

Stable sequential dynamics in prefrontal cortex represents subjective estimation of time

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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. By aligning long-term time-lapse datasets, we discovered that sequential patterns of time coding were stable over weeks, while cells with ramping activity patterns 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 might be a more relevant coding regime than the ramping activity in representing 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.

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.

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Introduction

Time estimation is an essential function in the brain^{1,2,3}, 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 space¹⁶⁻¹⁸. 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 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 (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).

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 sensor. These three types

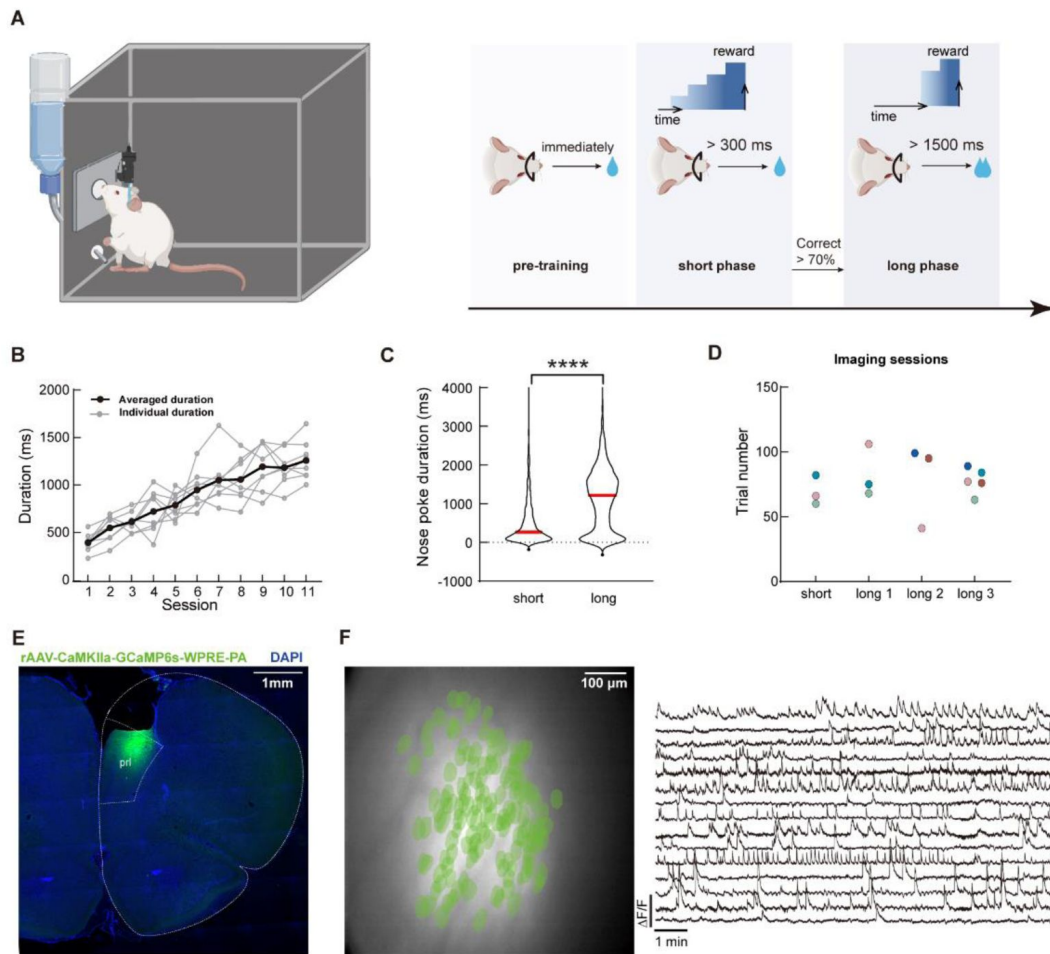


Figure 1.

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.
- 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).

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**).

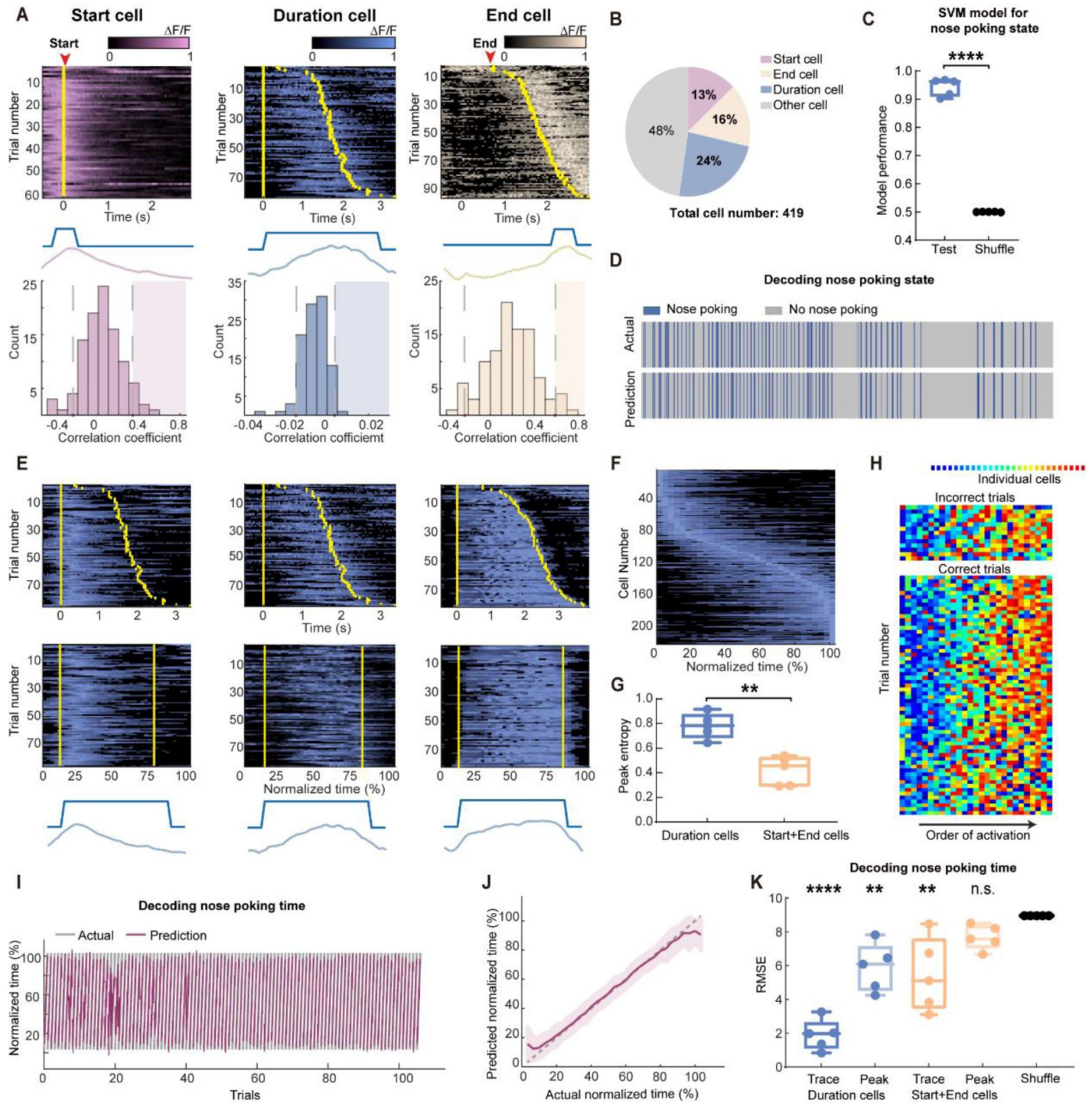


Figure 2.

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

- A.** The definition and examples of start (purple), duration (blue) and end (yellow) - 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 activity of all recorded duration cells in 5 rats, sorted by the ascending peak position in the normalized time.
- G.** Quantification of the peak entropy on the trial-average calcium traces as a measure of sequentiality. Two-tailed t test, **: $p < 0.01$, $n = 5$ rats.
- H.** 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.
- I-J.** 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 (**I**) and averaged trial (**J**). Results are represented as mean $\pm$ S.D.
- K.** 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.

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** [↗](#)). The duration cell activity exhibited robust sequentiality as measured by peak entropy ²⁵[↗](#),²⁶[↗](#), which was not seen in activities from start or end cells (**Figure 2G** [↗](#)). This activity sequence was stable across all trials within a session, regardless of whether rat reached

minimal reward threshold (**Figure 2H**[↗](#), **Supp. Figure 1B-C**[↗](#)). Furthermore, we were able to train a Gaussian process regression model to predict the progress of each trial with high accuracy (**Figure 2I-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 2K**[↗](#)). On the other hand, peak-extracted activities from duration cells maintained high decoding power, indicating a more reliable sequential activity pattern. 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 events²⁷[↗](#) and time points²⁴[↗](#). Interestingly, these sequences were highly dynamic, and the ordered pattern showed significant session-to-session variations²⁸[↗](#),²⁹[↗](#). 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 2**[↗](#)). 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. 2**[↗](#)).

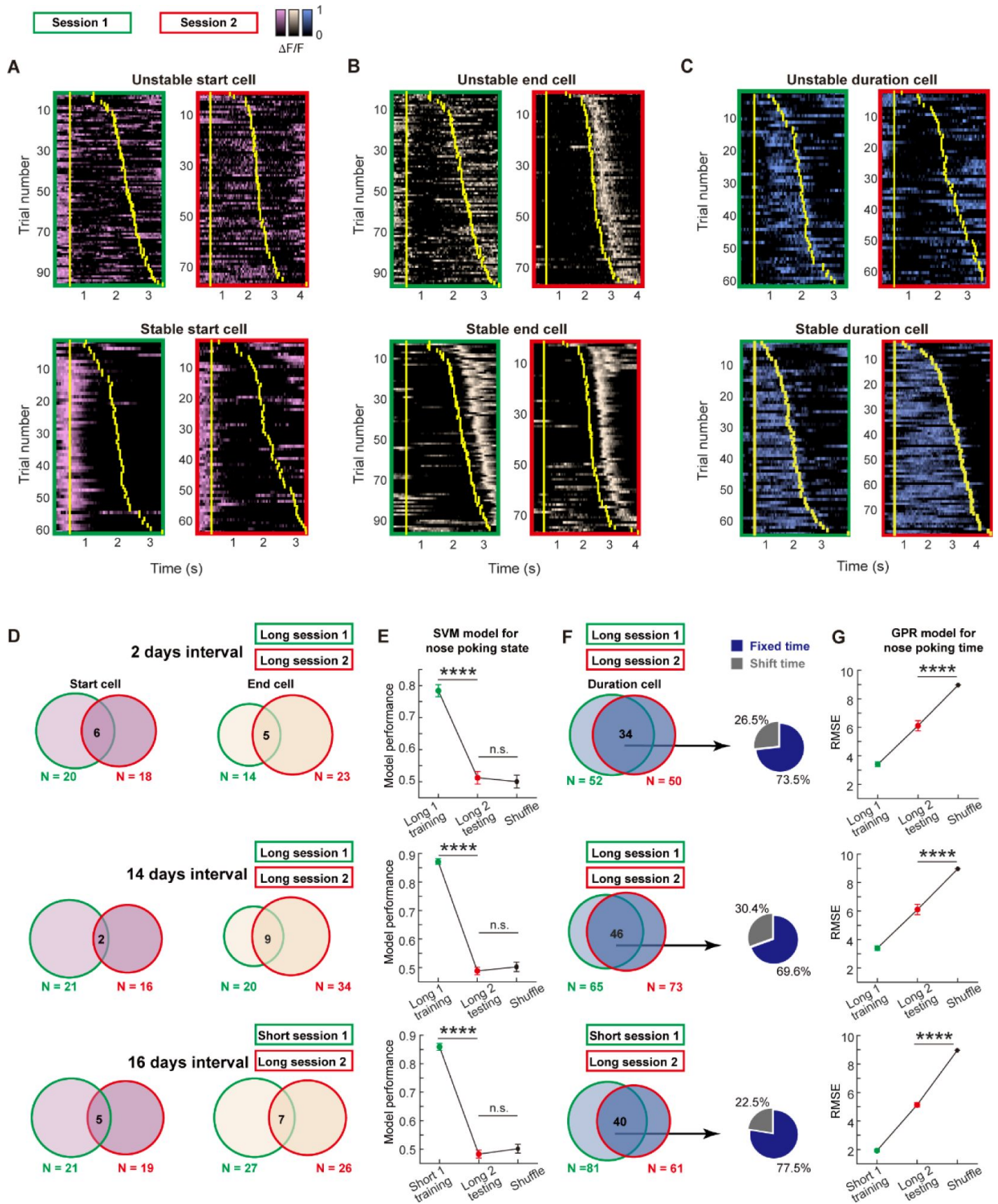


Figure 3.

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

A-C. Example traces of unstable or stable coding start cell (purple, **A**), end cell (yellow, **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.

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 hippocampus²⁹ and cortex³⁰ 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.

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 by partial least square regression of the neural activities in individual trials to normalized time, and found that almost all trials showed similar trajectories, indicating a lack of type I error described above (**Figure 4A** [↗](#)). In addition, we found that duration cells were more important for this trajectory pattern compared to other cells that we observed (**Figure 4B** [↗](#)). The constructed trajectories could explain ~52% of the variance in the normalized time, which was primarily contributed by the activities from duration cells (**Figure 4C** [↗](#)). When we examined the raw activities from duration cells in individual error trials, we also found only a small fraction showed the absence of sequential activities (**Supp. Figure 3** [↗](#)). Furthermore, we can find duration cells that showed stable coding timepoints regardless of correct or incorrect trials, and some duration cells with their activity modulated by trial types (**Figure 4D** [↗](#)), suggesting that precise time estimation from these sequential activities may be affected. We then quantified the time estimation errors between the predicted time from duration cell neural activity and actual time using our previously established GPR models (**Figure 4E** [↗](#)). Although we observed some fluctuations in the accuracy, in general we did not find significant difference between correct and incorrect trials, and we were able to train a common model that showed accurate time estimation for both trial types (**Figure 4F** [↗](#)), indicating a lack of type II error.

To estimate the scaling errors associated with the type III errors, we calculated the cumulative errors between GPR model predicted time based on neural activities and actual trial time. When we plotted these data against actual individual trial durations, we found that most trials were scattered around zero, indicating relatively high accuracy in the time estimation. Interestingly, incorrect trials showed a cluster centered below zero, and correct trials showed one above zero (**Figure 4G** [↗](#)), indicating that during the majority of the incorrect trials the rats timed too short. Surprisingly, the duration time at which the polarity of the cumulated 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 ^{31,32}, motor cortex ^{33,34}, sensory cortex ³⁵⁻³⁷, striatum ^{11,38-40}, thalamus ⁴¹, hippocampus ^{12,42,43}, and entorhinal cortex ^{30,44}. 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 ^{2,45}. 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. 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 experiments ⁴⁶, yet the low throughput and

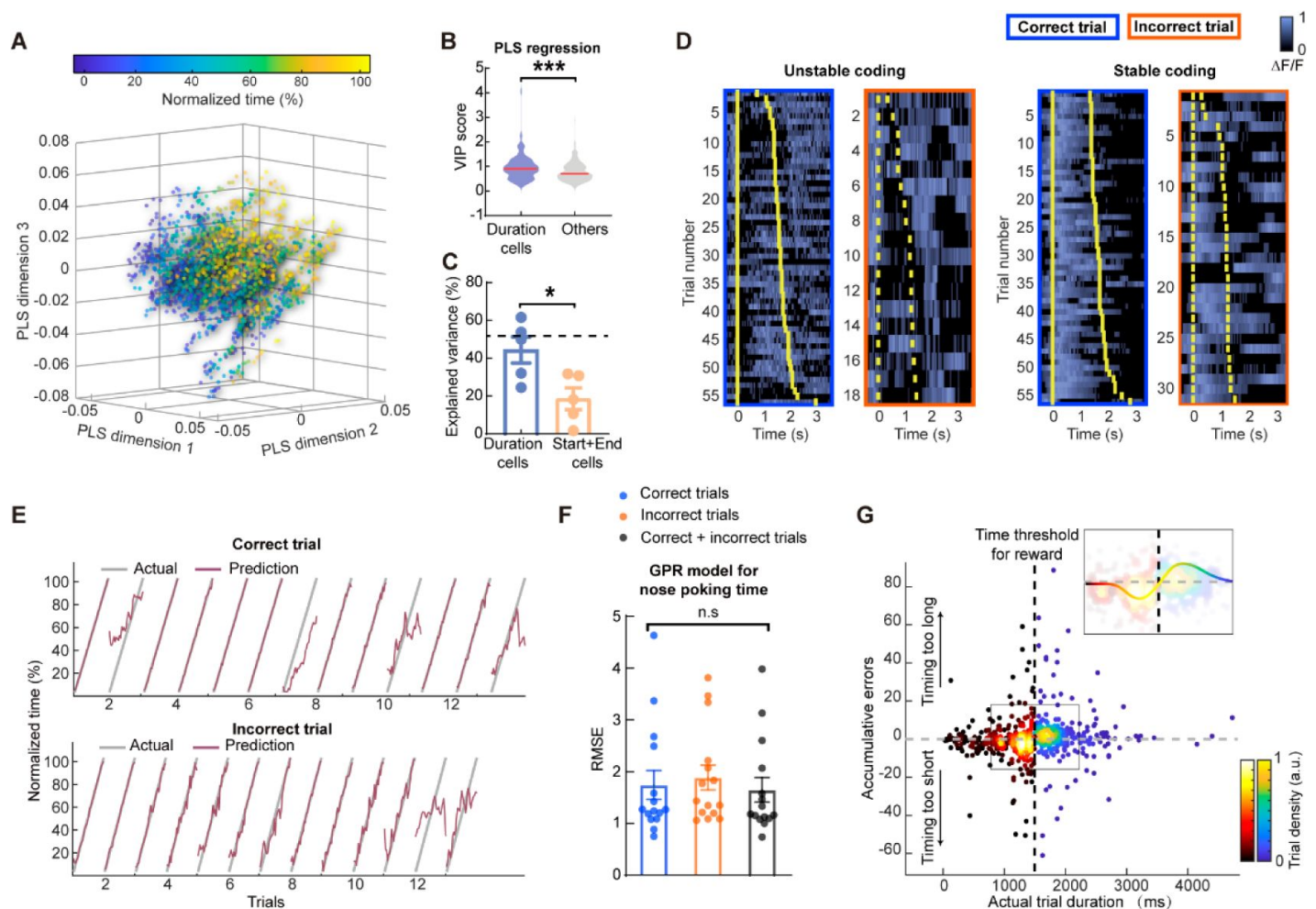


Figure 4.

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

A. Scatter plots of the first 3 dimensions of a partial least-square (PLS) regression model between neuron activities and normalized trial time.

B. Violin plot of the variable importance for projection (VIP) scores derived from the PLS regression in **A**. Two-tailed t test, ***: $p < 0.0001$.

C. 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.

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. 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.

F. 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.

G. 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.

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 durations²¹, 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. Future advances in single-cell manipulations may provide more definitive experimental evidence.

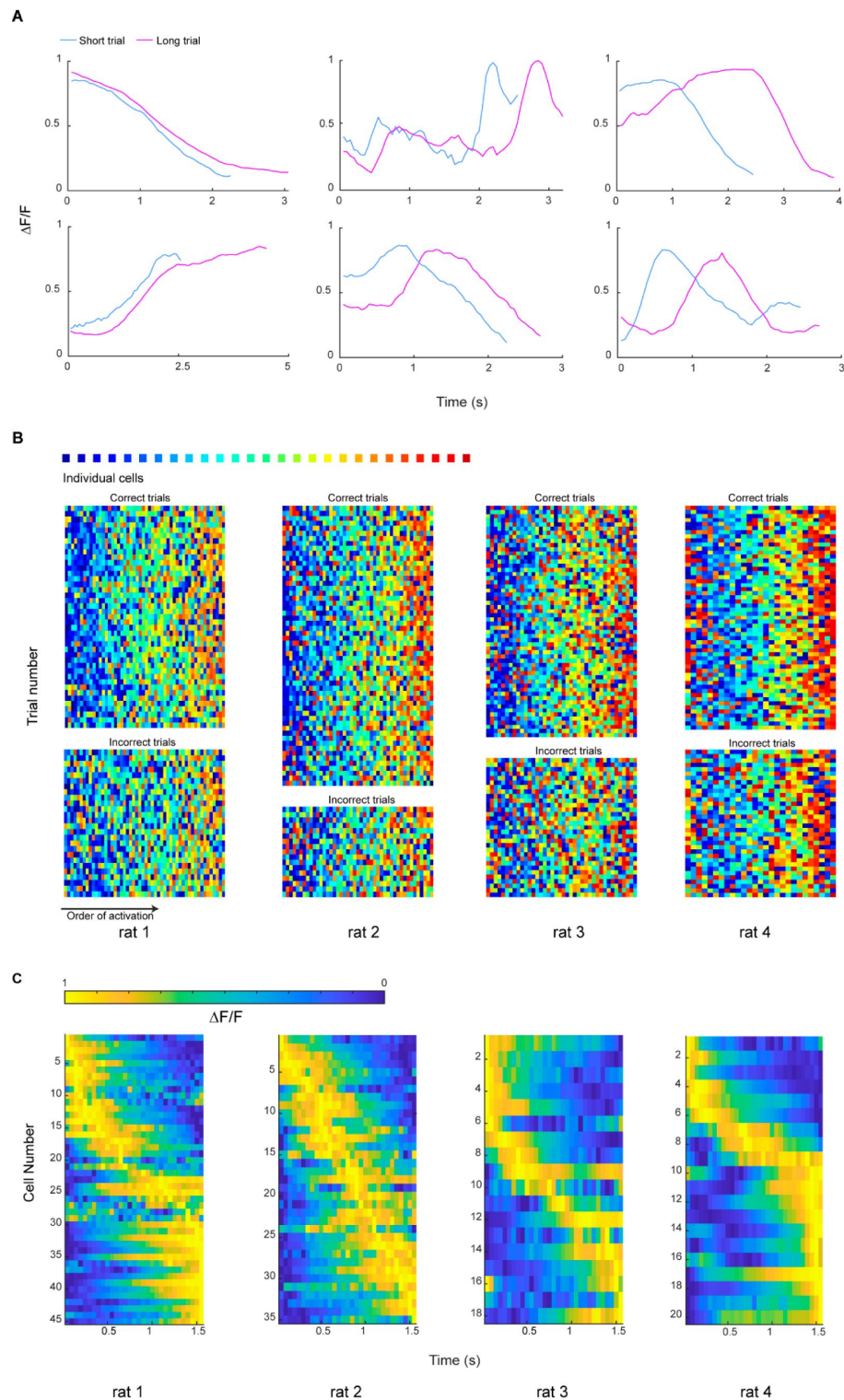
The second limitation in our understanding for the neural basis of time perception is the lack of biophysical mechanism for the emergence of sequential patterns and the scaling effect. In this regard, several theoretical models have been proposed, yet testing these models in biological systems is currently not possible. Our data demonstrated a unique stability of the duration cells and their sequential activity patterns for coding time. 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, and activities related to event trajectories are generally considered unstable. 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. Such feature 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.

Author contributions

CL.M. and BM.L. designed the study. YTL., WQ.L. and JW.L. performed the experiments. P.Y., SL.Z. and YTL. performed data analysis. YTL. drafted the manuscript. P.Y., CL.M. and BM.L. revised the manuscript.

Competing interest statement

The authors claim no competing interest.



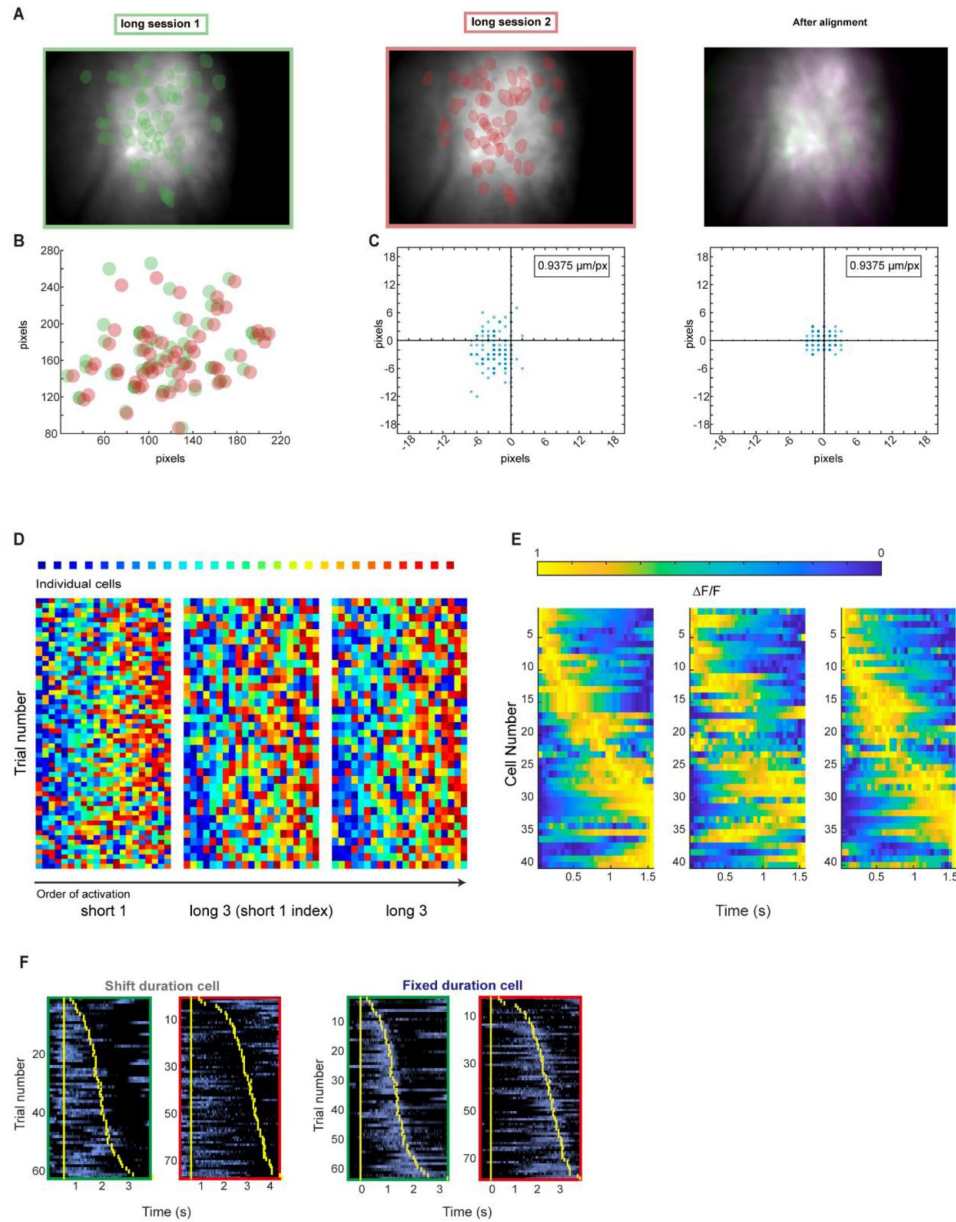
Supplementary Figure 1

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](#).

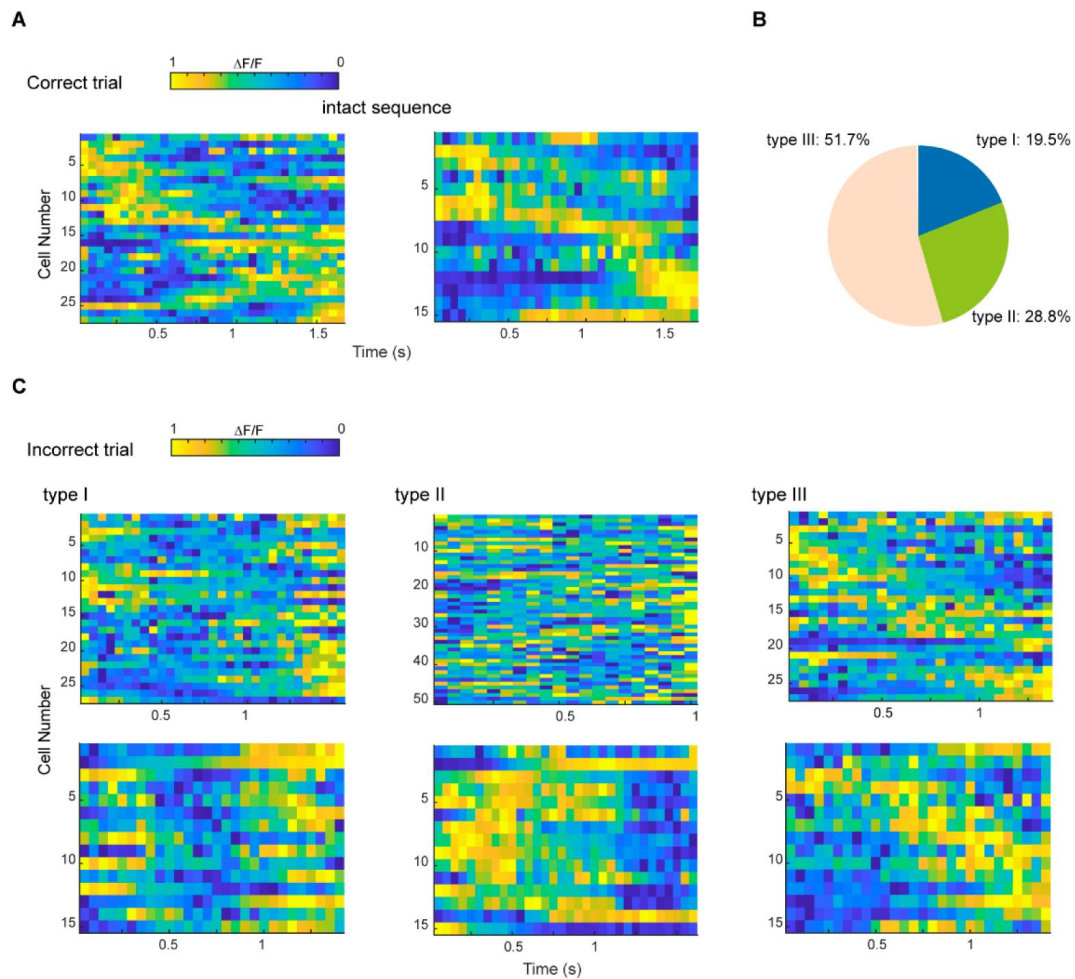
C. Individual rat's duration cell activity in trial-average form. Cells were sorted by their peak position in each graph.



Supplementary Figure 2

Additional information related to cross-session alignment.

- A.** Representative field of view for two sessions from the same rat. Green and red shades indicate extracted cells' position masks from each session. The right panel shows the matched cells' positions after alignment.
- B.** Scatter plots of the cell masks coordinates in two movies.
- C.** Scatter plots of the matched cell's distances between session before (left) and after (right) alignment.
- D.** 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.
- E.** Representative trial-averaged activities in normalized time scale from the same rat's two sessions, with the same sorting scheme described in **D**.
- F.** 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 3

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.

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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 homecage after waking up from anesthesia.

GRIN lens implantation

Our surgical method is based on the surgical procedure reported in mice⁴⁹[\[49\]](#). 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 2.5mm 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 homecage 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 NoRMCorre algorithm⁵⁰[\[48\]](#). 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^{51,52}. 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.

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 intrapolation. 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.

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

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

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

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.

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. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

0.001; **** $p < 0.0001$

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Editors

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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 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.

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."

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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.

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 as 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 cast 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 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).

(#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.

<https://doi.org/10.7554/eLife.96603.1.sa0>

Author response:

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 plan to perform 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 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.

We thank the reviewer for the comment. We would like to mention that the 18 cells plotted in Supplementary figure 1 were only from the duration cell category. To improve the clarity of our results, we are going to provide information regarding the number of cells from each rat in our revision. In general, we imaged more than 50 cells from each rat. We would also like to point to the data from individual trials in Supplementary figure 1B showing robust sequentiality.

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 are going to conduct the analysis as the reviewer recommended. We agree with the reviewer that better presentation of the neural activity will be helpful for the readers.

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.

We would like to mention that the prediction errors plotted in this graph were calculated from two types of trials. 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 suggested a possible use of this neural mechanism to time the action of the rats.

In addition, we are going to perform the analysis suggested by the reviewer in our revision. We agree that different ways of analyzing the data would provide better characterization of the scaling effect.

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 we have mentioned this caveat in our original manuscript. We are going to rephrase the sentence as the reviewer suggested during our revision.

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.

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 reflects 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 cast doubt on most of the conclusions of the study, there are also several major statistical issues.

Main Concerns

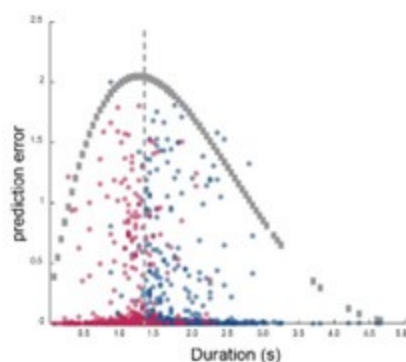
(1) The authors used a task in which rats must poke for a minimal amount of time (300 ms and then 1500 ms) to be able to obtain a drop of water delivered a few centimeters right below the nosepoke. They claim that their task is a time estimation task. However, they forget that they work with thirsty 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 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 the discussion goes 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 answer, 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 in 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 will perform a more comprehensive analysis of the behavior data at all the limbs and visible body parts of the rat during nose poke and analyze its periodicity among different trials, although the orofacial movements may not be visible to us.

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 durations cells only represents motivation, then the errors should distribute evenly across different trial times, or linearly modulated by trial durations. The unimodal distribution we observed (Figure 4G and see Author response image 1 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 will measure the time interval between exiting nose poke to the start of licking water reward as an independent measurement of motivation for each trial. We will analyze and report whether this measurement correlates with the nose poking durations in our data in the revision.

Author response image 1.



Furthermore, whether the scaling sequential activity we report represents behavioral timing or true time estimation, the reviewer would agree that these activities correlate with the animal's nose poking durations, and a previous study has showed that PFC silencing led to disruption of the mouse's timing behavior (PMID: 24367075). The main surprising finding of the paper 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 clue regarding whether they receive inputs from thirst or reward-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. There is undoubtedly variance among individual animals. One of the core reasons for statistical comparison is to compare the group difference with the variance due to sampling. It appears that the reviewer would like to require we conduct our analysis using each rat individually. We will conduct and report analysis with individual rat in Figure 1C, Figure 2C, G, K, Figure 4F in our revised manuscript.

(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 will incorporate 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 will modify 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 will cite and discuss this study in our revised paper.

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

We will provide this information as requested. The number of animals were 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 will label the analyzed sessions in Figure 1B during our revision.

(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 will modify the figure accordingly during revision.

(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 mPFC did not show observable different to mice without surgery regarding the acquisition of the task and the distribution of the nose-poke durations. Although we could not rule out the effect on other cognitive process, the mice appeared to be intact in the scope of our task. We will provide these behavior data during our revision.

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