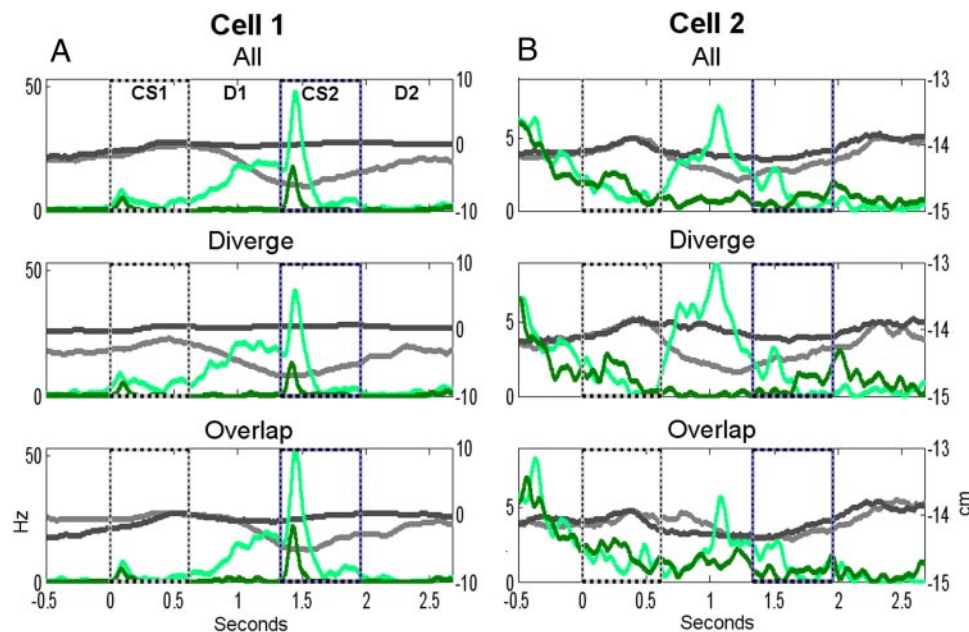


FIG. 11. Head position and selective delay activity. All plots in *A* and *B* indicate the average PETH responses of 2 neurons during the 2 sequences (light and dark green) in the predictive condition. PETHs present the average responses before segmentation (*top row*) and after segmenting trials into the “diverge” (position diverged the most between stimulus sequences) group and the “overlap” (position was most similar) groups. Dark and light green lines indicate the average firing rate response across trials (Hz), whereas black and gray lines indicate the median head position (cm) corresponding to each sequence (*right axis*). Delay activity for the cell in plot *A* was apparently not affected by the segmentation procedure. In contrast, the selective delay activity was almost entirely eliminated in the overlap condition for the cell in plot *B*, suggesting that delay activity was entirely modulated by position. It is important to note that even though the cell in plot *A* did not demonstrate an effect of the segmentation procedure, the explained variance between trial-by-trial changes in firing rate and head position (see METHODS) during the first sequence (light green) was very large ( $R^2 = 0.56$ ,  $P < 10^{-6}$ ), suggesting that the segmentation approach in this condition underestimates the potential contribution of head position to the firing activity of this neuron.

delay activity may become enhanced in the overlap condition, suggesting that large deviations in head position between the two sequences may interfere with sequence-selective activity.

Results of the trial-segmentation analysis over the population of selective cells are presented in Fig. 12. About 24% of selective cells demonstrated a significant ( $P < 0.05$ ) interaction effect between position and sequence. The strength of delay activity was significantly greater in the interaction group ( $P < 0.01$ , ANOVA), with the magnitude of the effect being more than twofold as large as the effect observed in any other group. This result suggests that the cells that expressed the strongest selective delay activity were also the cells that were the most sensitive to head position, a result that is in agreement with the results of the regression analysis presented in Fig. 9. This result is also contrary to the hypothesis that head position may interfere with the selective delay activity.

*Features of selective and nonselective cells*

**CELL TYPE.** The sensitivity of a cell to a stimulus sequence or to head position could be a function of the cell class (e.g., principal cell or interneuron). To examine this question, cells with selective delay activity were classified into putative interneurons (13% of cells) and into two classes of principal cells

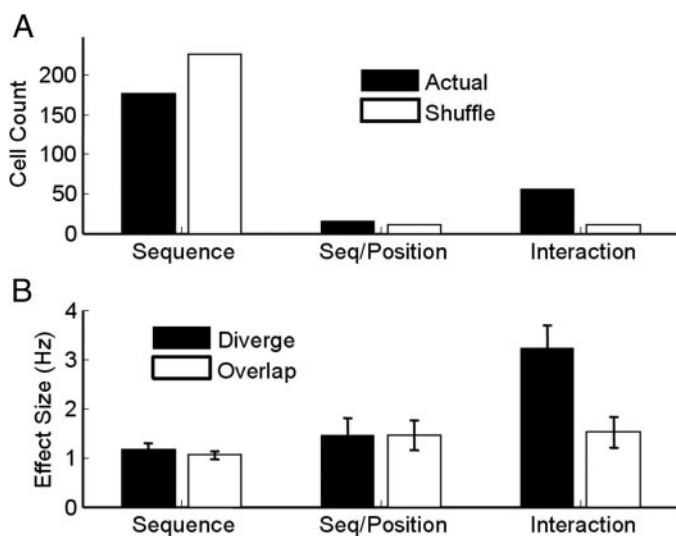


FIG. 12. Categories of selective cells. Cells that were sensitive to changes in position also expressed the strongest selectivity during the delay interval (D1). Trials were segmented according to the stimulus sequence and to the degree to which head position varied. Firing rate activity in these 2 conditions was analyzed (2-way ANOVA, factors: Sequence, Head-position; see METHODS). *A*, top row: number of cells in each delay-response category. Cells in the “Sequence” category (70% of selective cells) were cells with a significant effect of sequence but no effect of position and no interaction ( $P < 0.05$ ). “Seq/Position” cells (6% of selective cells) expressed a significant effect of sequence and position but with no interaction. “Interaction” cells (24% of selective cells) were those cells with an interaction between head position and sequence. Open bars indicate the cell counts that would be expected if position played no role in modulating neural activity (obtained by shuffling membership of trials in the position groups, but maintaining membership in the 2 sequence groups). These results indicate that about 25% of selective cells were clearly modulated by head position (Interaction). *B*: strength of the selective delay activity, measured as effect size (absolute difference in firing rate between the 2 sequences). Contrary to the hypothesis that head position may interfere with selective delay activity, the largest effect was observed in the interaction group, suggesting that cells with a sensitivity to position also exhibited the largest selective delay response ( $P < 0.01$ , ANOVA, with test for multiple comparisons).

[PK10 (32%), PK100 (55%)] by evaluating the waveform shape (Bartho et al. 2004) and the autocorrelogram (see Supplementary Materials and Supplementary Figs. 3 and 4). No difference in the degree of selectivity was identified between cell classes ( $P > 0.05$ , Kruskal–Wallis; Supplementary Fig. 4A). In addition, no cell type demonstrated a significant bias toward a particular sequence ( $P > 0.05$ , Kruskal–Wallis, Supplementary Fig. 4B). The PK10 neurons did exhibit a significantly lower degree of sensitivity to position ( $P < 0.05$ , Kruskal–Wallis); however, this effect may have been attributable to the notably lower baseline firing rate observed for the PK10 neurons (~1.0 Hz compared with ~4.0 and ~8.0 Hz for the PK100 and interneurons, respectively).

**DEPTH.** A growing body of evidence suggests that there is a high degree of functional segregation within the mPFC (Corbit and Balleine 2003; Gabbott et al. 2005; Haddon and Killcross 2005; Killcross and Coutureau 2003). As a result, it was important to investigate the relationship between the depth of the electrode and the degree of sequence selectivity and sensitivity to head position. Regression of the measure of sensitivity of cells to position ( $R^2$  of the regression of firing rate and head position) versus the depth of the electrode revealed a significant negative correlation ( $P < 0.01$ ). A significant negative correlation was also observed ( $P < 0.01$ ) for the regression of sequence selectivity (absolute value of the difference in firing rate) versus depth. These results indicate that both position sensitivity and selective delay activity decrease with increasing depth. Average values of position and sequence selectivity at various depths are presented in Fig. 13.

These results suggest that selective delay activity is strongest in the dorsal prelimbic and the anterior cingulate cortices and notably weaker in the ventral prelimbic cortex. This finding is consistent with the anatomy of the region because more dorsal regions of the prefrontal cortex are more strongly connected with the spinal cord and the dorsal striatum (Gabbott et al. 2005). It is also consistent with functional studies that suggest that ventral regions may be more involved in action selection and action sequencing (e.g., Ragozzino and Kesner 2001). It is conceivable that the effect of depth was influenced by the degree of training that the animal received in the task, in that depth did covary with the number of recording sessions (electrodes were typically lowered, not raised). This seems unlikely given the fact that the animals’ performance measures remained relatively constant through the course of the experiment. The animals were overtrained on this task, receiving at least 5 mo of pretraining and maintaining peak performance levels for  $\geq 1$  mo before recording.

DISCUSSION

The activities of multiple individual neurons in the mPFC were recorded as animals performed a complex sequence-discrimination task. The capacity of the stimuli in the sequences to predict reward was systematically varied. It was hypothesized that during training, associative connections among neurons that responded to the first or to the second stimulus would strengthen if both stimuli in the sequence contributed to the prediction of reward. This hypothesis was motivated by results from previous studies that suggest that neuromodulatory signals associated with neural plasticity and reward appear to be selectively activated during the presenta-

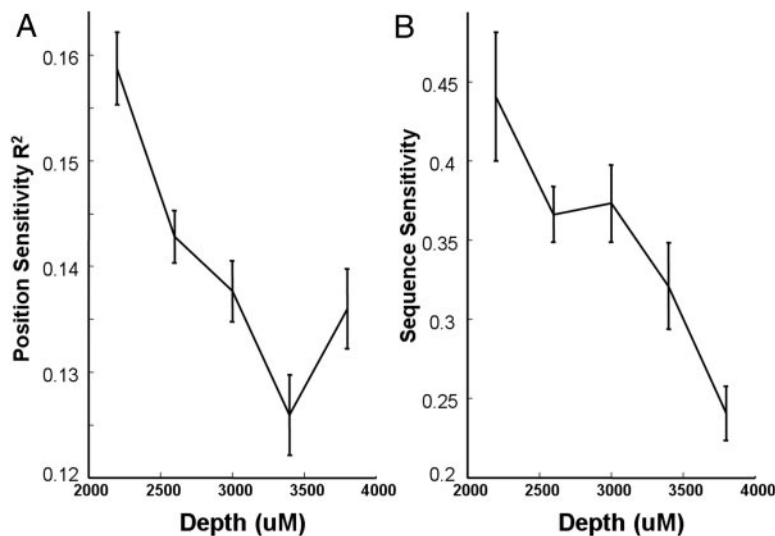


FIG. 13. Depth and sensitivity to position and stimulus sequence during delay intervals. Neurons with selective delay responses were segregated into groups according to depth (400- $\mu$ M increments). Average degree ( $\pm$ SE) of sensitivity of the neurons to variations in position ( $R^2$ , plot A) or sequence selectivity ( $|\text{seq1} - \text{seq2}|$ , plot B) is indicated on the y-axis. A significant negative correlation with increasing depth was observed in both conditions ( $P < 0.01$ ).

tion of cues that predict reward. Furthermore, it was hypothesized that a consequence of strengthened associative connections would be the activation of neurons that were responsive to the second stimulus immediately after presentation of the first stimulus (e.g., pattern completion) and that this activation would be observable during the delay interval as selective delay activity (Baeg et al. 2003; Batuev et al. 1990; Narayanan and Laubach 2006; Sakurai and Sugimoto 1986). In accord with these hypotheses, prospective delay activity was observed in 19% of neurons in the mPFC, with the majority of responding neurons being located in the dorsal region. In addition, this activity was strongest during sequences in which the first and second stimuli both contributed to the prediction of reward. Although these observations support the original hypotheses, a critical assumption was that the activities of neurons during delay intervals would be driven primarily by mnemonic processes intrinsic to the mPFC. Evaluation of the behavioral and neural data indicated that this assumption was not correct. Analysis of neural activity revealed that 42% of the selective neurons were significantly influenced by slight variations in head position. Furthermore, the head position of the animals varied as a function of the stimulus sequence, suggesting that the animals may not have used intrinsically sustained memory-guided activity to solve the task.

Although subsets of neurons in mPFC were extremely sensitive to head position, the degree to which this selectivity accounts for the reported selective delay responses is unclear. For example, it could be argued that the 42% of position-sensitive cells represents a unique subset of neurons that are tuned to sensorimotor parameters, whereas the remaining 58% are driven by the hypothesized stimulus-stimulus associations. It could also be argued that individual neurons in the mPFC combine extrinsic sensorimotor input with stored sensory-sensory associations. A number of factors argue against such interpretations and suggest that sensory-sensory associations may not be strongly stored in the mPFC and that sensitivity to sensorimotor variation may be the principal factor behind the reported selective delay response. First, the estimate of the contribution of sensorimotor activity most likely underestimates its true contribution. This seems likely because only two behavioral parameters (head position, EMG), were measured, ignoring the myriad other behavioral parameters (e.g., move-

ment of the hindquarters, vibrissae, tongue, etc.). Furthermore, the degree of sensitivity to position was significantly correlated with the strength of sequence-selective delay activity (Fig. 9B). In addition, in two separate analyses, the effect size in the group of cells that was modulated by position was twofold as large as the level observed in the unmodulated group (Figs. 10B and 12B), suggesting that the magnitude of selectivity was principally a function of head position. Finally, both selectivity during the delay intervals and position sensitivity covaried strongly with recording depth.

The conclusion that sensitivity to sensorimotor information is a major factor driving mPFC neurons is supported by the results of a number of recent studies. For instance, Euston and McNaughton (2006) observed strong spatial sequence-selective delay activity in the mPFC of the rat as the animal performed an open-field variant of the classic figure-eight delayed alternation test of working memory. After careful analysis of the animal's movement in the task, it was determined that the majority of this activity was a result of sensorimotor parameters related to slight variations in the animal's running path. The present findings extend these results to an experimental paradigm (go/no-go, "fixation," etc.) that shares much in common with traditional tests of delay activity. Also in accord with the results of the present study, Euston and McNaughton identified fewer neurons with selective delay activity or position sensitivity at deeper electrode locations. A study by Gemmell et al. (2002) also reported that neurons in the mPFC appear to be extremely sensitive to sensorimotor information. In that study, neural activity in the anterior cingulate and the dorsal prelimbic cortex was monitored as animals performed a foraging task. Although the authors observed activity that appeared to be related to allocentric spatial location, further analysis revealed that apparent spatial selectivity was principally a function of unique behaviors, such as grooming or rearing, performed at specific locations in the apparatus. Involvement of the mPFC in sensorimotor processing is further supported by the results of studies by Whishaw (1992), which suggest that the mPFC is involved in the control of skilled limb movement, and the results of stimulation studies of the dorsal mPFC by Hall (1974), which produce oculomotor responses.

An unexpected result from the present study was the observation that the sensitivity of neurons to position was greatest

during delay intervals in which the animal was presumably certain about the future delivery of reward (Fig. 9A). This observation is in accord with the results of other studies that report that neurons in the mPFC respond during the performance of actions related to future delivery of reward. For example, Chang et al. (1997, 2000) reported that neurons in the mPFC respond selectively to sequential behaviors that lead to the infusion of cocaine, Mulder et al. (2003) observed that delay activity in the mPFC was most sensitive to reward-related behaviors that were triggered by predictive cues, and results from Kargo et al. (2007) suggest that neurons in the far dorsal mPFC (precentral cortex) respond to action-reward contingencies. In the primate, neurons in the dlPFC respond to both anticipated actions and the rewards associated with those actions (Watanabe 1996). Similarly, neurons in the rostral cingulate motor area of the primate appear to respond to the selection of appropriate reward-related actions (Shima and Tanji 1998). These observations suggest that subregions of the PFC may link actions to rewarding outcomes. It is also possible that this function may extend to nonrewarding outcomes. For instance, previous studies in the primate have reported single-unit responses in the anterior cingulate region to errors (Gemba et al. 1986; Niki and Watanabe 1979). A detailed analysis of the error-related responses was not performed in the present experiment given the scarcity of error trials. As a result, future studies will be required to resolve whether neurons in the mPFC of the rat are primarily sensitive to behaviors related to rewarding outcomes or whether neurons in this region respond to actions related to both positive and negative outcomes.

Although neurons in the dorsal mPFC responded to slight changes in behavior, the utility of such behaviors is not entirely clear. It is possible, for instance, that these behaviors served no function. For instance, Skinner (1948) observed that pigeons naturally develop idiosyncratic and repetitive behaviors during delayed-reinforcement tasks, even when reinforcement rates and intervals are fixed and reinforcement is independent of the animal's actions. Alternatively, the observed postural adjustments may indicate an adaptive behavioral strategy, adopted to sense anticipated stimuli (e.g., lean to feel the vibration motor or tilt head to better hear an auditory stimulus). It is also possible that the animal solved the present task by using body posture as a mnemonic cue. For example, at the time of the presentation of the second CS, the animal could compare its current posture with the delivered stimulus and make a decision based on a few simple rules (e.g., leaning left + CS2 stay, leaning left + no CS2 withdraw, leaning left + Foil withdraw). Granted, such decision rules must also be learned and maintained through delay intervals; however, such behavior is likely the product of a gradual learning process acquired through the course of training (Mulder et al. 2003).

There are a number of advantages of using behavioral strategies to solve highly repetitive short-interval delay tasks. For instance, such strategies may be less prone to interference from external stimuli because, once initiated, changes in body position or running trajectory have a physical inertia that is difficult to disrupt aside from physically moving the animal. Furthermore, storing information in the "egocentric" reference frame of the body may be ideal for tasks that have an egocentric component such as delayed-alternation tasks that are often used as tests of working memory in the rat. The usefulness of embodied strategies is also illustrated by evidence that animals

use "mediating" behaviors to estimate the duration of delay intervals (Hodos et al. 1962; Wilson and Keller 1953). Furthermore, it has been suggested that animals can and do use the body and environment to link neural computations to an allocentric reference frame to support processes such as working memory (Ballard et al. 1997). Whether the mPFC is necessary for organizing such embodied strategies is an open question and resolving this issue will require future inactivation or lesion studies.

Embodied strategies are clearly not advantageous in all short-term delay tasks. For example, such strategies could easily be disrupted during tasks that utilize long timescales (e.g., >1 min) in which intervening behaviors (e.g., grooming) may disrupt any ongoing behavioral strategy. In such tasks, it may be far more advantageous for memories to be stored through active maintenance processes or through short-term synaptic plasticity; however, almost all published studies of delay activity involve intervals of <1.5 s and most short-duration (<10 s) memory tasks in the rat do not necessarily encourage nonbehavioral strategies (e.g., by disrupting stereotyped behaviors during the delay interval). It is therefore suggested that great care must be taken when interpreting delay responses that appear to be driven by intrinsic mnemonic processes, such as those proposed by the active maintenance hypothesis of prefrontal function.

In conclusion, the reported results suggest that quite subtle changes in head position can result in significant changes in the activity of medial prefrontal neurons. This activity was very similar to selective delay activity and could be easily misinterpreted as memory driven, even in tasks that impose restrictive controls on head and body position. Furthermore, responses were sensitive to the degree of expectancy regarding the future delivery of reward. These findings are in accord with the powerful inputs this region receives from the sensory system and its connection with the motor cortex, basal ganglia, and the VTA. It is also in accord with evidence that suggests the involvement of the anterior cingulate cortex in the organization of goal-relevant behavioral sequences. Finally, and from a technical perspective, the present results suggest that the separation of intrinsic and memory-guided processes from sensorimotor effects poses a significant challenge for the study of delay activity, especially if animals readily develop subtle idiosyncratic behaviors or adopt embodied strategies to solve highly repetitive short-term delay tasks.

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