

Stable sequential dynamics in prefrontal cortex represents subjective estimation of time

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eLife Assessment

This **useful** study reports how neuronal activity in the prefrontal cortex maps time intervals during which animals wait to reach a reward, with this mapping remaining consistent across days. While most claims are supported by **solid** evidence, the study could have benefitted from an improved experimental design to more clearly disambiguate correlations between neuronal patterns and not only time but also stereotypical behaviors and restraint from impulsive decisions. This study will be of particular interest to neuroscientists focused on decision-making and motor control.

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Abstract

Time estimation is an essential prerequisite underlying various cognitive functions. Previous studies identified “sequential firing” and “activity ramps” as the primary neuron activity patterns in the medial frontal cortex (mPFC) that could convey information regarding time. However, the relationship between these patterns and the timing behavior has not been fully understood. In this study, we utilized *in vivo* calcium imaging of mPFC in rats performing a timing task. We observed cells that showed selective activation at trial start, end, or during the timing interval. By aligning long-term time-lapse datasets, we discovered that sequential patterns of time coding were stable over weeks, while cells coding for trial start or end showed constant dynamism. Furthermore, with a novel behavior design that allowed the animal to determine individual trial interval, we were able to demonstrate that real-time adjustment in the sequence procession speed closely tracked the trial-to-trial interval variations. And errors in the rats’ timing behavior can be primarily attributed to the premature ending of the time sequence. Together, our data suggest that sequential activity maybe a stable neural substrate that represents time under physiological

conditions. Furthermore, our results imply the existence of a unique cell type in the mPFC that participates in the time-related sequences. Future characterization of this cell type could provide important insights in the neural mechanism of timing and related cognitive functions.

Introduction

Time estimation is an essential function in the brain^{1–3}, since many crucial cognitive functions implicitly require a record of time, such as motor control^{4,5} or memory^{6,7}. The neural substrate for time estimation in the brain has been studied for decades, and several modes of time coding emerged from these studies: 1) Time can be represented by the gradual change of activity levels in certain cells (ramping)^{8–10}; 2) Individual cells show selective activation at a specific time point (sequential)^{11–15}; and 3) Population coding that showed complex patterns but stable dynamics in latent space^{16–18}. Interestingly, by training the animal to learn different lengths of waiting periods, several groups found that these “time codes” exhibit scaling properties so that the number of cells for coding different lengths of time remains constant, while the activity can be compressed or stretched according to the duration of target time^{11,19–21}. While these findings provide strong evidence for a neural mechanism of time coding in the brain, true causal evidence at single-cell resolution remains beyond reach due to technical limitations. Although inhibiting certain brain regions (such as medial prefrontal cortex, mPFC,²²) led to disruption in the performance of the timing task, it is difficult to attribute the effect specifically to the ramping or sequential activity patterns seen in those regions as other processes may be involved.

Lacking direct experimental evidence, one potential way of testing the causal involvement of “time codes” in time estimation function is to examine their correlation at a finer resolution. However, a limitation in the experimental protocols of previous studies is that the animal learns a fixed time target, so that the scaling phenomenon is observed at the group level. Thus, there is a lack of evidence regarding whether the scaling happens rapidly at single-trial level to support time estimation.

In this study, we utilized a novel timing task in rat that allowed the animal to control the waiting period on its own will, so that we can observe the correlation of “time code” scaling with behavioral waiting responses at individual trial resolution. We found robust sequential activities in the mPFC when the rats performed the task. To the best of our knowledge, we provide the first piece of evidence that the scaling effect is dynamic to account for the variation of waiting periods at individual trial level. And the rats were capable of subjectively adjusting the scaling factor for accurate time estimation. Intriguingly, we found that cells coding for the start or end of the waiting period undergo cross-session shifts, while the sequential time code remains stable over weeks. This surprising stability of the time code suggests an underlying mechanism that is different from apparently similar sequential activities seen in place coding or time coding in hippocampus CA1^{23,24}, which showed dynamic shifts across sessions. Altogether, our study provides strong evidence for the online utilization of sequential time code in rats mPFC during a timing task. The unique rapid scaling and cell-identity stability of these sequential time code suggest a designated cell population for coding time in this region.

Results

Calcium imaging in mPFC during rats perform the timing task

We trained eight rats to perform a modified version of the timing task used in previous studies²¹. In order to get the water rewards, water-deprived rats must poke their nose in a designated hole and maintain position (**Figure 1A**). The rat was free to start and end the nose poke at its own will, but only when the duration of the nose poke was above the minimal threshold would the rat receive a water reward. The rat did not receive additional punishment for nose poke duration below the minimal threshold, and can start a new trial when it was ready. Importantly, once the nose poke duration was over the minimum threshold, the amount of water reward was proportional to the total length of the nose poke duration. We trained the rat to perform this task in two phases. After the initial shaping for rats to associate nose poke with water rewards, the rats were trained on a short duration phase in which the minimal threshold was set to be 300 ms. When the rats' correct performance reached above 70%, they advanced into a long duration phase and the threshold was set to be 1500 ms (**Figure 1A**).

Rats that underwent the training paradigm described above showed gradual increase in their nose poke durations (**Figure 1B**). And the median durations in each phase were slightly above the respective threshold in that phase (**Figure 1C**), indicating that the rats were capable of learning the different minimal threshold. Notably, there was a wide distribution of the nose poking durations in both phases of the task, which allowed us to study the trial-to-trial variation of the timing behavior.

We recorded neuronal activities from 4 different sessions from 5 rats (1 from short phase and 3 from long phase, **Figure 1D**). To obtain neuronal activity, we injected adeno-associated viruses expressing calcium sensor GCaMP6S in the medial prefrontal cortex (mPFC) of the rat and installed a 1.8 mm diameter GRIN lens after aspirating the cortical tissue on top (**Figure 1E**). Using a miniaturized microscope, we were able to record calcium activities from pyramidal neurons in the mPFC (**Figure 1F**), while the rats can freely move and perform the timing task. Our preparation yielded stable imaging data with good signal-to-noise ratio and cell counts (ranging from 50 to 150 cells per session, **Supp. Figure 1**).

Scaling of the mPFC time sequences at single-trial resolution

We started our analysis by examining the neural activities correlated with different features of the nose-poking behavior. To this end, we aligned calcium traces by nose-poking events for each cell, and calculated the Pearson's correlation between the averaged activity trace to the binarized event trace (start, during and end). To determine the statistical significance of the correlation, we performed random shuffles of the calcium traces and repeated the calculations above, generating a distribution of correlation coefficients for each cell. If the actual correlation coefficient reached beyond 95% confidence interval of this distribution, we then assigned this cell to the group that codes for the specific feature of the nose-poking event. With this method, we identified neurons that showed selective activation at the beginning, the middle, or the end of the nose poke events (**Figure 2A**), which we designated as "start cell", "duration cell" and "end cell", respectively. A small portion of these coding cells (~10%) showed significant correlation between two features of the task ("start" and "duration"; "end" and "duration"). In later analysis, we excluded these cells from the duration cell group. Notably, we observed gradual decays of GCaMP signal in start cells and sometimes gradual rise in end cells. However, we cannot definitively separate these "ramping-like" activities from the potential artifact due to slow kinetics of calcium sensor²⁵. These three types of cells constituted more than half of the observed neurons (**Figure 2B**). By training a support-vector machine classifier using activities from the start cells and end cells, we were able to predict nose-poking events with above 90% accuracy, indicating that their activities were highly specific to the nose-poking events (**Figure 2C, D**).

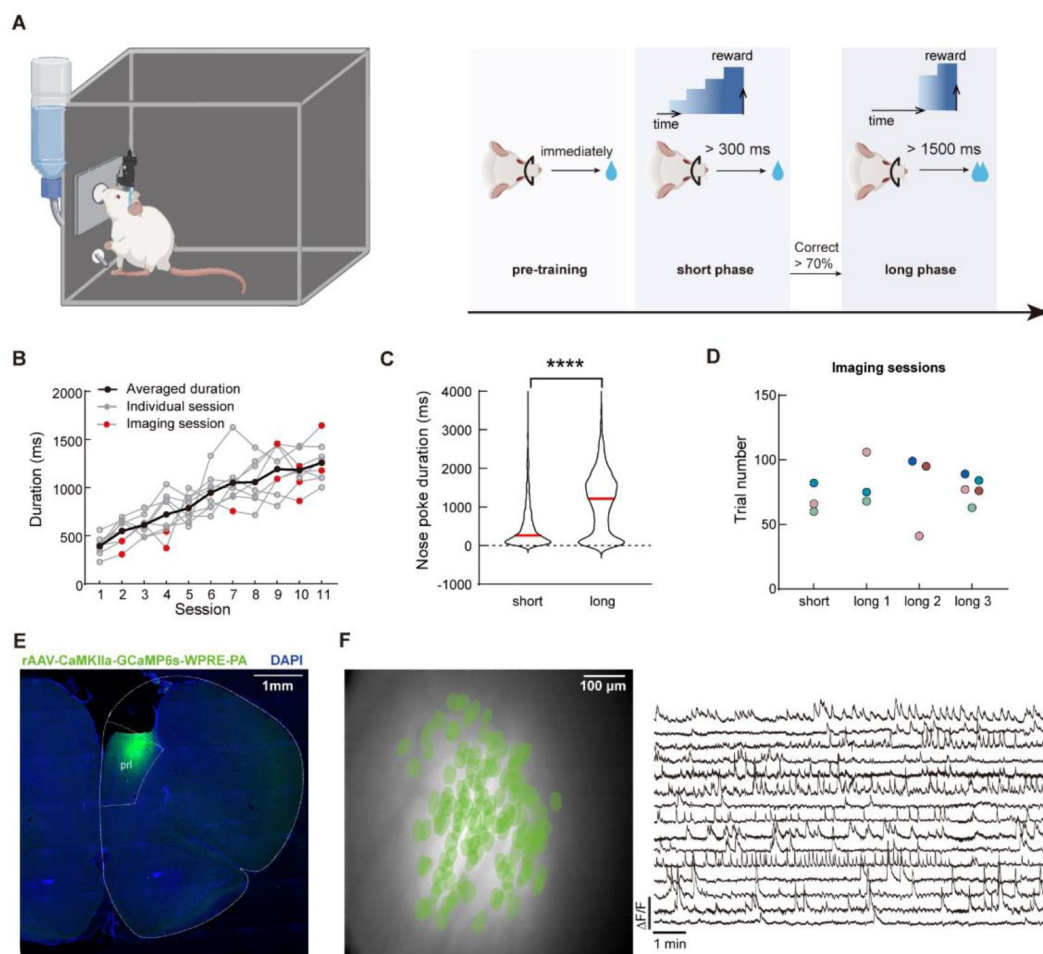


Figure 1.

Calcium imaging during the timing task in rat mPFC.

- A.** Schematics of the timing tasks. Rats undergo behavior training in three phases. Pre-training phase: rat received water reward immediately after nose poking action. Short phase: rat had to maintain nose poking position for at least 300 ms to receive reward. And long phase: rat had to maintain nose poking position for at least 1500 ms to receive reward. Additional water reward would be provided for every additional 500 ms of holding the nose poke.
- B.** Quantification of the rats' average nose poking durations in each session. Gray lines indicate each rat's performance, and the black line represents the averaged performance. Red dots indicate sessions with calcium imaging.
- C.** Violin plot for the nose poking duration in two phases of the task. Red lines indicate the median value in each group. Two-tailed t test, ****: $p < 0.0001$, $n = 5$ rats.
- D.** Sessions with calcium imaging data included in this study. The same color of dots indicated data from the same rat.
- E.** Representative histological section of mPFC from an imaged rat. The white dotted line indicates borders of brain structures.
- F.** Representative imaging field of view (left panel) with green masks of extracted cells' positions and example GCaMP fluorescent traces (right panel).

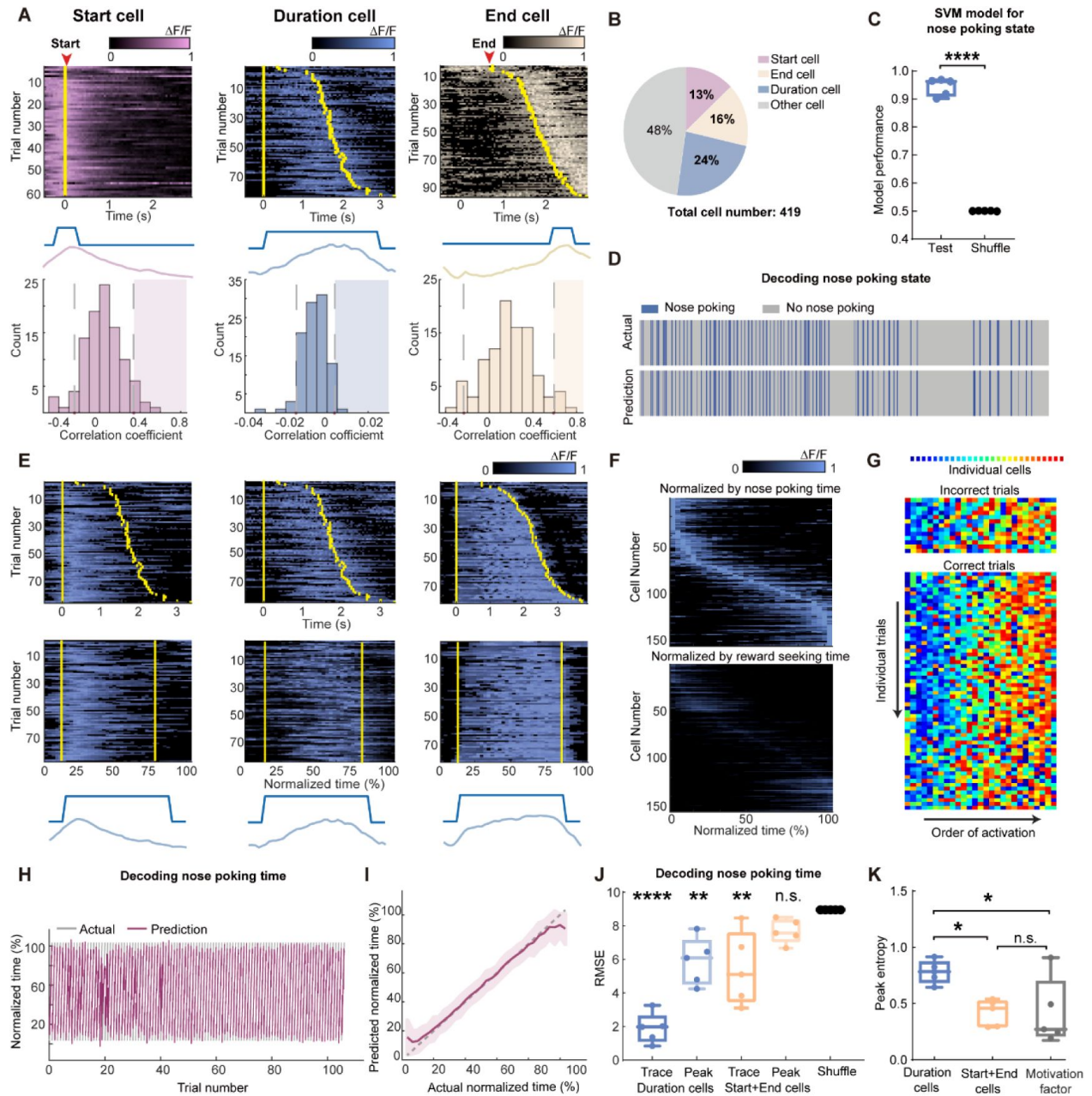


Figure 2.

Scaling of the mPFC sequential activity by duration at individual trial resolution.

A. The definition and examples of start (pink), duration (blue) and end (beige) -coding cells. To define a cell's coding identity, we calculated the correlation between its activity and the specific event (middle diagrams), and compared to the null distribution generated from shuffled data from the same cell (bottom graphs). Statistical significance was determined if the actual correlation fell into the right 95th percentiles of the null distribution (shaded areas). Top panels show calcium activities from representative coding cells. Trials were sorted in ascending total durations. Yellow bars indicate the start and end of the nose poking event.

B. The proportion of cell coding types in the observed neurons from 5 rats in the long3 session.

C. Quantification of SVM classifiers in predicting the nose-poking state using neuron activities from the start and end cells, from the long3 session. Two-tailed t test, ****: $p < 0.0001$, $n = 5$ rats.

D. Representative actual nose poking events and predicted events using SVM models.

E. Three examples of duration cell with their raw traces (top panels) and traces in normalized time (middle panels). Yellow bars indicate the start and end of the nose poking event. Bottom panels show nose poking periods and averaged traces in normalized time scale.

F. Traces of averaged normalized activity of all recorded duration cells in 5 rats, sorted by the ascending peak position in the normalized time (upper panel) or the water reward-seeking time (lower panel).

G. Representative rainbow plot of the activity peak position ranks from duration cells in one session. Each color indicates an individual duration cell and the x-axis is sorted by the order of each cell's peak activity appearance on individual trials.

H-I. Representative results of predicted trial progression and actual trial progression by a GPR model fitted from duration cells activity in one session. Results are plotted in individual trials (**H**) and averaged trial (**I**). Results are represented as mean $\pm$ S.D.

J. Quantification of the GPR model performance. Each dot indicates averaged RMSE from one rat. Models were trained using duration cells or start and end cells activities, with raw calcium traces or extracted peaks. Data are represented as medium $\pm$ S.D. One-way ANOVA test with Dunnett's multiple comparisons post-hoc test, ****: $p < 0.0001$, **: $p < 0.01$, n.s.: not significant, $n = 5$ rats.

K. Quantification of the peak entropy on the trial-average calcium traces as a measure of sequentiality. The motivation factor group indicates duration cells' activities normalized with the water reward-seeking time. One-way ANOVA test with Dunnett's multiple comparisons post-hoc test, *: $p < 0.05$, n.s.: not significant, $n = 5$ rats.

We then examined the activity patterns of the duration cells. We found that many duration cells showed activation during a certain proportion of the nose-poking events, and the timing of the activation seemed to be modulated by the total length of the events (**Figure 2E** [↗](#), **Supp. Figure 1A** [↗](#)). When we normalized the actual time to the total length of each nose-poking event and transformed the calcium traces accordingly, we found that many duration cells showed selective activation at a fixed point in the normalized time scale (**Figure 2E** [↗](#)), consistent with the view that their actual activity was scaled by the total event duration ^{11,18}. The ensemble activities from duration cells tiled across the normalized nose-poking time, showing a sequential activation pattern (**Figure 2F** [↗](#)). This activity sequence was stable across individual trials within a session, regardless of whether rat reached minimal reward threshold (**Figure 2G** [↗](#), **Supp. Figure 1B-C** [↗](#)).

These data suggest that the sequential activities of the duration cells may be representing nose-poking time. To examine other possible interpretations of these data, we first measured the time interval between exiting nose poke to licking the water reward as indicators for the rat's motivation (**Supp. Figure 2**). While nose-poking durations were correlated with this reward-seeking time, normalizing the duration cells' activities according to this motivation factor showed poor sequential patterns (**Figure 2F**), suggesting that the sequences were not representing the rat's motivation for water rewards. In addition, we measured the rat's head movements during the nose poke and found that the duration cells' activities were not modulated by these movements (**Supp. Figure 2**). Furthermore, we were able to train a Gaussian process regression model to predict the progress of each trial with high accuracy (**Figure 2H-J**). While activities from start and end cells can also decode time, this might be due to slow calcium dynamics arising from either ramping activity or GCaMP kinetics as peak-extracted traces showed no decoding power (**Figure 2J**). On the other hand, peak-extracted activities from duration cells maintained high decoding power, indicating a more reliable sequential activity pattern. Consistently, the trial-length normalized duration cell activity exhibited robust sequentiality as measured by peak entropy^{26,27}, which was not seen in activities from start or end cells, or when duration cells normalized with motivation factors (**Figure 2K**). Together, these results indicated that distinct cell groups existed in the mPFC to code for different features of the nose-poking timing task. Duration cells showed selective activation towards specific timepoints in the normalized time scale, and their actual trial-by-trial activity was scaled by total event duration.

mPFC sequential time code remains stable over weeks

Previous studies reported that sequential activity patterns in the hippocampus can represent temporally ordered events²⁸ and timepoints²⁴. Interestingly, these sequences were highly dynamic, and the ordered pattern showed significant session-to-session variations^{29,30}. Therefore, we then examined whether the sequential time code we observed in the mPFC showed similar instability across different sessions. To achieve this, we aligned imaging data from multiple sessions based on matching unique blood vessel patterns in the field of view (**Supp. Figure 3**). This allowed us to examine the coding properties in the aligned cells. We were able to see cells that maintained their coding features in different sessions (**Figure 3A-C**). And particularly for duration cells, we found that cells can represent a fixed timepoint in the duration across sessions, or shifted to another timepoint while still being a duration cell (**Figure 3C**, **Supp Fig. 3**).

We next formally quantified these dynamics. Below 30% of the start cells or end cells continued to maintain the same type of coding in the next session (**Figure 3D**). When we used the activities from the aligned start and end cells to classify the nose poking state, we found chance level performance for cross-session decoding despite of high within-session accuracy (**Figure 3E**). These results indicated that cells code for nose poking start and end are highly dynamic and they did not form stable representation over days. In contrast, around 70% of the duration cells kept their identity in the next session. And within these stable duration cells, more than 70% of them coded for a fixed timepoint in the normalized time scale (**Figure 3F**). Using activities from aligned duration cells, we were able to make GPR models that show good cross-session decoding of the nose poking time (**Figure 3G**), suggesting a stable code in the duration cells regarding the sequential activity pattern and time. We found largely the same results from three pairs of sessions: two sessions in the long phase with 2-day interval, two sessions in the long phase with 14-day interval and one short phase session and one long phase session with 16-day interval. Thus, the dynamism and stability we observed were not influenced by elapsed time or task structure, but may be a reflection of intrinsic properties of those cells. Given that previous studies in the hippocampus³⁰ and cortex³¹ all showed unstable sequential pattern across days, to the best of our knowledge our data for the first time demonstrated a stable time code in the brain.

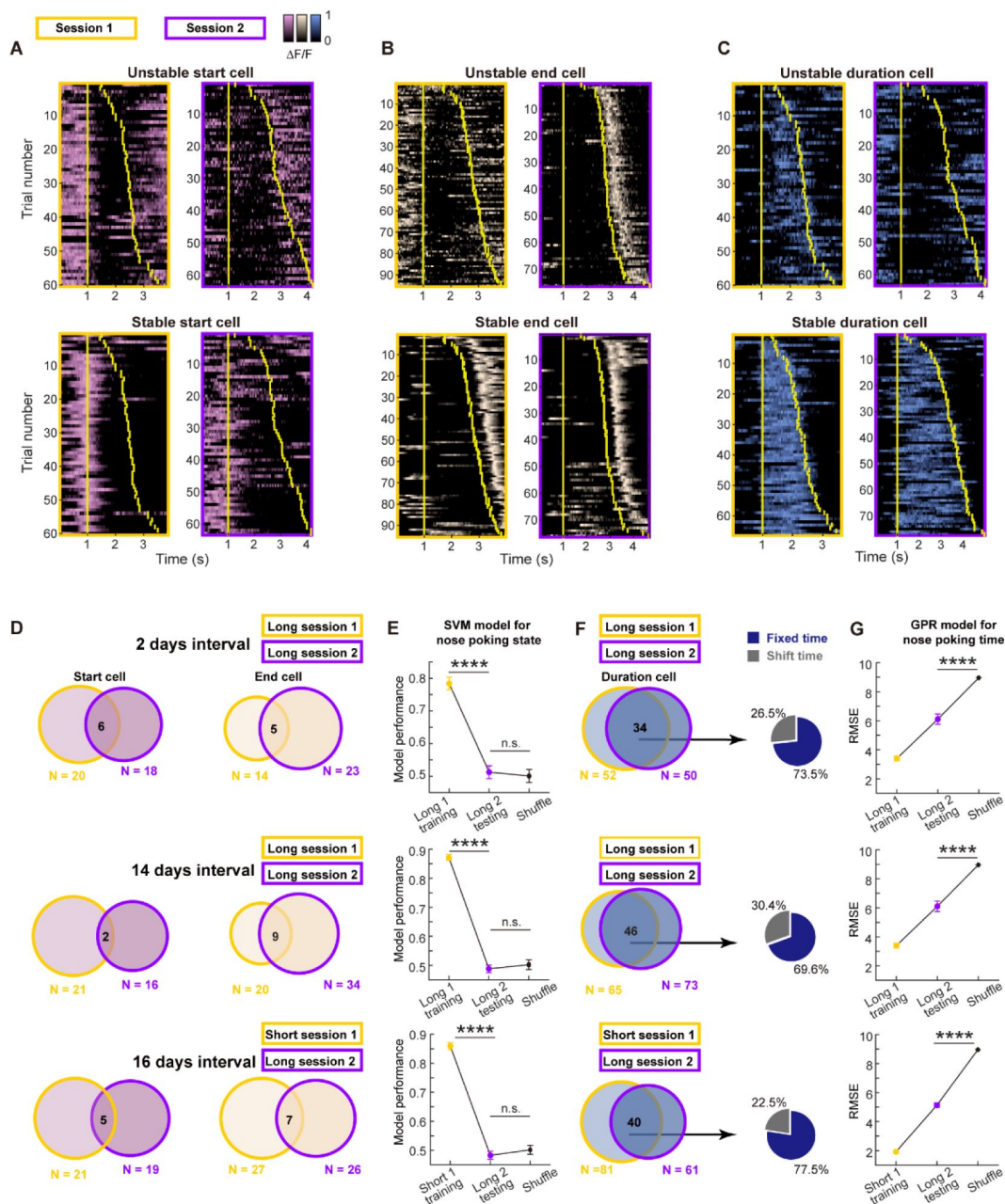


Figure 3.

Long-term stable time coding by mPFC duration cell sequences.

A-C. Example traces of unstable or stable coding start cell (pink, **A**), end cell (beige, **B**) and duration cell (blue, **C**) in two sessions. Yellow bars indicate the onset and offset of the nose poking event.

D. Venn diagrams of dynamics in start cells and end cells across sessions with different intervals.

E. Quantification of within-session and cross-session performance of the SVM models trained by start cells and end cells from one session for classifying nose poking states. For each group, 3 rats' data were pooled together. Data are presented as mean $\pm$ S.D. One-way ANOVA test with Dunnett's multiple comparisons post-hoc test, ****: $p < 0.0001$, ***: $p < 0.001$, n.s.: not significant, $n = 50$ random sampling.

F. Venn diagrams of dynamics in start cells and end cells across sessions with different intervals. And pie charts show shifted cell and fixed cell within the stable duration cells.

G. Quantification of within-session and cross-session performance of the GPR models trained by duration cells from one session for predicting normalized nose poking time. For each group, 3 rats' data were pooled together. Data are presented as mean $\pm$ S.D. Two-tailed t test, ****: $p < 0.0001$, $n = 50$ random sampling.

Active scaling of the mPFC sequences represents subjective time estimation

Having found that mPFC showed time-associated stable sequential activities in the duration cells, we next investigated whether this activity could serve as a neural substrate for time estimation in rats performing our task. Previous studies showed that cooling of the mPFC in rats disrupted their performance in tasks that require timing^{21,22}. However, in that study it was difficult to separate the influence of other cognitive functions affected by cooling the mPFC. Lacking the technology of single-neuron manipulation in freely-moving rats, we argue that further evidence on the necessity of mPFC sequential activities for time estimation could come from the analysis of the trials in that rats made errors. If the sequential time code we observed is causally linked with time estimation function, we should see reflection of behavior errors in the neural activity in some form. We speculated that there might be three types of coding errors in the mPFC: type I, absence of sequential activities that could be due to inattention or disengagement of the rats; type II, disordered sequences that may lead to errors in time estimation; and type III, scaling error with intact sequential code but wrong target time.

We then examined the above error types in detail. We performed a decomposition of the neural activity with principal component analysis in individual trials and found that almost all trials showed similar trajectories, indicating a lack of type I error described above (**Figure 4A**). Interestingly, we found that trajectories from correct trials appeared to be more expanded than those from the incorrect trials (**Figure 4B and C**), suggesting a difference in scaling between these two types of trials. We next utilized a partial least square regression model to examine the neural trajectory directions specifically aligned to trial durations (**Figure 4D**). Again, we observed that correct and incorrect trials in general follow the same direction during nose poke. In addition, we found that duration cells were more important for this trajectory pattern compared to other cells that we observed (**Figure 4E**). The constructed trajectories could explain ~52% of the variance in the normalized time, which was primarily contributed by the activities from duration cells (**Figure 4F**). These results indicated a lack of type I or type II errors. Consistent with this view, when we examined the raw activities from duration cells in individual error trials, we found only a small fraction showed the absence of sequential activities (**Supp. Figure 4**), indicating that type III errors (error in activity scaling) are the primary source of coding error. Indeed, we can find some duration cells showed trial type-modulated activities (**Supp. Figure 4D**), suggesting that precise time estimation from these sequential activities may be affected.

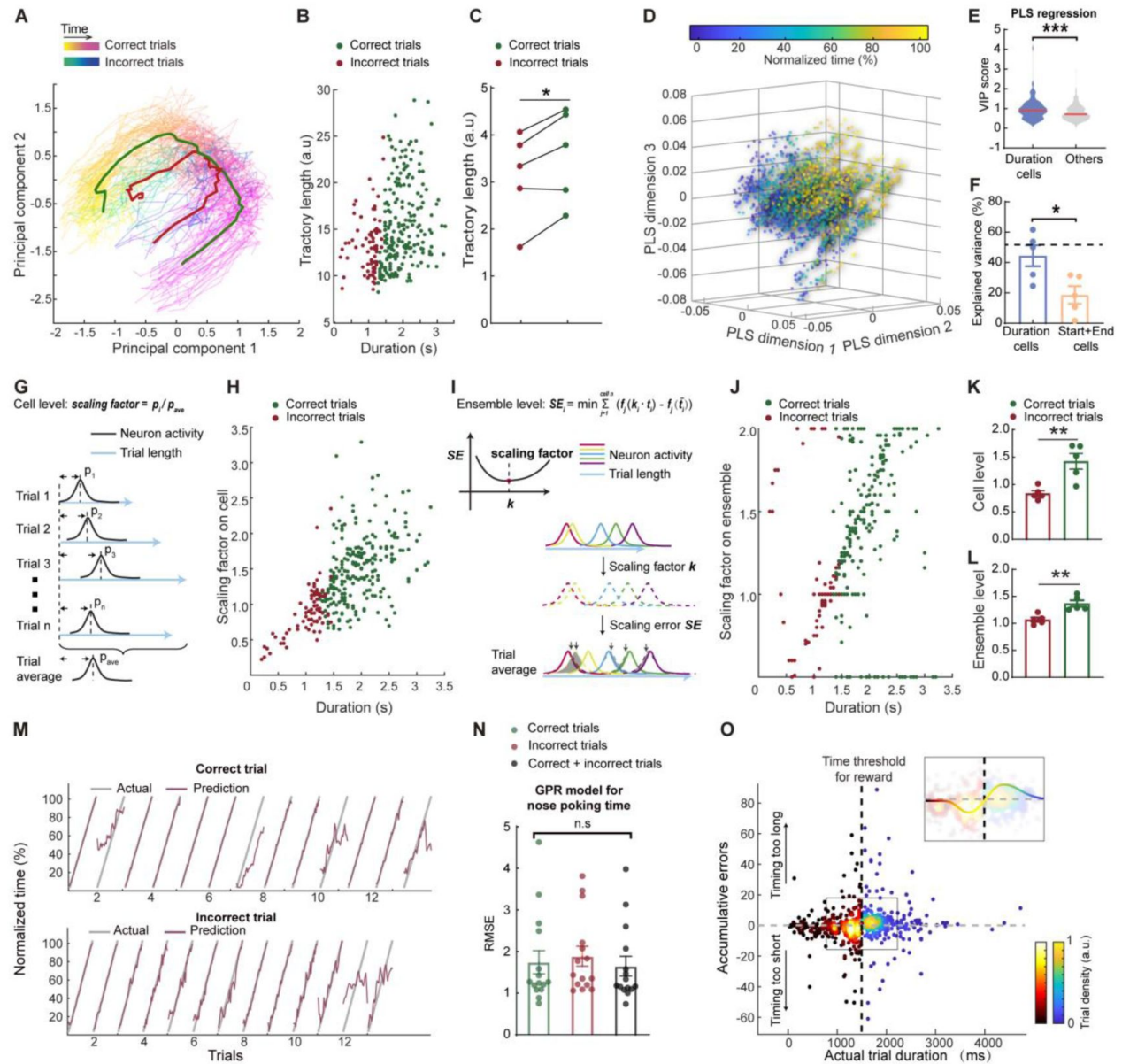


Figure 4.

Behavioral errors in time estimation can be attributed to miscalculation in scaling the mPFC sequences.

- A.** Representative trajectories of neuronal activities from correct (spring colored) and incorrect (winter colored) trials. The red and green curves indicate averaged trajectories from incorrect and correct trials, respectively.
- B.** Scatter plot of the trajectories lengths from individual trials, plotted against trial durations.
- C.** Quantification of averaged correct and incorrect trajectory lengths. $N = 5$ rats. Paired t test, *: $p < 0.05$.
- D.** Scatter plots of the first 3 dimensions of a partial least-square (PLS) regression model between neuron activities and normalized trial time.
- E.** Violin plot of the variable importance for projection (VIP) scores derived from the PLS regression in **A**. Two-tailed t test, ***: $p < 0.0001$.
- F.** Quantification of the explained variance in the normalized time by neuron activities from duration cells or start and end cells. Dashed line indicated explained variance from all cells. Data are represented as mean $\pm$ S.D. Two-tailed t test, *: $p < 0.05$. $n = 5$ rats.
- G.** Diagram of the scaling factor calculation process based on individual cell activities.
- H.** Scatter plot of the single-cell level scaling factor plotted against trial durations.
- I.** Diagram of the scaling factor calculation process based on population cell activities.
- J.** Scatter plot of the population level scaling factor plotted against trial durations.
- K and L.** Quantification of the averaged scaling factors on single-cell (**K**) or population (**L**) level. $N = 5$ rats. Two-tailed t test, **: $p < 0.01$.
- M.** Representative model prediction of normalized trial time for correct trials and incorrect trials. Each segment indicated on trial. The GPR model was trained on balanced correct and incorrect trials.
- N.** Quantification of GPR model performance for predicting different types of trials. In each group the model was trained on one type of trials and tested on the same trial type. Data are presented as mean $\pm$ S.D. One-way ANOVA test, n.s., not significant.
- O.** Scatter plots of cumulative prediction errors and actual trial duration. Correct and incorrect trials were colored differently but both by trial densities. There is a cluster of incorrect trials with below 0 cumulative errors and one for correct trials above 0. The insert shows the same data from the region in the box with a trend line color-coded with the trial densities.

To estimate the scaling errors associated with the type III errors, we calculated a scaling factor of the sequential activities on individual trials. To this end, we first calculated a scaling factor with individual duration cells' activities by comparing the peak position of each cell's activity on individual trial to the expected position from the averaged template (**Figure 4G**). We found that correct trials showed significantly larger scaling factors compared to those from the incorrect trials (**Figure 4H** and **4K**), supporting the view that scaling errors are the major source for behavior errors. Furthermore, we computed a scaling factor following a previously reported

method²¹, by using population activities from the duration cells. We hypothesized that a singular multiplier could affect all duration cells in individual trials to prolong or compress the activity sequences, and the scaling factor for the trial should be the multiplier that minimizes the difference between the scaled activity and the averaged template (**Figure 4I**). The scaling factors derived this way again showed larger values in correct trials compared to incorrect ones (**Figure 4J** and **4L**), further corroborating the importance of scaling errors.

Furthermore, we quantified the time estimation errors between the predicted time from duration cell neural activity and actual time using our previously established GPR models (**Figure 4M**). In general we did not find significant difference in prediction errors between correct and incorrect trials, and we were able to train a common model that showed accurate time estimation for both trial types (**Figure 4N**), again indicating a lack of type I or type II error. To estimate the scaling errors, we calculated the cumulative errors between the model predicted time and actual trial time. When we plotted these data against actual individual trial durations, we found that interestingly, incorrect trials showed cumulative errors clustered below zero, and correct trials showed errors above zero (**Figure 4O**), indicating that during the majority of the incorrect trials the rats timed too short from the expected sequences. Surprisingly, the duration time at which the polarity of the cumulative error reversed was 1500 ms, exactly the minimal time threshold required for the rats to receive reward. This result strongly suggests that the rat was capable of perceiving the minimal time threshold and adjusting trial-by-trial scaling factor of the mPFC sequential activities during the task, and that errors in the online scaling accounted for most of the incorrect trials that we observed.

Discussion

The neural basis of time representation is a fundamental question in neuroscience. As timing is closely intertwined with various cognitive functions, such as short-term memory, decision-making, etc., it is perhaps not surprising that neuron activities correlated with time were found throughout the brain. Previous studies established that sequential or ramping activity can be feasible neural code for time representation. Indeed, time-related sequential or ramping patterns of neuron activity have been reported in prefrontal cortex^{32,33}, motor cortex^{34,35}, sensory cortex^{36–38}, striatum^{11,39–41}, thalamus⁴², hippocampus^{12,43,44}, and entorhinal cortex^{31,45}. A fascinating feature of time coding is the capability of scaling, in which the same sequential or ramping patterns were compressed or stretched to represent different durations of time^{2,46}. While these previous findings set forth substantial insights into the neural substrate of time in the brain, two major limitations exist in the field.

One aspect is the lack of direct causal evidence regarding the neuron activity and the perception of time. Resolving this would not be an easy task as the causal evidence between similar sequential patterns of place cell firing and spatial perception was not fully demonstrated either, although the phenomenon was described about 50 years ago. Recent advances in the technology indicated a possibility of single-cell optogenetic control for such experiments⁴⁷, yet the low throughput and the difficulty for inhibitory control still greatly limit its application. While lacking direct causal evidence, results from our current study provides a finer correlation between sequential firing and timing function. As previous studies generally trained the animal to learn distinct categories of durations^{21,48}, the scaling effect of sequential activity was usually described as a group effect. We discovered that scaling is correlated with durations at individual trial level, and that failure in reaching the minimal timing threshold for reward can be primarily attributed to scaling errors, in which the sequential pattern prematurely reached the end. Furthermore, the probability of high scaling error was disproportionally increased when the duration of the trial is close to the timing threshold for reward, suggesting that the animal provides trial-by-trial adjustment of the scaling factor that reflected its estimation of the timing threshold. The real-time scaling of sequential activity, and the enrichment of scaling errors around the timing threshold strongly

suggest that the animal actively uses this mechanism, and thus the sequential activity we observed is likely to be the neural code for time estimation. We have also provided evidence excluding the effect of motivation or motion. Future advances in single-cell manipulations may provide more definitive experimental evidence.

The second limitation in our understanding for the neural basis of time perception is the lack of biophysical mechanism for the emergence of sequential patterns and the scaling effect. In this regard, several theoretical models have been proposed^{18,26,49}, yet testing these models in biological systems is currently not possible. Our data demonstrated a unique stability of the duration cells and their sequential activity patterns for coding time (**Supp. Figure 5**). Large proportions of duration cells' coding type and their sequential order remained unchanged over weeks, even when the target time threshold was changed. In contrast, within the same field of view, cells that code for the start or end phase of the nose poke showed dynamic changes, although we could not separate the start and end-coding cells from time-coding cells with ramping patterns and could not assess the long-term stability of time coding by ramping activities. Previous studies of sequential activity did not report such stability over time, and activities related to event trajectories are generally considered unstable^{29,30}. It is possible that this difference reflects different mechanisms underlying sequence emergence in different brain regions. This prolonged stability of duration cells suggests that in the mPFC, participation in the sequential pattern was unlikely a result of input-driven flexible coding, but a reflection of certain intrinsic properties. In other words, our data implied the existence of predetermined factors such as a genetic program for time coding neural population in the mPFC. Similarly, a recent study reported stable time sequences across different modalities in premotor cortex⁵⁰. A designated neural substrate for timing could be advantageous so that time coding can be a relatively independent module and can be inert to constant plastic changes happened in the brain. Future studies isolating molecular signatures of the time-coding population could provide a powerful tool in dissecting the neural mechanism of time representation in the brain.

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Additional information

Author contributions

CL.M. and BM.L. designed the study. YT.L., WQ.L. and JW.L. performed the experiments. P.Y., SL.Z. and YT.L. performed data analysis. YT.L. drafted the manuscript. YT.L. and X.W. prepared figures. P.Y., CL.M. and BM.L. revised the manuscript.

Competing interest statement

The authors claim no competing interest.

Materials and Methods

Subjects

Our study used Sprague Dawley rats. Equal number of male and female rats were used in the study. The environment temperature was controlled at 25-degree Celsius with 12/12 light-dark cycle. All the rats were raised in single-housing after surgery with ad libitum access to food and water. During behavior training, water access was restricted according to an experimental procedure, in which the body weight loss was monitored and kept under 20%. Animal procedures were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals. All experiments were approved and monitored by the Ethical Committee of Animal Experiments at the Institute of Life Science, Nanchang University.

Apparatus

Behavioral training and testing were conducted in a 40×20×30 cm plastic chamber. One side of the chamber had a semicircular hole (2.5 cm diameter) which was 13 cm above the floor and an infrared detector was placed to detect the nose poking, this hole was called as the operant hole. Under the operant hole is a reward hole where rats can get a certain volume of water, and water delivery is controlled by a solenoid valve. If the detector had detected a nose poking longer than the minimal time threshold from a rat, the solenoid valve will deliver a certain amount of water. We had written scripts to control those processes and record the timestamp of the nose poking.

Virus injection

The rat was anesthetized with isoflurane and placed into a stereotactic apparatus. Rats were injected with carprofen solution (5 mg/kg) and dexamethasone solution (0.2 mg/kg) to minimize inflammation. We then shaved the head area and disinfected 3 times with alternating iodophors and alcohol. An opening on the skull was made with a dental drill. Then we unilaterally microinjected 500 nl of rAAV-CaMKIIa-GCaMP6S-WPRE-PA virus (1.37×10^{12} vg/ml, BrainVTA) at 80 nl/min into the prelimbic cortex with the following stereotactic coordinates: 3.7 mm anterior to bregma, 0.5 mm lateral to the midline, and -3.2 mm ventral to the skull surface. After the virus injection, we waited 10 minutes to remove the microelectrode from the prelimbic cortex, and then sutured the scalp and disinfected. The rat was placed back in the home cage after waking up from anesthesia.

GRIN lens implantation

Our surgical method is based on the surgical procedure reported in mice⁵¹. Two weeks after the virus injection, the GRIN lens was implanted above the previous injection site. For the procedure, after disinfecting the head area, we removed the scalp with scissors and cleaned up the skull with hydrogen peroxide and saline, and then inserted three skull screws on the skull. The skull screws were placed in a way that would not block the lens. Then, we used saline to clean up the fragment, and drilled a hole with 1.9 mm diameter on top of the virus injection site. We slowly aspirated brain tissue above the prelimbic cortex by connecting a syringe needle with the vacuum pump. The aspiration stopped at 2.5 mm below brain surface. We then inserted a GRIN lens (1.8 mm diameter, N.A: 0.54, Edmund) to the target position with the micromanipulators. The placement was checked using a miniscope (Labmaker) and adjusted to maximize the fluorescence signal.

We then removed excess liquid with the vacuum pump, and glued the GRIN lens with skull and skull screw using cyanoacrylate glue. After the glue was fully cured, we then applied black dental cement and covered the top of the lens with Kwik-Sil (WPI) after the dental cement is dry. The

rat was placed back to their home cage and amoxicillin was added to their drinking water for 7 days.

After GRIN lens implantation, we waited 4 to 6 weeks to install baseplate. The rat was anesthetized with isoflurane again, and then we used forceps to remove the Kwik-Sil from the top of the lens, cleaned the lens with wet lens paper. We then installed a miniscope with a micro-manipulator holder and found the best view with clear vasculature and cells. At this position, we applied glue around the baseplate. After the glue was set, we continued to cover the larger area with dental cement. When the dental cement is dry, we removed the miniscope. Finally, we covered the plastic cap on the top of the baseplate and fixed it with a screw.

The timing task

To motivate the rats to learn the task, their drinking water was limited during the training period, so that rats could only obtain water through task operation. Specifically, the rats need to probe their nose into the operant hole and stay longer than the minimal threshold time. After rats withdrew their nose from the hole, they can get reward from the reward hole. If the rats performed this action and acquired water reward, we called this as a correct trial. Conversely, an incorrect trial is when the rats started a nose poke but did not reach designated time for reward.

The training was separated into three phases. In the pre-training phase, once rats produced the nose poking action, they could immediately acquire water reward at a small amount (60 μ l). The rats moved on to the short phase after they mastered the nose poking action. In this phase, rats would acquire the reward only if they kept the action of nose poking for at least 300 ms. Rats received 50 μ l water with 300 ms duration. The rats graduated from this phase when their performance reached above 70% correct. In the long phase, the nose poking duration must reach at least 1500 ms. Rats received 100 μ l water with 1500 ms duration. In these two phases, with each additional 500 ms nose poke time in a trial, the reward was increased by 60 μ l.

Calcium image recording

We did not anesthetize the rat for installing miniscope. Instead, we hand-held the rat to maintain its position and installed the miniscope and fixed it with screws. We monitored the calcium signal so that if the recording was not stable, the experiment would be interrupted. The calcium imaging of animals was recorded using a UCLA miniscope (V3), and the animal behavior was recorded using a webcam. Data acquisition software recorded the timestamps of the behavior camera and miniscope at the same time in order for subsequent alignment. Video streams were recorded at 20 frames per second. And excitation was set at ~ 4.5 mW. In our experiment, we used an MCX adapters to link the coaxial cable to ensure the animals can move freely and avoid the winding of cables.

Extracting calcium activity

All analyses of the calcium imaging data were performed using custom software written in MATLAB. First, the raw imaging data was motion corrected using a previously published the NoRMCorr algorithm⁵². The corrected movies have been processed by median filter and down-sampled prior to extract the calcium traces. Here, to accurately extract the calcium signals of individual cells, we applied a supervised and robust the EXTRACT algorithm^{53,54}. Extracted units were checked manually to exclude artifacts.

The identification of cell types

To identify cells that encode different periods of the nose-poking event, we calculated the Pearson's correlation coefficients between calcium traces and binarized event traces. In our task, we focused on three types of events, the start of nose poking was defined as the 5 frames prior to the detection of the nose-poking event start. The end of nose probing was defined as the 5

frames after the rat began to withdraw from the operant hole. The duration of the nose poke was defined as the interrupted time of the infrared sensor in each trial. In order to calculate the statistics of the correlation coefficients, we generated a null hypothesis for each cell by shuffling the calcium traces with random delays for one hundred times, and calculated the resulted correlation coefficients. A cell was deemed to respond to certain events when the observed coefficient reached above the 95th percentiles of the corresponding coefficient distribution from the shuffled dataset. Those neurons responding specifically the three events were called respectively the start cells, the end cells and the duration cells.

Calculation of peak entropy

PE (peak entropy) measures the entropy of peak times distribution across the population. Peak times are determined by analyzing the activity of the duration cells during normalized intervals for each trial. For a group of trials, the frequency of peak times is then calculated within M bins, where M represents the number of bins used to estimate the peak time distribution (30 frames for neural dynamics in normalized durations). Here, p_j is the proportion of units peaking in time bin j relative to the total number of units. We did not further normalize the peak entropy with cell numbers since the numbers between groups were close to each other.

$$PE = \sum_{j=1}^M -p_j \log(p_j) / \log(M)$$

The alignment of calcium imaging cross sessions

We performed cross-session alignment by manually select unique blood-vessel or neuron patterns as fixed control points, and then calculated the transformation between sessions by linear interpolation. We then applied the transformation to the centroid coordinates of each extracted cell to obtain the aligned cell positions in the target session. By calculating the pair-wise distances between the aligned centroids and that from the extracted cells in that session, we selected the minimal distance for each cell as the most likely candidate matches. And if this distance is below 5 pixels, which is about the radius of a cell, we recorded these two cells as a matched pair. In order to maximize the number of aligned cells, in all cross-session analysis, we used matched paired between two sessions.

Support vector machine model for classification

We trained a linear kernel support vector machine (SVM) model using the activities of the start and end cells to predict the binarized nose poking state for each timepoint. In order to avoid biases caused by the imbalance of the samples, we chose random subsamples of recorded timepoints with equal number of nose-poking and non-nose-poking states. From this subsample, we then selected 80% of the timepoints for model training, and used the remaining 20% to evaluate the performance of the model. For performance measurement, we tabulated the differences between the model's predicted states from the actual states and calculated percentages of accurate predictions. In order to avoid variations introduced by sampling, we repeated our random sampling process for 100 times for each dataset and recorded the average performance. For generating a null hypothesis, the actual states were randomly shuffled during the training process, and the resulted model was then tested on real testing dataset. For testing cross-session performance, the within-session training and testing procedures were similar as described above, but using only cells that can be matched to the target session as model input. After training, the model was tested on the timepoints of the target session using activities from the activities of the matched neurons, and accuracy calculation was the same as that described above.

Gaussian process regression model

Neuron activities from each nose poking trial were isolated by taking the frames of each nose-poking bouts detected by the sensors and the amplitude of each cell's activity was normalized into a 0-1 scale within the trial. We then used linear interpolation algorithm (MATLAB “interp1” function) to make each trial into the same number of “normalized” timepoints. In our case we used 30 frames as the normalized trial length. Before model fitting, we selected subsamples of the same number of correct and incorrect trials, and used 80% of the trials for model training and saved 20% for testing. We then fitted a Gaussian process regression (GPR) model with a principal analysis pre-processing with a rational quadratic kernel function to describe the relationship between neuron activities and the progression of trial in normalized timepoints.

Rational Quadratic Kernel:

$$k(x_i, x_j | \theta) = \sigma_f^2 \left(1 + \frac{r^2}{2\alpha\sigma_f^2} \right)^{-\alpha}$$

$$r = \sqrt{(x_i - x_j)^T (x_i - x_j)}$$

where σ is the characteristic length scale, α is a positive-valued scale-mixture parameter, r is the Euclidean distance between x_i and x_j .

We feed the testing set data to the fitted model to calculate the predicted normalized time. The performance was defined as the root mean square error (RMSE) between the actual normalized time and the predicted time across all tested trials. Similar to the SVM model, we repeated this process for 100 times to eliminate the sampling variations and shuffled the normalized time in the training set to generate a null hypothesis. It is notable that in this case, the null model output was generally around 15, which is at the middle of our normalized trial length. While this number gave no prediction power, the RMSE (~9) was lower than a random number set between 1 and 30 (~12).

Partial least square regression

The VIP score was computed to authenticate the importance of neuronal ensembles on time estimation. VIP score is a measure used in PLS regression to identify the most important variables that predict a response variable. VIP score values range from 0 to 1, where higher values indicate greater importance of the variable in predicting the response. To calculate VIP score using the Partial Least Squares Regression method, several steps are involved. First, the dataset is divided into a training set and a test set. The training set is used to build the PLS regression model, while the test set is used to evaluate its performance. Next, the PLS regression model is trained using the training set. During this step, the model identifies the most relevant variables that explain the variance in the response variable. The VIP score for each variable is then calculated based on its contribution to explaining the variance in the response variable.

The VIP score is importance in predicting the response variable.

$$W_0 = W_i / \sqrt{\sum_{i=1}^n W_i^2}$$

$$sumSq = \sum_{i=1}^n X S_i^2 * \sum_{i=1}^n Y L_i^2$$

$$vip score = \sqrt{n * \sum_{j=1}^m (sumSq_{ij} * (W_0^2)) / \sum_{j=1}^m sumSq_{ij}}$$

XS is an orthonormal matrix including n time points by m components. Each row of XS corresponds to one time point, and each column corresponds to one component. YL is an n -by-one matrix, where n is the number of response variables and one is the number of PLS components.

Each row of YL contains coefficients that define a linear combination of PLS components approximating the original response variables. W is n -by- m components matrix of PLS weights.

Principal components analysis (PCA) for duration cells

We applied the PCA function of matlab to determine the *score* of correct trials. The covariance matrix was calculated for duration cell activities that included only the normalized time in the correct trials. Next, eigenvalue decomposition of the covariance matrix was performed to obtain eigenvalues and eigenvector matrices. The feature vectors were sorted according to the numerical magnitude of the feature values, and the top three feature vectors were selected as the principal components. Finally, the projection of the dataset on the selected principal components is calculated, that is, the score matrix is calculated. And the scores in incorrect trials were determined as the Principal component coefficient $coeff_{CT}$ and estimated means μ_{CT} from the data on the correct trials.

Incorrect trials:

$$score_{IT} = (activity_{IT} - \mu_{CT}) * coeff_{CT}$$

To quantify the trajectories lengths of duration cell activities, we calculated the length of the average trajectory on the correct trials and the average trajectory on the incorrect trials using the Euclidean distance. Here, $then$ means that there are multiple time points in the average trajectory

$$trajectorylength = \sum_{i=1}^n \sqrt{(x_i - x_{i+1})^2 + (y_i - y_{i+1})^2 + (z_i - z_{i+1})^2}$$

Scaling factor

The scaling factor k at the cellular level is calculated by dividing the location p_i of the peak of activity of a single cell during each actual trial duration by the location p_{ave} of the peak of average activity. The average activity here is taken as the number of trials with the highest frequency of nose poking time within a session, and the activity of individual neurons during these trials is averaged.

Cell level:

$$k = p_i / p_{ave}$$

To determine the optimal scaling factor k_i at the ensemble level (where k_i yields the minimum difference after scaling at ensemble level), we linearly compressed or stretched the binary activities of each duration cell within the same actual trial t_i using various scaling factors ranging from 0.5 to 2. We calculated the standard error (SE) between the binary activities of each duration cell and their corresponding normalized time trial $\bar{t}_i$. The scaling factor k_i corresponding to the i th trial that resulted in the minimum SE was selected as the best scaling factor. As the following equation: Ensemble level:

$$SE_i = \min \sum_{j=1}^{cell\ n} (activity_j(k_i * t_i) - activity_j(\bar{t}_i))$$

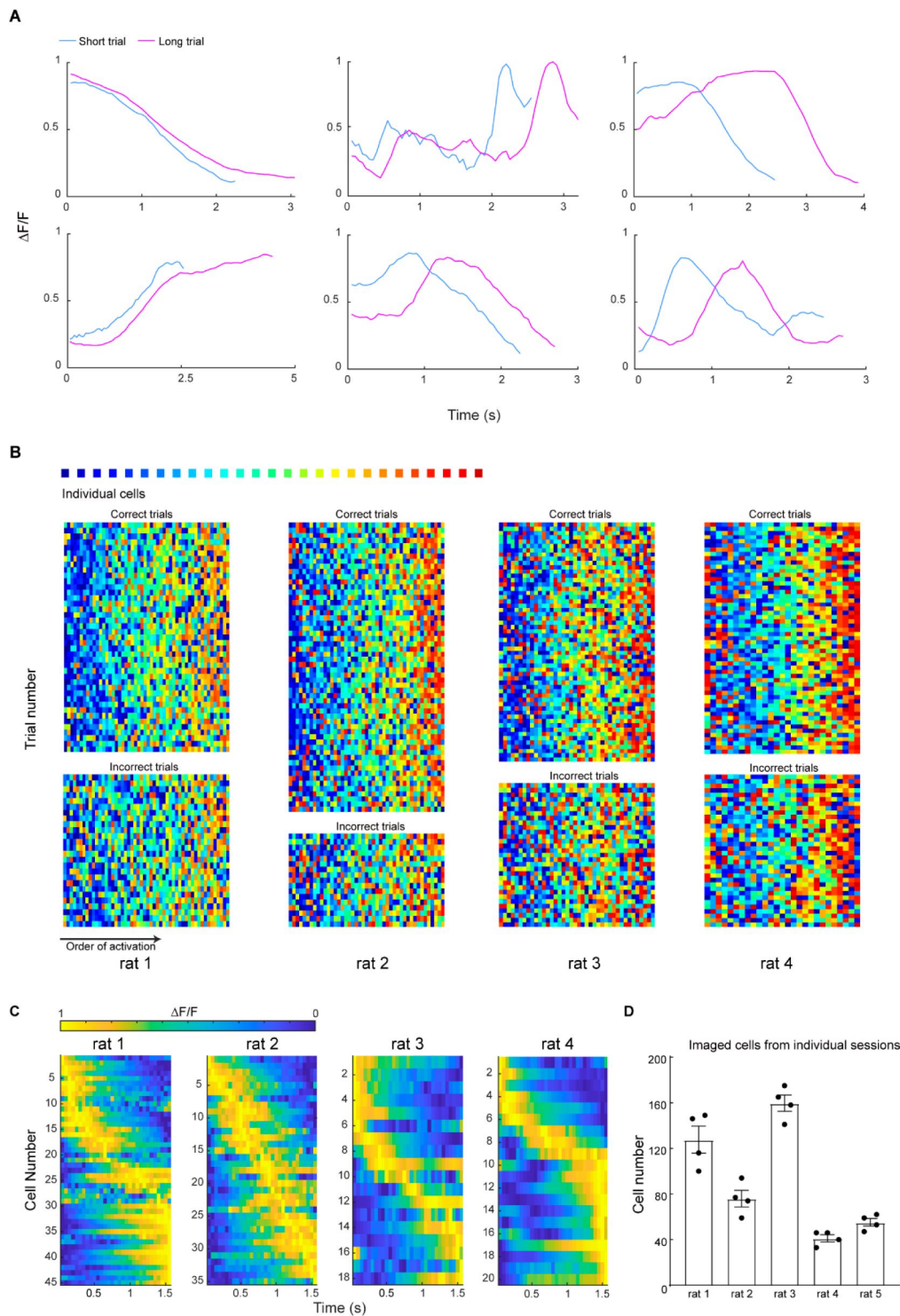
Motion analysis

To explore the effect of motion on regulating neural activity during the timing task, we utilized the popular software DeepLabCut^{55,56} for motion analysis based on transfer learning with deep neural networks. Using DeepLabCut, we successfully obtained information on the rat's head position. Initially, we created minimal training data from raw behavioral pictures where markers were manually placed on the rat's head. This labeled training data was then used with deep neural

networks (DNNs) to track changes in the rat's head movement throughout the entire behavioral video. Specifically, movements during nose poking were extracted to analyze the duration of cell activity in relation to head movements.

Statistics analysis

GraphPad Prism version 9.00 was used for statistical analyses. All data are presented as mean $\pm$ standard error (SE). Statistical significance was assessed by two-tailed and non-parametric Student's t-tests. $p < 0.05$ was considered statistically significant. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$



Supplementary Figure 1

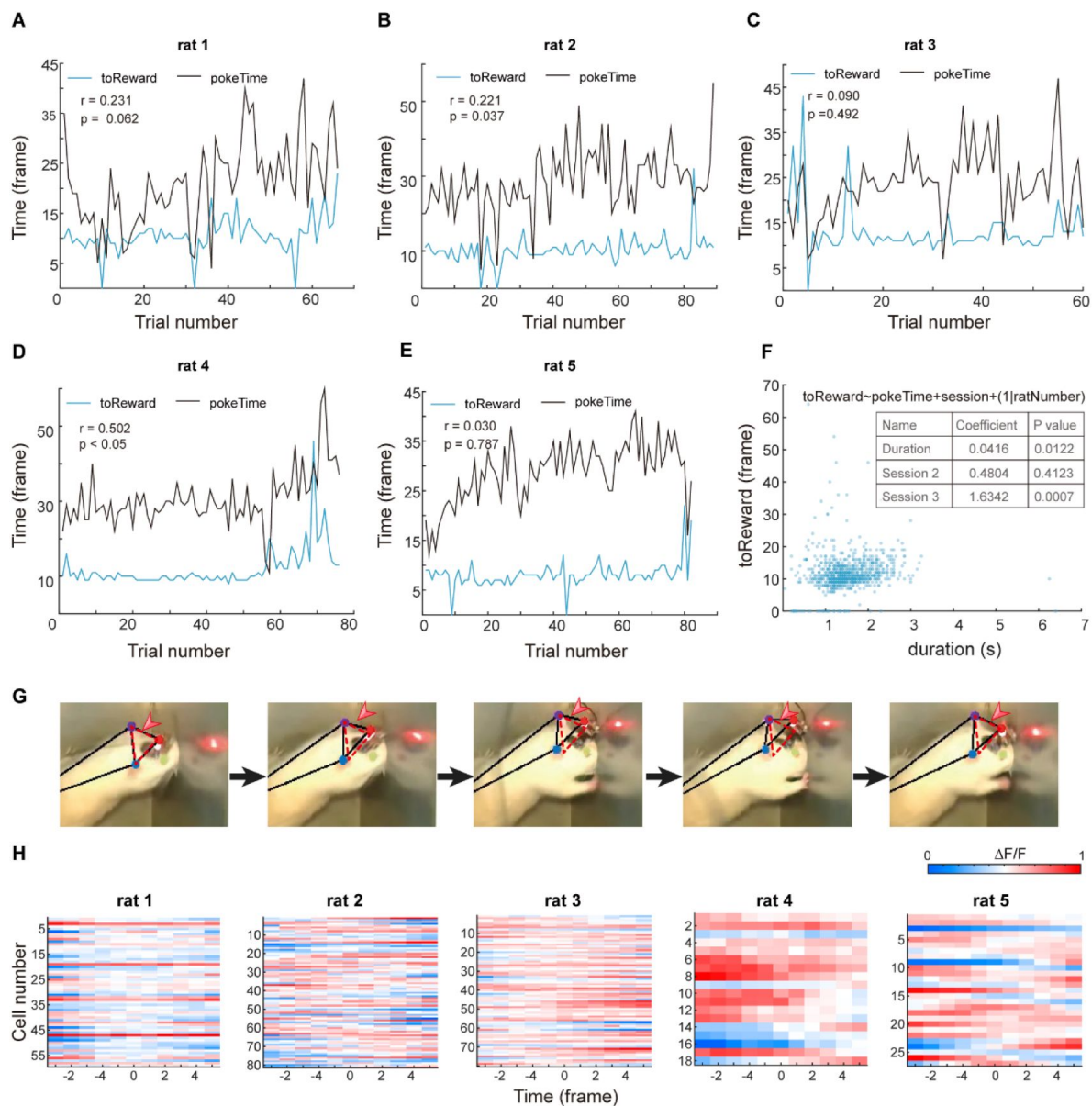
Additional examples of time-related sequential activities in mPFC.

A. Representative trial-average calcium traces from cells in short phase and long phase sessions. Each graph shows an individual cell's responses.

B. The rest of the imaged rats of their peak position rank maps in rainbow plot format, similar to [Figure 2G](#).

C. Individual rat's duration cell activity in trial-average form. Cells were sorted by their peak position in each graph.

D. Number of cells recorded from individual sessions of each rat. Each dot indicates an imaging session.



Supplementary Figure 2

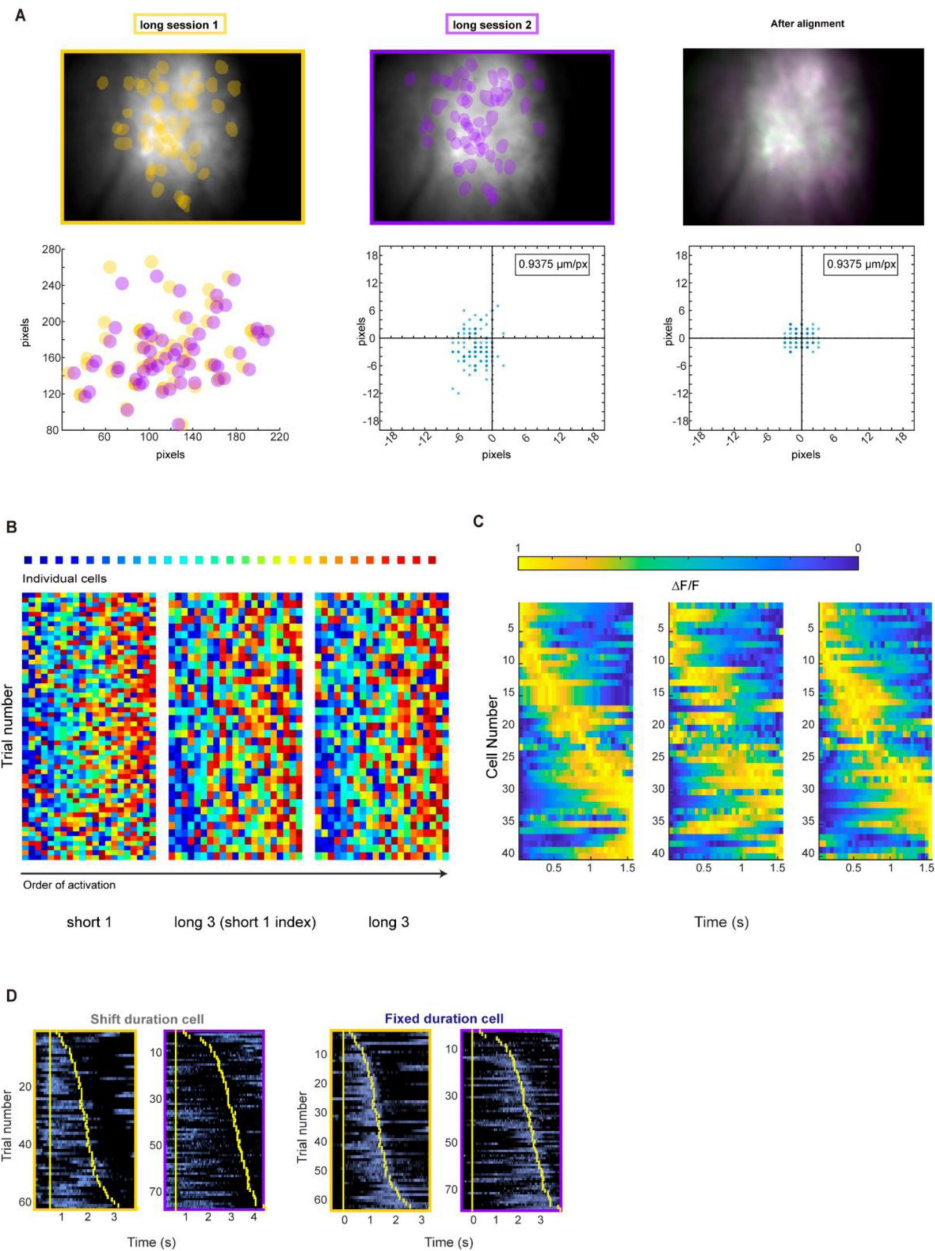
Analyses of rats' motivation and motions.

A-E. Plots of each trial's nose poking duration (black) and interval time between exiting nose poke to licking water reward (blue) for individual rats. Pearson's correlation analysis was used.

F. A mixed-effect general linear regression model for nose poking duration and reward-seeking time from all data. The formula was indicated above the graph. The coefficient for the "Duration" parameter indicates a significant correlation between nose poke duration and reward-seeking time.

G. Representative images for head motion. Three positions (red arrow head) were identified using DeepLabCut software and used for quantify head motion: left ear (purple dot), right ear (blue dot) and a reflective part on the miniscope (red dot). Red dashed triangle showed the head position at the first time point.

H. Traces of averaged activity of all recorded coding cells in 5 rats, aligned by the start of head motion (time 0).



Supplementary Figure 3

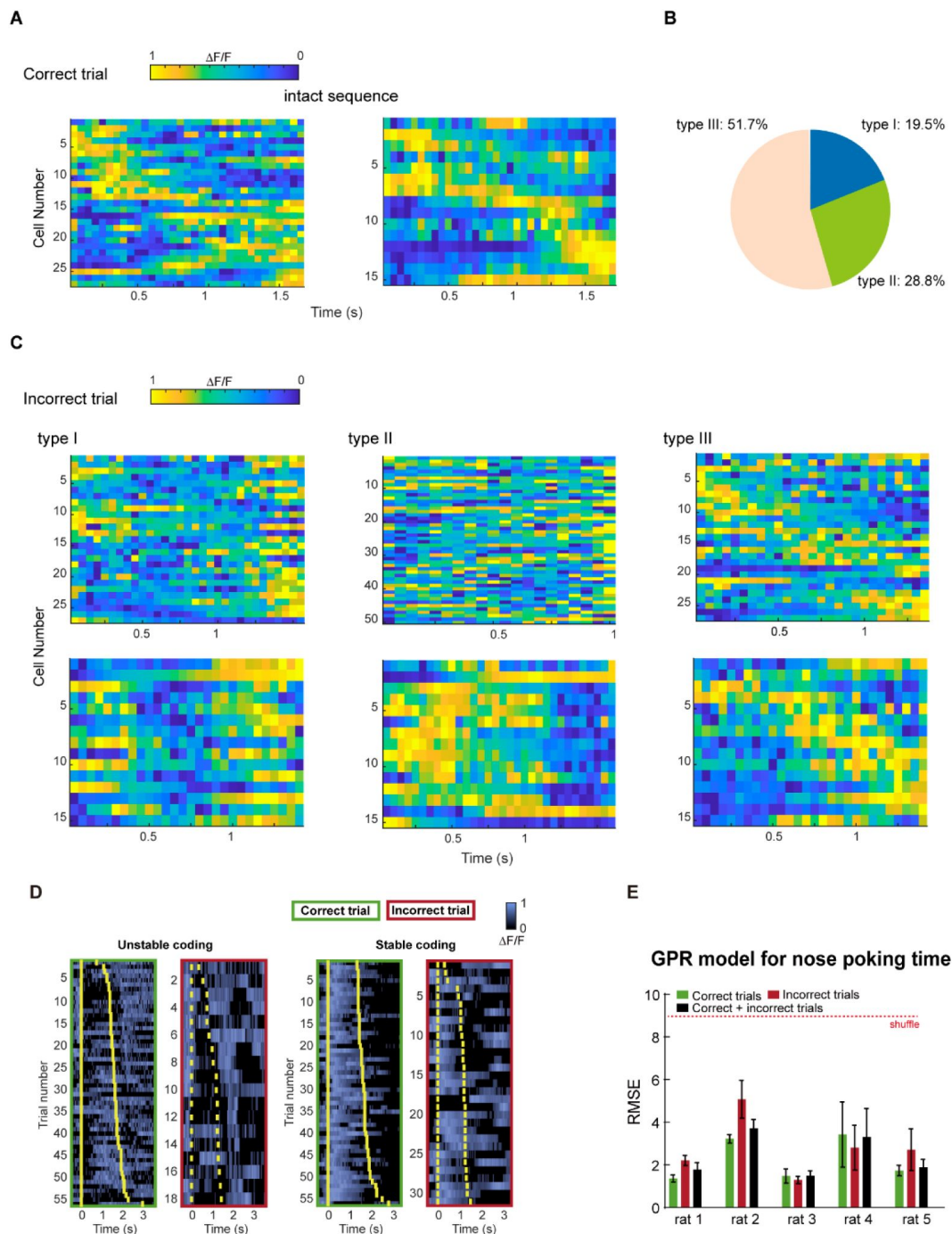
Additional information related to cross-session alignment.

A. Representative field of view for two sessions from the same rat. Yellow and purple shades indicate extracted cells' position masks from each session. The right panel shows the matched cells' positions after alignment. The scatter plot in the left bottom corner shows the cell masks coordinates in two movies. The scatter plots on the right side show matched cell's distances between session before (middle) and after (right) alignment.

B. Representative rainbow plot of the same rat's duration cells of their peak position rank maps in different sessions. Left panel shows data from short1 session sorted by the peak order in short1 session. Right panel shows data from long3 session sorted by the peak order in long3 session. Middle panels show data from long3 session sorted by the peak order in short1 session.

C. Representative trial-averaged activities in normalized time scale from the same rat's two sessions, with the same sorting scheme described in **B**.

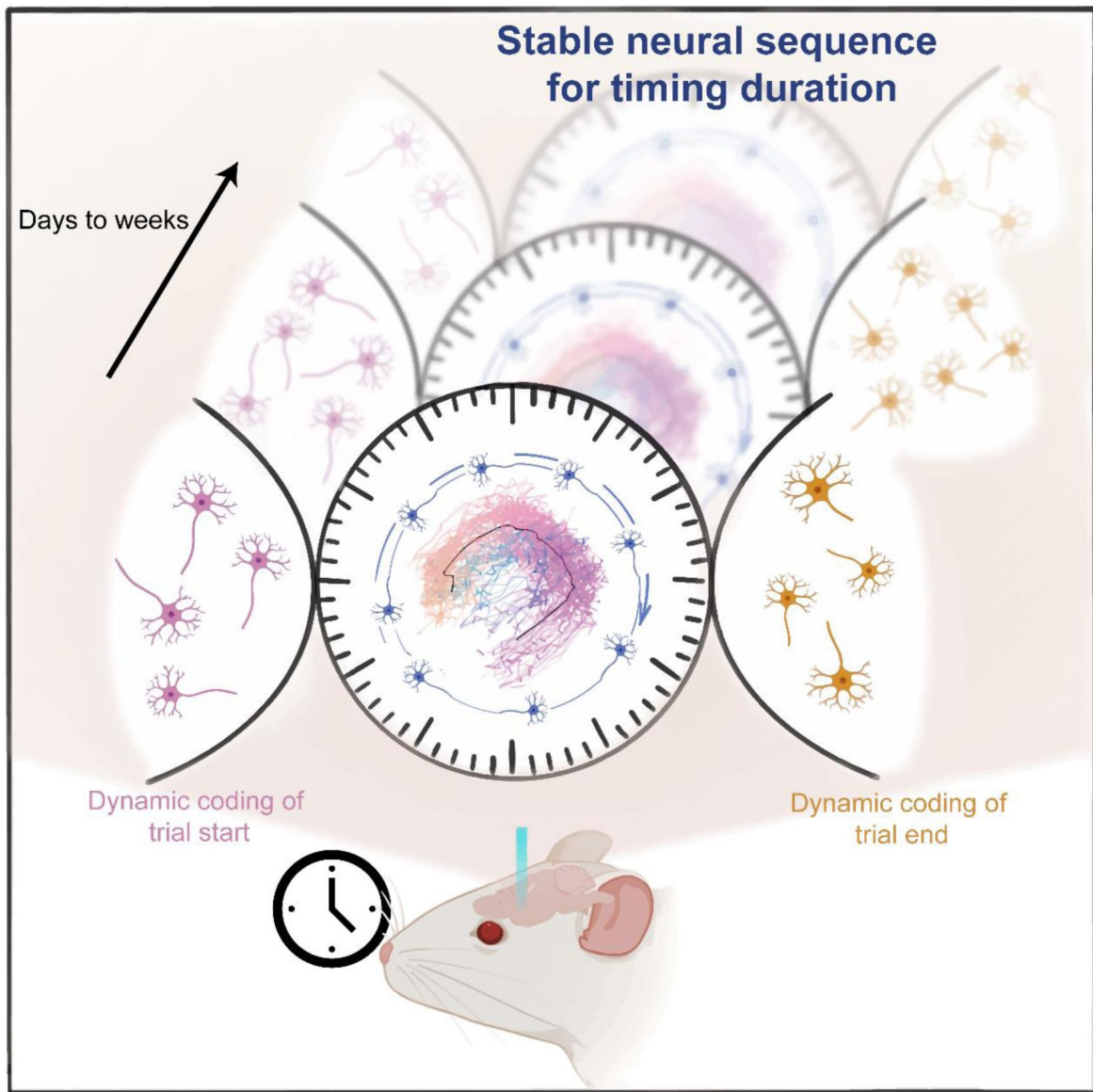
D. Example traces of stable duration cells that show shifted preferred position and fixed preferred duration. Yellow bars indicate the onset and offset of the nose poking event.



Supplementary Figure 4

Different types of incorrect types based on changes in mPFC time sequences.

- A.** Representative activities of duration cells in correct trials.
- B.** Pie chart of the prevalence of the 3 types of incorrect trials.
- C.** Example duration cell activities in the 3 types of incorrect trials.
- D.** Example traces of duration cells that show correctness-modulated (left) or correctness-independent coding (right). Yellow bars indicate the onset and offset of the nose poking event.
- E.** Quantification of GPR model performance for predicting different types of trials for individual rats. In each group the model was trained on one type of trials and tested on the same trial type. Data are presented as mean $\pm$ S.D. Red dotted line indicates errors derived from a randomly shuffled dataset.



Supplementary Figure 5

Graphic summary of the findings in current study.

We described a group of neurons in the rat mPFC that represent time with activity sequences. The sequential procession can be rapidly stretched or compressed to track the total time durations on individual trial level. The identity of these time-coding cells and their sequential code remain stable over weeks, which is not seen in neurons coding for trial start or end.

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Reviewer #1 (Public review):

Summary:

This paper investigates the neural population activity patterns of the medial frontal cortex in rats performing a nose poking timing task using in vivo calcium imaging. The results showed neurons that were active at the beginning and end of the nose poking and neurons that formed sequential patterns of activation that covaried with the timed interval during nose poking on a trial-by-trial basis. The former were not stable across sessions, while the latter tended to remain stable over weeks. The analysis of incorrect trials suggests the shorter non-rewarded intervals were due to errors in the scaling of the sequential pattern of activity.

Strengths:

This study measured stable signals using in vivo calcium imaging during experimental sessions that were separated by many days in animals performing a nose poking timing task. The correlation analysis on the activation profile to separate the cells in the three groups was effective and the functional dissociation between beginning and end, and duration cells was revealing. The analysis on the stability of decoding of both the nose poking state and poking time was very informative. Hence, this study dissected a neural population that formed sequential patterns of activation that encoded timed intervals.

Weaknesses:

It is not clear whether animals had enough simultaneously recorded cells to perform the analyzes of Figures 2-4. In fact, rat 3 had 18 responsive neurons which probably is not enough to get robust neural sequences for the trial-by-trial analysis and the correct and incorrect trial analysis. In addition, the analysis of behavioral errors could be improved. The analysis in Figure 4A could be replaced by a detailed analysis on the speed, and the geometry of neural population trajectories for correct and incorrect trials. In the case of Figure 4G is not clear why the density of errors formed two clusters instead of having a linear relation with the produce duration. I would be recommendable to compute the scaling factor on neuronal population trajectories and single cell activity or the computation of the center of mass to test the type III errors.

Due to the slow time resolution of calcium imaging, it is difficult to perform robust analysis on ramping activity. Therefore, I recommend downplaying the conclusion that: "Together, our data suggest that sequential activity might be a more relevant coding regime than the ramping activity in representing time under physiological conditions."

Comments on revisions:

The authors responded properly to my initial comments. However, I have three additional recommendations for the reviewed manuscript.

First, the paper urgently needs proofreading by a professional English editor. Second, Figure 4 must be divided in 2, it has too many panels and the resolution of the figure is low. Finally, please consider that what is called scaling factor in Figure 4G should be called something like neural sequence position index. A scaling factor in the timing literature implies that the pattern of activation of a cell contracts or expands according to the timed interval.

<https://doi.org/10.7554/eLife.96603.2.sa2>

Reviewer #2 (Public review):

In this manuscript, Li and collaborators set out to investigate the neuronal mechanisms underlying "subjective time estimation" in rats. For this purpose, they conducted calcium imaging in the prefrontal cortex of water-restricted rats that were required to perform an action (nose-poking) for a short duration to obtain drops of water. The authors provided evidence that animals progressively improved in performing their task. They subsequently analyzed the calcium imaging activity of neurons and identify start, duration, and stop cells associated with the nose poke. Specifically, they focused on duration cells and demonstrated that these cells served as a good proxy for timing on a trial-by-trial basis, scaling their pattern of activity in accordance with changes in behavioral performance. In summary, as stated in the title, the authors claim to provide mechanistic insights into subjective time estimation in rats, a function they deem important for various cognitive conditions.

This study aligns with a wide range of studies in system neuroscience that presume that rodents solve timing tasks through an explicit internal estimation of duration, underpinned by neuronal representations of time. Within this framework, the authors performed complex and challenging experiments, along with advanced data analysis, which undoubtedly merits acknowledgement. However, the question of time perception is a challenging one, and caution should be exercised when applying abstract ideas derived from human cognition to animals. Studying so-called time perception in rats has significant shortcomings because, whether acknowledged or not, rats do not passively estimate time in their heads. They are constantly in motion. Moreover, rats do not perform the task for the sake of estimating time but to obtain their rewards are they water restricted. Their behavior will therefore reflect their motivation and urgency to obtain rewards. Unfortunately, it appears that the authors are not aware of these shortcomings. These alternative processes (motivation, sensorimotor dynamics) that occur during task performance are likely to influence neuronal activity. Consequently, my review will be rather critical. It is not however intended to be dismissive. I acknowledge that the authors may have been influenced by numerous published studies that already draw similar conclusions. Unfortunately, all the data presented in this study can be explained without invoking the concept of time estimation. Therefore, I hope the authors will find my comments constructive and understand that as scientists, we cannot ignore alternative interpretations, even if they conflict with our a priori philosophical stance (e.g., duration can be explicitly estimated by reading neuronal representation of time) and anthropomorphic assumptions (e.g., rats estimate time as humans do). While space is limited in a review, if the authors are interested, they can refer to a lengthy review I recently

published on this topic, which demonstrates that my criticism is supported by a wide range of timing experiments across species (Robbe, 2023). In addition to this major conceptual issue that casts doubt on most of the conclusions of the study, there are also several major statistical issues.

Main Concerns

(1) The authors used a task in which rats must poke for a minimal amount of time (300 ms and then 1500 ms) to be able to obtain a drop of water delivered a few centimeters right below the nosepoke. They claim that their task is a time estimation task. However, they forget that they work with thirsty rats that are eager to get water sooner than later (there is a reason why they start by a short duration!). This task is mainly probing the animals ability to wait (that is impulse control) rather than time estimation per se. Second, the task does not require to estimate precise time because there appear to be no penalties when the nosepokes are too short or when they exceed. So it will be unclear if the variation in nosepoke reflects motivational changes rather than time estimation changes. The fact that this behavioral task is a poor assay for time estimation and rather reflects impulse control is shown by the tendency of animals to perform nose-pokes that are too short, the very slow improvement in their performance (Figure 1, with most of the mice making short responses), and the huge variability. Not only do the behavioral data not support the claim of the authors in terms of what the animals are actually doing (estimating time), but this also completely annihilates the interpretation of the Ca^{++} imaging data, which can be explained by motivational factors (changes in neuronal activity occurring while the animals nose poke may reflect a growing sense of urgency to check if water is available).

(2) A second issue is that the authors seem to assume that rats are perfectly immobile and perform like some kind of robots that would initiate nose pokes, maintain them, and remove them in a very discretized manner. However, in this kind of task, rats are constantly moving from the reward magazine to the nose poke. They also move while nose-poking (either their body or their mouth), and when they come out of the nose poke, they immediately move toward the reward spout. Thus, there is a continuous stream of movements, including fidgeting, that will covary with timing. Numerous studies have shown that sensorimotor dynamics influence neural activity, even in the prefrontal cortex. Therefore, the authors cannot rule out that what the records reflect are movements (and the scaling of movement) rather than underlying processes of time estimation (some kind of timer). Concretely, start cells could represent the ending of the movement going from the water spout to the nosepoke, and end cells could be neurons that initiate (if one can really isolate any initiation, which I doubt) the movement from the nosepoke to the water spout. Duration cells could reflect fidgeting or orofacial movements combined with an increasing urgency to leave the nose pokes.

(3) The statistics should be rethought for both the behavioral and neuronal data. They should be conducted separately for all the rats, as there is likely interindividual variability in the impulsivity of the animals.

(4) The fact that neuronal activity reflects an integration of movement and motivational factors rather than some abstract timing appears to be well compatible with the analysis conducted on the error trials (Figure 4), considering that the sensorimotor and motivational dynamics will rescale with the durations of the nose poke.

(5) The authors should mention upfront in the main text (result section) the temporal resolution allowed by their Ca^{+} probe and discuss whether it is fast enough in regard of behavioral dynamics occurring in the task.

Comments on the revised version

I have read the revised version of the manuscript and the rebuttal letter. My major concern was that the task used is not a time estimation task but primarily taps into impulse control and that animals are not immobile during the nose-poking epoch. I provided factual evidence for this (the animal's timing performance is poor and, on average, animals struggle to wait long enough), and I pointed to a review that discusses the results of many studies congruent with the importance of movement/motivation, not only in constraining the timing of reward-oriented actions during so-called time estimation tasks but also in powerfully modulating neuronal activity.

The authors' responses to my comments are puzzling and unconvincing. First, on the one hand, they acknowledge in their rebuttal letter the difficulty of demonstrating a neuronal representation of explicit internal estimation of time. Then, they seem to imply that this issue is beyond the scope of their study and focus in the rebuttal on whether the neuronal activity they report shows signs of being sensitive to movement and motivation, which they claim is independent of movement and motivation. This leads the authors to make no major changes in their manuscript. Their title, abstract, introduction, and discussion are largely unchanged and do not reflect the possibility that there are major confounding factors in so-called time estimation (rodents are not disembodied passive information processors) that may well explain some of the neuronal patterns. Evidently, the dismissive treatment by the authors is not satisfying. I will briefly restate my comments and reply to their responses and their new figure, which not only is unconvincing but raises new questions.

My comments were primarily focused on the behavioral task. The authors replied: "Studying the neural representation of any internal state may suffer from the same ambiguity [by ambiguity they meant that it is difficult to know if animals are explicitly estimating time]. With all due respect, however, we would like to limit our response to the scope of our results. According to the reviewer, two alternative interpretations of the task-related sequential activity exist." The authors imply that my comments are beyond the scope of their study. That is not true. My comments were targeted at the behavior of the animals, behavior they rely on to title their study: "Stable sequential dynamics in prefrontal cortex represents a subjective estimation of time." When I question whether the task and behavioral data presented are congruent with "subjective estimation of time," my comments are not beyond the scope of the study—they directly tackle the main point of the authors. Other researchers will read the title and abstract of this manuscript and conclude: "Here is a paper that provides evidence of a mechanism for animals estimating duration internally (because subjective time perception is assumed to be different from using clocks)." Still, there is a large body of literature showing that the behavior of animals in such tasks can be entirely explained without invoking subjective time perception and internal representation. How can the authors acknowledge that they can't be sure that mice are estimating time and then have such an affirmative title and abstract?

In my opinion, science is not just about forcing ideas (often reflecting philosophical preconceptions) on data and dismissing those who disagree. It is about discussing alternative possibilities fairly and being humble. In their revised version, I see no effort by the authors to investigate the importance of movement and motivation during their task or seriously engage with this idea. It's much easier to dismiss my comments as being beyond the scope of their results. According to the authors, it seems that movements and motivations play no role in the task. Still, the animals are water-restricted, and during the task, they will display decreased motivation (due to increased satiety), and their history of rewarded vs. non-rewarded trials will affect their behavior. This is one of the most robust effects seen across all behavioral studies. Moreover, the animals are constantly moving. Maybe the authors used a special breed of mice that behave like some kind of robots? I acknowledge that this is not easy to investigate, but if the authors did not use high-quality video recording or an experimental paradigm that allows disentangling motivational confounds, then they should

refrain from using big words such as subjective time estimation and discuss alternative representations by acknowledging the studies that do find that movement and motivation are present during reward-based timing tasks and do in fact modulate neuronal activity, even in associative brain regions.

To sustain their claim that what they reported is movement-independent, the authors provided a supplementary figure in which they correlated neuronal activity and head movement tracked using DeepLabCut. I have to say that I was particularly surprised by this figure. First, in the original manuscript, there was absolutely no mention of video recording. Now it appears in the methods section, but the description is very short. There is no information on how these video recordings were made. The quality of the images provided in Figure S2 is far from reassuring. It is unclear whether the temporal and spatial resolution would be good enough to make meaningful correlations. Fast head/orofacial movements that occur during nose-poking can be on the order of 20 Hz. To be tracked, this would require at least a 40 Hz sampling rate. But no sampling information is provided. The authors should explain how they synchronized behavioral and neuronal data acquisition. Could the authors share behavioral videos of the 5 sessions shown in Figure S2 so we can judge the behavior of the animals, the quality of the video, and the possibility of making correlations?

Figure S2A-F: I am not sure why the authors correlated nose-poking duration (time estimation) and the duration between upper and lower nose-pokes (reward-oriented movement). It is not relevant to the issue I raised. Without any information about video acquisition frame rate, the y-axis legend (frame) is not very informative. Still, in Figure S2A-F, Rat 5 shows a clear increase in nose-poke duration, which is congruent with decreased impulsivity. Is the time coding different in this rat compared to other rats? There are some similar trends in other animals (Rat 1 and maybe Rat 3), but what is surprising is the huge variability (big downward deflections in the nose-poke duration). I would not be surprised if those deflections occurred after a long pause in activity. Could the authors plot trial time instead of trial number? How do the authors explain such a huge deflection if the animals are estimating time?

Regarding Figure S2H: I don't see how it addresses my concern. My concern is that some of the Ca activity recorded during nose-poking reflects head movements. The authors need to show if they can detect head movement during nose-poking. Aligning the Ca data relative to head movement should give the same result as when aligning the data relative to the time at which the animals pull out of the upper nose-poke.

Minor comments:

In their introduction, the authors wrote: "While these findings [correlates of time perception] provide strong evidence for a neural mechanism of time coding in the brain, true causal evidence at single-cell resolution remains beyond reach due to technical limitations. Although inhibiting certain brain regions (such as medial prefrontal cortex, mPFC,22) led to disruption in the performance of the timing task, it is difficult to attribute the effect specifically to the ramping or sequential activity patterns seen in those regions as other processes may be involved. Lacking direct experimental evidence, one potential way of testing the causal involvement of 'time codes' in time estimation function is to examine their correlation at a finer resolution."

This statement is inaccurate at two levels. First, very good causal evidence has been obtained on this topic (see Monteiro et al., 2023, Nature Neuroscience), and see my News & Views on the strengths and weaknesses of this paper. Second, their proposal is inaccurate. Looking at a finer correlation will still be a correlative approach, and the authors will not be able to disentangle motor/motivation confounds.

<https://doi.org/10.7554/eLife.96603.2.sa1>

Author response:

The following is the authors' response to the original reviews.

eLife Assessment

This useful study reports how neuronal activity in the prefrontal cortex maps time intervals during which animals have to wait until reaching a reward and how this mapping is preserved across days. However, the evidence supporting the claims is incomplete as these sequential neuronal patterns do not necessarily represent time but instead may be correlated with stereotypical behavior and restraint from impulsive decision, which would require further controls (e.g. behavioral analysis) to clarify the main message. The study will be of interest to neuroscientists interested in decision making and motor control.

We thank the editors and reviewers for the constructive comments. In light of the questions mentioned by the reviewers, we have performed additional analyses in our revision, particularly aiming to address issues related to single-cell scalability, and effects of motivation and movement. We believe these additional data will greatly improve the rigor and clarity of our study. We are grateful for the review process of eLife.

Public Reviews:**Reviewer #1 (Public Review):***Summary:*

This paper investigates the neural population activity patterns of the medial frontal cortex in rats performing a nose poking timing task using in vivo calcium imaging. The results showed neurons that were active at the beginning and end of the nose poking and neurons that formed sequential patterns of activation that covaried with the timed interval during nose poking on a trial-by-trial basis. The former were not stable across sessions, while the latter tended to remain stable over weeks. The analysis on incorrect trials suggests the shorter non-rewarded intervals were due to errors in the scaling of the sequential pattern of activity.

Strengths:

This study measured stable signals using in vivo calcium imaging during experimental sessions that were separated by many days in animals performing a nose poking timing task. The correlation analysis on the activation profile to separate the cells in the three groups was effective and the functional dissociation between beginning and end, and duration cells was revealing. The analysis on the stability of decoding of both the nose poking state and poking time was very informative. Hence, this study dissected a neural population that formed sequential patterns of activation that encoded timed intervals.

We thank the reviewer for the positive comments.

Weaknesses:

It is not clear whether animals had enough simultaneously recorded cells to perform the analyses of Figures 2-4. In fact, rat 3 had 18 responsive neurons which probably is not enough to get robust neural sequences for the trial-by-trial analysis and the correct and incorrect trial analysis.

We thank the reviewer for the comment. Our imaging data generally yielded 50-150 cells in each session. The 18 neurons mentioned by the reviewer are from the duration cell category. We have now provided the number of imaged cells from each rat in the new Supplementary figure 1D. In addition, we have plotted the duration cells' sequential activity of individual trials for each rat in new Supplementary figure 1B and 1C. These data demonstrate robust sequential activities from the duration cells.

In addition, the analysis of behavioral errors could be improved. The analysis in Figure 4A could be replaced by a detailed analysis on the speed, and the geometry of neural population trajectories for correct and incorrect trials.

We thank the reviewer for the suggestions. We have now performed analyses of the neural population trajectories as the reviewer suggested. We have calculated the neural population trajectories using the first two principal components of the neural activities during nose poke events. While both correct and incorrect trials show similar shapes of the trajectories, correct trials show more expanded paths, with longer lengths on average. These new results are now updated in Figure 4. Since type I or type II errors would likely generate trajectories not following the general direction which is different from our observations, these results are consistent with our conclusion that scaling errors contribute to the incorrect behavior timing in these rats.

In the case of Figure 4G is not clear why the density of errors formed two clusters instead of having a linear relation with the produce duration. I would be recommendable to compute the scaling factor on neuronal population trajectories and single cell activity or the computation of the center of mass to test the type III errors.

To clarify the original Figure 4G, the correct trials tended to show positive time estimation errors while the incorrect trials showed negative time estimation errors. We believe that the polarity switch between these two types suggests a possible use of this neural mechanism to time the action of the rats.

In addition, we have performed the analysis suggested by the reviewer in our revision. We calculated two types of scaling factors. On individual cell level, we computed the peak position of individual trials to the expected positions from averaged template. And on neural population level, we searched for a scaling multiplier to resample the calcium activity data and minimized the differences between scaled activity and the expected template. Using these two factors, we found that correct trials show significantly larger scaling compared to incorrect trials, consistent with our original interpretation that behavior errors are primarily correlated with scaling errors in the neural activities (type III error). These new results are now incorporated in Figure 4 and we have also updated the main text for the descriptions.

Due to the slow time resolution of calcium imaging, it is difficult to perform robust analysis on ramping activity. Therefore, I recommend downplaying the conclusion that: "Together, our data suggest that sequential activity might be a more relevant coding regime than the ramping activity in representing time under physiological conditions."

We agree with the reviewer, and have now modified this sentence in the abstract.

Reviewer #2 (Public Review):

In this manuscript, Li and collaborators set out to investigate the neuronal mechanisms underlying "subjective time estimation" in rats. For this purpose, they conducted calcium imaging in the prefrontal cortex of water-restricted rats that were required to perform an action (nosepoking) for a short duration to obtain drops of water. The authors provided evidence that animals progressively improved in performing their task. They

subsequently analyzed the calcium imaging activity of neurons and identify start, duration, and stop cells associated with the nose poke. Specifically, they focused on duration cells and demonstrated that these cells served as a good proxy for timing on a trial-by-trial basis, scaling their pattern of activity in accordance with changes in behavioral performance. In summary, as stated in the title, the authors claim to provide mechanistic insights into subjective time estimation in rats, a function they deem important for various cognitive conditions.

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Main Concerns

(1) The authors used a task in which rats must poke for a minimal amount of time (300 ms and then 1500 ms) to be able to obtain a drop of water delivered a few centimeters right below the nosepoke. They claim that their task is a time estimation task. However, they forget that they work with thirsty rats that are eager to get water sooner than later (there is a reason why they start by a short duration!). This task is mainly probing the animals ability to wait (that is impulse control) rather than time estimation per se. Second, the task does not require to estimate precisely time because there appear to be no penalties when the nosepokes are too short or when they exceed. So it will be unclear if the variation in nosepoke reflects motivational changes rather than time estimation changes. The fact that this behavioral task is a poor assay for time estimation and rather reflects impulse control is shown by the tendency of animals to perform nose-pokes that are too short, the very slow improvement in their performance (Figure 1, with most of the mice making short responses), and the huge variability. Not only do the behavioral data not support the claim of the authors in terms of what the animals are actually doing (estimating time), but this also completely annihilates the interpretation of the Ca⁺⁺ imaging data, which can be explained by motivational factors (changes in neuronal

activity occurring while the animals nose poke may reflect a growing sense of urgency to check if water is available).

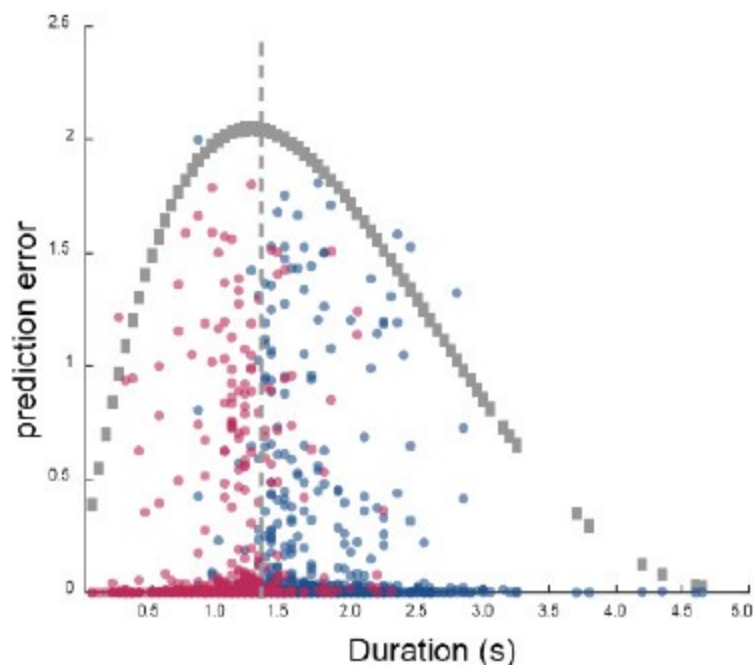
We would like to respond to the reviewer's comments 1, 2 and 4 together, since they all focus on the same issue. We thank the reviewer for the very thoughtful comments and for sharing his detailed reasoning from a recently published review (Robbe, 2023). A lot of discussions go beyond the scope of this study, and we agree that whether there is an explicit representation of time (an internal clock) in the brain is a difficult question to be answered, particularly by using animal behaviors. In fact, even with fully conscious humans and elaborated task design, we think it is still questionable to clearly dissociate the neural substrate of "timing" from "motor". In the end, it may as well be that as the reviewer cited from Bergson's article, the experience of time cannot be measured.

Studying the neural representation of any internal state may suffer from the same ambiguity. With all due respect, however, we would like to limit our response to the scope of our results. According to the reviewer, two alternative interpretations of the task-related sequential activity exist: 1, duration cells may represent fidgeting or orofacial movements and 2, duration cells may represent motivation or motion plan of the rats. To test the first alternative interpretation, we have now performed a more comprehensive analysis of the behavior data at all the limbs and visible body parts of the experimental rats during nose poke and analyzed its periodicity among different trials. We found that the coding cells (including duration, start and end cells) activities were not modulated by these motions, arguing against this possibility. These data are now included in the new Supp. Figure 2, and we have added corresponding texts in the manuscript.

Regarding the second alternative interpretation, we think our data in the original Figure 4G argues against it. In this graph, we plotted the decoding error of time using the duration cells' activity against the actual duration of the trials. If the sequential activity of duration cells only represents motivation, then the errors should be linearly modulated by trial durations. The unimodal distribution we observed (Figure 4G and see graph below for a re-plot without signs) suggests that the scaling factor of the sequential activity represents information related to time. And the fact that this unimodal distribution centered at the time threshold of the task provides strong evidence for the active use of scaling factor for time estimation.

In order to further test the relationship to motivation, we have measured the time interval between exiting nose poke to the start of licking water reward as an independent measurement of motivation for each trial. We found that this reward-seeking time was positively correlated with the trial durations, suggesting that the durations were correlated with motivation to some degree. And when we scaled the activities of the duration cells by this reward-seeking time, we found that the patterns of the sequential activities were largely diminished, and showed a significantly lower peak entropy compared to the same activities scaled by trial durations. The remaining sequential pattern may be due to the correlation between trial durations and motivation (Supp. Figure 2), and the sequential pattern reflects timing more prominently. These analyses provide further evidence that the sequential activities were not coding motivations. These data are included in Figure 2F, 2K and supp. Figure 3 in revised manuscript.

Author response image 1.



Regarding whether the scaling sequential activity we report represents behavioral timing or true time estimation, we did not have evidence on this point. However, a previous study has shown that PFC silencing led to disruption of the mouse's timing behavior without affecting the execution of the task (PMID: 24367075), arguing against the behavior timing interpretation. The main surprising finding of our present study is that these duration cells are different from the start and end cells

in terms of their coding stability. Thus, future studies dissecting the anatomical microcircuit of these duration cells may provide further clues regarding whether they are connected with reward-related or motion-related brain regions. This may help partially resolve the “time” vs.

“motor” debate the reviewer mentioned.

(2) A second issue is that the authors seem to assume that rats are perfectly immobile and perform like some kind of robots that would initiate nose pokes, maintain them, and remove them in a very discretized manner. However, in this kind of task, rats are constantly moving from the reward magazine to the nose poke. They also move while nose-poking (either their body or their mouth), and when they come out of the nose poke, they immediately move toward the reward spout. Thus, there is a continuous stream of movements, including fidgeting, that will covary with timing. Numerous studies have shown that sensorimotor dynamics influence neural activity, even in the prefrontal cortex. Therefore, the authors cannot rule out that what the records reflect are movements (and the scaling of movement) rather than underlying processes of time estimation (some kind of timer). Concretely, start cells could represent the ending of the movement going from the water spout to the nosepoke, and end cells could be neurons that initiate (if one can really isolate any initiation, which I doubt) the movement from the nosepoke to the water spout. Duration cells could reflect fidgeting or orofacial movements combined with an increasing urgency to leave the nose pokes.

(3) The statistics should be rethought for both the behavioral and neuronal data. They should be conducted separately for all the rats, as there is likely interindividual variability in the impulsivity of the animals.

We thank the reviewer for the comment, yet we are not quite sure what specifically was asked by the reviewer. It appears that the reviewer requires we conduct our analysis using each rat individually. In our revised manuscript, we have conducted and reported analyses with individual rat in the original Figure 1C, Figure 2C, G, K, Figure 4F.

(4) The fact that neuronal activity reflects an integration of movement and motivational factors rather than some abstract timing appears to be well compatible with the analysis conducted on the error trials (Figure 4), considering that the sensorimotor and motivational dynamics will rescale with the durations of the nose poke.

(5) The authors should mention upfront in the main text (result section) the temporal resolution allowed by their Ca⁺ probe and discuss whether it is fast enough in regard of behavioral dynamics occurring in the task.

We thank the reviewer for the suggestion. We have originally mentioned the caveat of calcium imaging in the interpretation of our results. We have now incorporated more texts for this purpose during our revision. In terms of behavioral dynamics (start and end of nose poke in this case), we think calcium imaging could provide sufficient kinetics. However, the more refined dynamics related to the reproducibility of the sequential activity or the precise representation of individual cells on the scaled duration may be benefited from improved time resolution.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

(1) Please refer explicitly to the three types of cells in the abstract.

We have now modified the abstract as suggested during revision.

(2) Please refer to the work of Betancourt et al., 2023 Cell Reports, where a trial-by-trial analysis on the correlation between neural trajectory dynamics in MPC and timing behavior is reported. In that same paper the stability of neural sequences across task parameters is reported.

We have now cited and discussed the study in the discussion section of the revised manuscript.

(3) Please state the number of studied animals at the beginning of the results section.

We have now provided this information as requested. The numbers of rats are also plotted in Figure 1D for each analysis.

(4) Why do the middle and right panels of Figure 2E show duration cells.

Figure 2E was intended to show examples of duration cells' activity. We included different examples of cells that peak at different points in the scaled duration. We believe these multiple examples would give the readers a straight forward impression of these cells' activity patterns.

| (5) Which behavioral sessions of Figure 1B were analyzed further.

We have now labeled the analyzed sessions in Figure 1B with red color in the revised manuscript.

| (6) In Figure 3A-C please increase the time before the beginning of the trial in order to visualize properly the activation patterns of the start cells.

We thank the reviewer for the suggestion and have now modified the figure accordingly in the revised manuscript.

| (7) Please state what could be the behavioral and functional effect of the ablation of the cortical tissue on top of mPFC.

We thank the reviewer for the question. In our experience, mice with lens implanted in the mPFC did not show observable difference with mice without surgery in the acquisition of the task and the distribution of the nose-poke durations. In our dataset, rats with the lens implantation showed similar nose-poking behavior as those without lens implantation (Figure 1B). Thus, it seems that the effect of ablation, if any, was quite limited, in the scope of our task.

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