

**Fig. 3.** Comparative expression levels of K3 in wild-type (BK64) (5) versus YSH44 (6) *P. pastoris*. Analysis of the reporter protein K3 by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (6).

This was further substantiated by the introduction of GnTII, which preempted the action of  $\alpha$ -1,2-mannosyltransferase and led to the formation of uniform GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub> structures (Fig. 2, D and E).

In the glycosylation process, multiple enzymes compete for the same transient glycan structures, which typically leads to heterogeneous mixtures of glycoforms. Here the fungal glycosylation pathway was reengineered to mirror the processing of human *N*-glycan structures. However, unlike the human pathway, which typically results in an array of glycoforms, this system yielded essentially homogeneous glycoforms.

To better understand the relation between *N*-glycosylation and protein function, generating uniform glycoproteins with specific *N*-glycan structures will prove to be of great utility (8). At present, the interpretation of these relations is compromised by the heterogeneity of the glycoform pools that can be generated from mammalian sources. These pools are typically created by expressing glycoproteins in specific glycosylation mutants or by *ex vivo* enrichment, such as lectin chromatography or enzymatic treatment. Moreover, even when a particular structure is identified, it is difficult to produce these structures at a commercial scale. Being able to engineer human glycosylation pathways into yeast strains able to express proteins with uniform *N*-glycan structures offers a more practical solution to this problem. When single proteins from a library of genetically engineered yeasts can be expressed and when each produces a defined and uniform glycoform, glycoprotein libraries can be generated to elucidate specific structure-function relations and to identify the most efficacious glycoform for a particular biological function. Once identified, a particular glycoform can be readily produced at industrial scale because of the rapid and well-established scale-up of yeast fermentations.

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Materials and Methods

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# Representation of Action Sequence Boundaries by Macaque Prefrontal Cortical Neurons

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Complex biological systems such as human language and the genetic code are characterized by explicit markers at the beginning and end of functional sequences. We report here that macaque prefrontal cortical neurons exhibit phasic peaks of spike activity that occur at the beginning and endpoint of sequential oculomotor saccade performance and have the properties of dynamic start- and end-state encoders accompanying responses to sequential actions. Sequence bounding may thus reflect a general mechanism for encoding biological information.

Speaking, hitting a fastball, and playing the flute are premier examples of behaviors requiring meticulous sequencing of actions. These are learned skills, but the ability to carry out sequences of movements is fundamental to most normal behaviors of humans and other animals (1). These sequential behaviors extend over time, so that temporal order as well as spatial order must be regulated. Many levels of the neuraxis have been implicated in the control of sequential behaviors (2–6). The prefrontal cortex, however, is a key region involved in the planning and automatization of the sequential behaviors that enable us to produce a smooth flow of actions or thoughts across time (7–11).

Implicit in the structure of such sequences is that they have a clear beginning and end, and explicit designations of start and end states have proven useful in models of sequential behavior (12). To test for such representations of start and end states, we recorded from multiple neurons in the prefrontal cortex and, for comparison, in the frontal eye field (FEF), as highly trained macaque monkeys performed up to seven sequential saccades (eye movements to redirect the line of sight) in blocks of trials (13) (Fig.

1A). Successive trials had a clear beginning (the illumination of a central red fixation point), a clear movement period (in which the monkey made saccades to successively presented red visual targets), and a final reward delivery period (in which the monkeys received juice or water for correct performance). In the standard task, different sequences were presented in pseudo-random order within trial blocks. We analyzed neuronal spike activity in relation to successive task events throughout the trials and during the intertrial intervals (13).

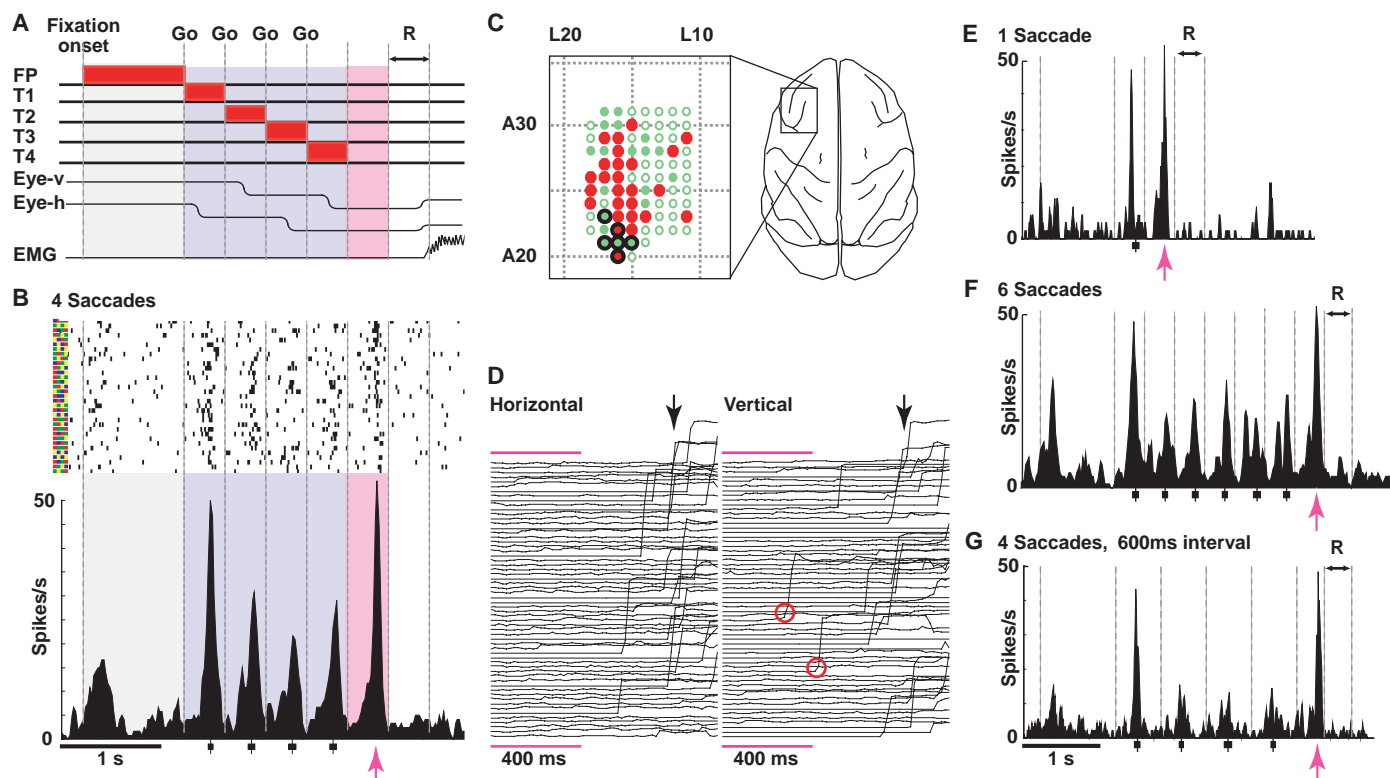
Neurons in the FEF responded selectively to saccades made in particular directions (14, 15), and some showed sequence selectivity. Many neurons in the prefrontal cortex exhibited similar properties, but in addition they responded to multiple phases of the task and often exhibited tonic firing during the entire trial or during the movement period (13, 16). Figure 1B illustrates typical prefrontal responses during the four-saccade task, with peaks of activity for each saccade during the movement period and a smaller peak after initiating fixation.

Nearly half of the task-related prefrontal neurons (295 of 658), but only rare FEF neurons (13 of 116), had an additional phasic peak in firing 270 to 280 ms after the sequence of saccades was completed. This “extra” peak occurred no matter how many saccades were made during the previous movement period (Fig. 1, B, E, and F) (fig. S1) (13). Even for a single saccade, there

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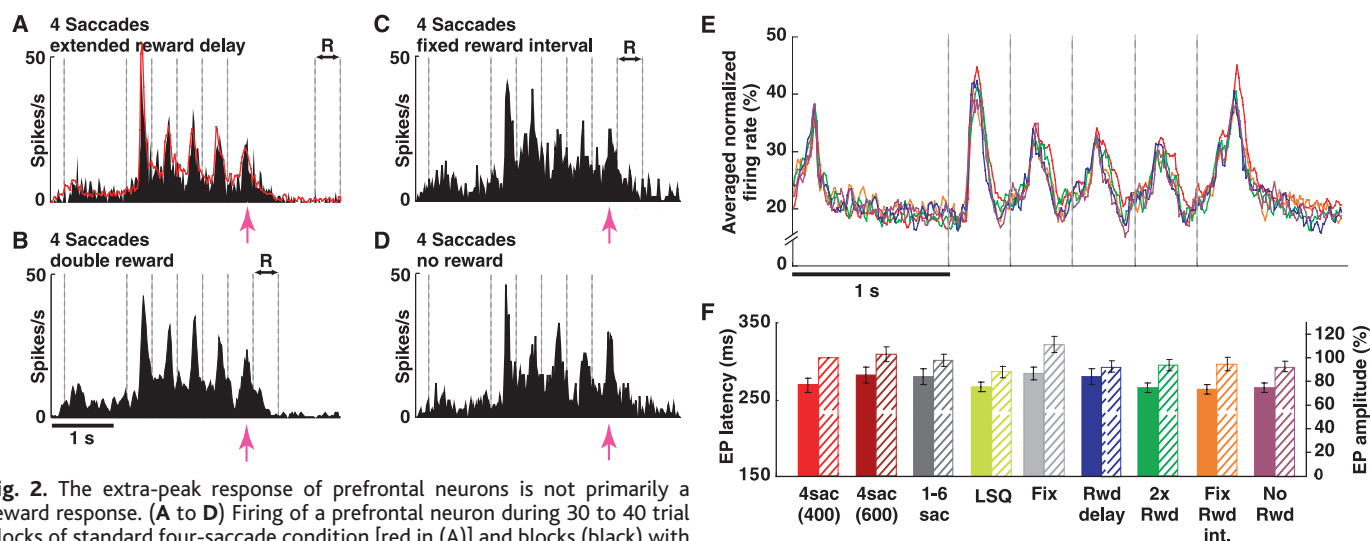
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**Fig. 1.** Prefrontal neurons exhibit enhanced firing at the start and end of sequential saccade performance. (A) Standard four-saccade task (73), with example of vertical and horizontal (Eye-v, Eye-h) eye position traces and digastric electromyographic (EMG) activity accompanying licking. R, 400-ms reward delivery window. (B) Dot raster and peristimulus time histogram (PSTH) of activity of a single prefrontal neuron during 35 trials of this task [events aligned with those in (A)]; saccade directions indicated to left (yellow, down; red, up; green, left; blue, right). Pink denotes 400-ms extra-peak window; pink arrow indicates extra peak. Mean saccade onset times (vertical ticks) and standard deviations (black boxes) are shown below x axis. (C)

Recording and stimulation map of monkey M7 (A, anterior; L, lateral). Black circles are sites at which microstimulation elicited saccades. Colored circles show tracks with extra-peak activity (solid red), with task-related but not extra-peak activity (solid green), or lacking task-related activity (open green). (D) Rectified eye position traces recorded during 60 consecutive trials of the four-saccade task [35 trials in (B)]. Pink bars mark 400-ms extra-peak window, during which saccades occurred only in two trials (red circles). Black arrow, 811 ms after last target off. (E to G) Responses of the same neuron shown in (B) for blocks of one (E) and six (F) saccade sequences, and for four-saccade task with 600-ms target intervals (G).



**Fig. 2.** The extra-peak response of prefrontal neurons is not primarily a reward response. (A to D) Firing of a prefrontal neuron during 30 to 40 trial blocks of standard four-saccade condition [red in (A)] and blocks (black) with reward (A) delayed by 1 s; (B) doubled; (C) increased by a factor of 5, but delivered in 1:5 fixed-interval schedule; and (D) absent. Pink arrows indicate extra peak. (E) Overlay of five averaged normalized PSTHs made from 84 prefrontal neurons recorded during tasks indicated in (A) to (D); colors as in (F). (F) Extra-peak response latency (solid bars) and amplitude relative to that in standard four-saccade task (hatched bars), with standard errors. From left to right: four-saccade tasks with 400- and 600-ms target intervals, randomly varied one- to six-saccade sequences, and repeating overlearned sequence (LSQ); fixation task; and reward manipulation tasks as in (A) to (D). The latencies and amplitudes did not vary significantly across tasks ( $P > 0.05$ , one-way analysis of variance with post hoc test).

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were two peaks of activity, not one (Fig. 1E). It was as though the extra-peak cells fired  $n + 1$  times for target sequences of length  $n$ .

To examine this  $n + 1$  activity at the end of the sequences, we examined a series of alternative hypotheses to account for its presence. We first tested the possibility that the extra peak was related to the forthcoming reward by manipulating reward parameters in trial blocks (Fig. 2). Neither postponing reward delivery by 1 s (Fig. 2A), nor doubling the amount of reward (Fig. 2B), nor quintupling the amount of reward for 20% of the trials (Fig. 2C), nor even omitting reward altogether (Fig. 2D) significantly altered the timing of the extra peak. Nor did these manipulations significantly alter the amplitude of the extra-peak response of most (~70%) of the prefrontal neurons (Fig. 2F). Behavioral performance remained at ~93% correct through these manipulations, as in the standard task. It is thus unlikely that the extra-peak firing was primarily related to reward, but the small changes in extra-peak timing (~4%) and amplitude (~7%) could be related to reward expectation (13, 17, 18).

A second alternative was that the monkeys made saccades at about the time of the extra peak to release fixation of the last target light. If so, the additional peak would not be an “extra” peak but a response related to these saccades. However, the monkeys only rarely made saccades during the extra-peak time window (Fig. 1D). The average latency of the release saccade after the last target presentation in the standard four-saccade task was  $811 \pm 322$  ms. This also suggested that the extra peak was not related to attentional release, because the monkeys continued to fixate throughout the extra-peak period. Nor was the extra peak time-locked to licking movements associated with the liquid reward delivery, which occurred on average  $811 \pm 287$  ms after the end of the last target presentation (last target off).

To test whether the extra peak reflected the rhythmicity or specific sequence configurations of the task, we introduced trial blocks with different target presentation intervals, and with randomized sequence lengths (one to six targets) and target durations (400 to 800 ms) (Fig. 2F). In these conditions, the extra peak still occurred ~270 to 280 ms after the last target off (Fig. 2F). The extra peak was also present ( $260 \pm 52$  ms) during blocks of “overlearned” sequences on which the monkeys had been trained for more than a year (LSQ, Fig. 2F), which suggests that the extra peak did not reflect expectation of a next target.

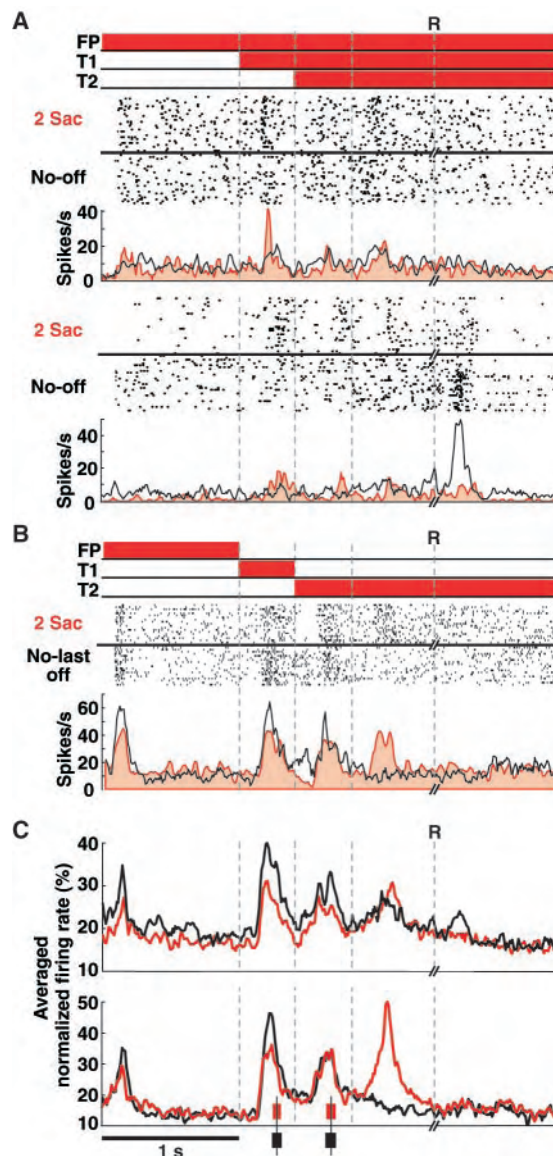
When we extended the duration of the last target light from 400 ms to 600 or 800 ms, the extra peak occurred with nearly the same ~270- to 280-ms latency after the last

target off (Figs. 1G and 2F). We therefore tested for an off response in the extra-peak neurons by having the monkeys perform a fixation task in which the behavioral sequence required was simply to acquire the fixation point and to fixate for 1 s. A large peak of activity occurred ~280 ms after the fixation point was extinguished (Fig. 2F) (13), which suggested that the extra peak could be a curious late-appearing visual off response (19, 20). Our next experiment, however, suggested that this is not so.

We introduced two conditions in which blocks of two-saccade sequences were used to test for the effects of turning off the target on the extra-peak response (Fig. 3). In one condition, there were no visual off signals at all (no-off, Fig. 3A); in the other, the last target was not extinguished (no-last-off, Fig. 3B). If the extra peak were a visual off response, the neurons should lack extra-peak responses in both conditions. Yet more than

half of the extra-peak cells tested in the no-off condition (55%, 62 of 113) and in the no-last-off condition (65%, 73 of 112) retained extra peaks (Fig. 3, A and C, top panels;  $P > 0.05$ , two-tailed  $t$  test). Thus, the extra peak cannot be a pure visual off response, at least for half or more of the extra-peak neurons. In both conditions, roughly 20% of the neurons now had a new peak that occurred at reward delivery (Fig. 3A, bottom; Fig. 3C, top). This new “reward” response is interesting, given that reward is the last event in the entire task. Eliminating the last target off also, in roughly 15% of the neurons, led to increases in the first response of the movement period.

These experiments demonstrate that a robust, phasic response occurs in the prefrontal cortex at the end of learned oculomotor task performance, and that this response is not a simple motor, visual, or reward response, nor is it sensitive to sequence length or pace. In



**Fig. 3.** The extra-peak response of prefrontal neurons is not a simple visual off response. Neural activity in prefrontal cortex was recorded during a standard two-saccade condition and in two-saccade conditions in which off signals were manipulated. **(A)** No-off two-saccade condition. Neither the fixation point (FP) nor the targets (T1, T2) were extinguished until the end of the 1500-ms intertrial interval. Plots are aligned from left on fixation onset and are realigned at R on reward delivery (800 to 1200 ms after T2 on). Data are shown for two prefrontal neurons during standard task (orange) and no-off task (black). Upper panel: A neuron that retained an extra peak in the no-off condition (~30 ms earlier) but lost an accentuated first saccade response. Lower panel: A neuron that lost an extra peak but gained a response after reward delivery. **(B)** No-last-off two-saccade condition. The neuron illustrated lost its extra-peak response when the last target (T2) was not turned off. **(C)** Averaged normalized PSTHs of 112 extra-peak cells recorded in the no-last-off task (black) and standard task (red), of which 73 retained (top) and 39 lost (bottom) their extra peak. Saccade onset times are as in Fig. 1 for the standard and no-last-off tasks.

all but the no-off/no-last-off saccade tasks, the end of the task was signified by the conjunction of last target off and no new target on. If the extra peak were a response to such an end signal, indicating that the sequential action was finished, it should be influenced by giving the monkeys an explicit signal that the sequence had ended. We tested this in monkey M7 by introducing conditions in which the last target was yellow instead of red (Fig. 4) (fig. S2).

This simple change had a striking effect. The extra peak decreased, and the response to the last (now yellow) target increased. The decreased extra-peak response occurred in 34% of the extra-peak neurons, and the increased response to the last target occurred in 43%. This response switch was not simply a novelty response, because it was present after more than 2 months of recording and did not habituate within trial blocks. Moreover, in 10% of the neurons, there was also an increased response to the first target of the task, and in 12% an increase at fixation onset (Fig. 4, bottom). Thus, changing the cue at task end led to changes in neural responses not only at task end, but also at the beginning of the task (13). Trick trials suggested that the yellow target did signify “task end” to the monkey: When suddenly either the second or third target was yellow, the monkey simply

stopped the task when the yellow target appeared in 70% of the trials of the first trick trial series (13 trials) and in 54% of the second trick trial series (37 trials), conducted 10 weeks later.

Our findings suggest that some neurons in the prefrontal cortex carry explicit signals that mark the completed performance of learned behaviors. The fact that we could change the extra-peak responses by changing physical end signals suggests that the extra-peak neurons may be true “end encoders” dynamically linking external physical cues to neural representations of end states. Most of these neurons showed differentially strong phasic responses at the start as well as at the end of sequential behavior, and, commonly, the start and end responses showed linked changes when the physical end signals were modified. This linkage could represent a neural correlate of action-sequence chunking, a segmentation of complex behavior into action subsets that, like memory segments, are called chunks (21–26).

Explicit end-state designators are coding features characteristic of complex biological sequences such as those in DNA and in human language (27, 28). In the brain, such designators could be part of a computational mechanism for representation of learned behaviors. By analogy to

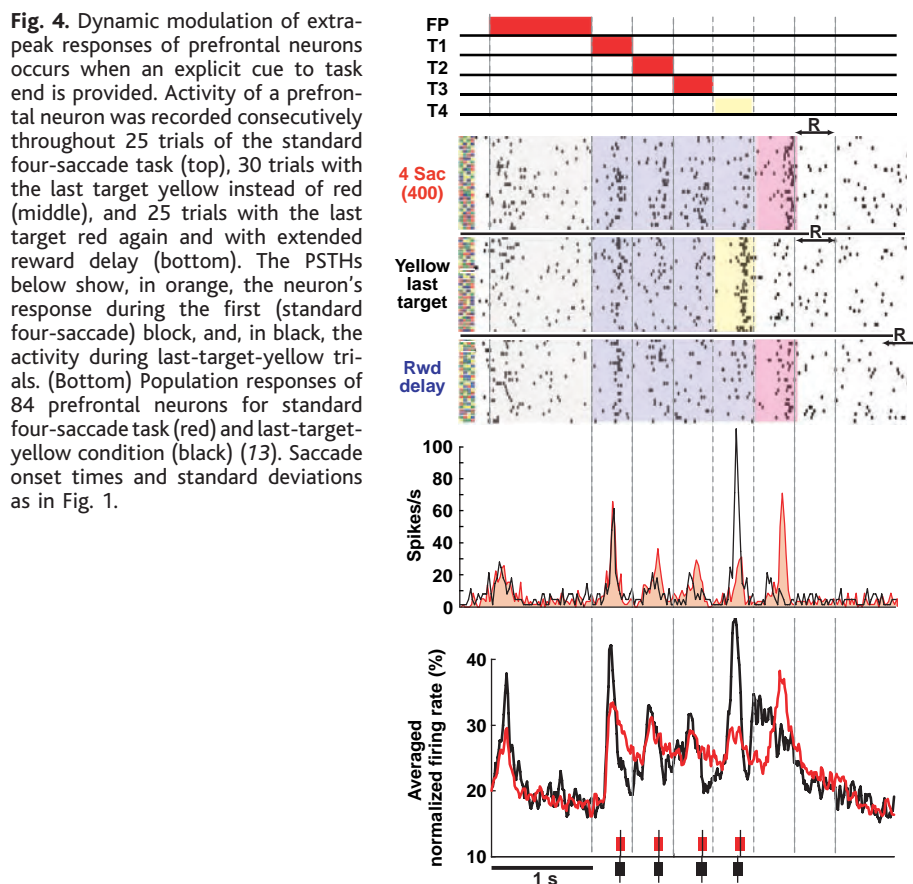
finite-state machine and hidden Markov models used to analyze genomic and linguistic sequences (4, 5, 29), such explicit designators could be used to reset prefrontal neurons and could be used by these and other brain networks in conjunction with state designators for sequence recognition, behavioral parsing, memory encoding, or creation of sparse codes for action motifs.

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**Fig. 4.** Dynamic modulation of extra-peak responses of prefrontal neurons occurs when an explicit cue to task end is provided. Activity of a prefrontal neuron was recorded consecutively throughout 25 trials of the standard four-saccade task (top), 30 trials with the last target yellow instead of red (middle), and 25 trials with the last target red again and with extended reward delay (bottom). The PSTHs below show, in orange, the neuron’s response during the first (standard four-saccade) block, and, in black, the activity during last-target-yellow trials. (Bottom) Population responses of 84 prefrontal neurons for standard four-saccade task (red) and last-target-yellow condition (black) (13). Saccade onset times and standard deviations as in Fig. 1.

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