

## **Spontaneous behavior is a succession of self-directed tasks**

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## SUMMARY

Animals achieve high-level goals by sequencing low-level actions. This transformation is best understood in structured tasks that impose a specific mapping between goals and actions. However, it remains unclear whether spontaneous behavior is similarly organized in the service of identifiable goals, or how it might be supported by brain regions responsible for goal-oriented behavior such as prefrontal cortex (PFC). Here we show that low-level actions in freely exploring mice are hierarchically organized into seconds-long behavioral states that correspond to task-like programs of behavior. These persistent states structure neural activity in PFC, which preferentially encodes the identity of states relative to low-level behavioral features, and shapes which states are expressed in a given context. These findings argue that spontaneous behavior is organized as a succession of self-directed tasks and identify principles of neural control common to structured tasks and spontaneous exploration.

## INTRODUCTION

The brain translates goals into actions. Systems neuroscience typically studies this transformation through structured tasks in which animals generate specific actions (e.g., licking left or right) to earn rewards<sup>1</sup>. In this framework, movements linked to reward are considered purposive<sup>2,3</sup>. Evidence from such tasks suggests that goal-oriented behaviors rely on the prefrontal cortex (PFC), which encodes key information about context-, task-, and value-related variables<sup>4-15</sup>. In contrast, when animals act spontaneously — as they do most of the time in the natural world — it is not obvious whether actions are organized around specific goals or whether PFC similarly encodes task-related information<sup>16</sup>.

Inferring the purpose of behavior is challenging because most low-level actions are compatible with many high-level goals<sup>17,18</sup>. For example, mice exploring an open field can establish home bases, plan escape routes, and attend to novel objects and odors — all of which employ common actions (e.g., running, sniffing, rearing) that might also occur in the absence of a specific goal<sup>19-22</sup>. Consistent with the idea that spontaneous behavior is not always goal-oriented, PFC lesions have little effect on open field exploration, at least when measured using conventional metrics<sup>23-27</sup>.

One strategy for connecting low-level actions to high-level goals arises from ethology, which uses the grouping of actions over time to illuminate their functions<sup>28,29</sup>. An animal might crouch, stalk and sprint while hunting, for example, but burrow and manipulate bedding to build a nest<sup>29</sup>. This organization leaves a statistical signature: frequently co-occurring movements are likely to serve the same goal, and sudden shifts between groups of movements can mark when one goal succeeds another<sup>30</sup>. A recent explosion of machine learning tools has given us unprecedented access to animal's low-level movements, including the sub-second behavioral motifs (or "syllables") that compose mouse behavior<sup>31-33</sup>. However, we currently lack tools for identifying behavioral states on longer timescales<sup>34</sup>.

Given such a tool, several criteria would, if fulfilled, indicate that the identified states reflect goal-directed tasks. First, each state should include movements that are *prima facie* related to a task. Second, the states expressed in a given context should depend on available affordances<sup>35</sup> (i.e., elements of the environment that allow some form of interaction). Finally, information about states and corresponding task-related variables should be present in brain regions that support goal-directed behavior, such as PFC<sup>4,11,12,14,15,36</sup>. Indeed it is sometimes possible to infer the structure of a task *a priori* from PFC recordings, as recently demonstrated in rats trained to play hide-and-seek<sup>37</sup>. In social contexts, PFC neurons represent spatial location, social proximity, and

conspecific actions, all of which are key to effective social behavior<sup>38-44</sup>. However, there have been few recordings of PFC outside of structured tasks or social interactions (but see<sup>45,46</sup>), so whether PFC encodes hierarchical features of spontaneous behavior remains largely untested.

Here we test whether spontaneous behavior is organized into self-directed tasks that are legible in PFC activity. To do so, we develop a novel hierarchical model that identifies seconds-to-minutes-long behavioral states from patterns of syllables. We find that dorsomedial PFC (dmPFC) activity is dominated by behavioral states rather than low-level movements. Lesions demonstrate that dmPFC promotes the expression of less-used behavioral states when appropriate in a given context. These results suggest that spontaneous behavior consists of self-initiated tasks whose appropriate selection depends on dmPFC and establishes common principles shared by experimenter-defined tasks and self-guided exploration.

## RESULTS

To test whether spontaneous behavior is organized into task-like states anchored to neural activity, we performed calcium imaging in dmPFC<sup>47</sup> while monitoring behavior with depth camera-based Motion Sequencing (MoSeq). MoSeq is an unsupervised machine learning approach for segmenting behavior into sub-second syllables like rears, turns or sniffs<sup>32,48-50</sup>. We recorded male mice in an open field arena that was alternately empty or supplemented with a male conspecific belonging to a strain (C57) that is less prone to aggression in novel environments (Figures 1A and S1A–S1C; Videos S1 and S2)<sup>51</sup>. In this setting MoSeq identified 72 behavioral syllables, including various forms of locomotion, rearing, grooming, and investigation (Figures 1B and S1D–S1F)<sup>52</sup>.

### Spontaneous behavior is organized into higher-order behavioral states

Ethologists recognize structure in animal behavior by analyzing how actions cluster over time; behavior possesses higher-order structure when this clustering cannot be explained through simple (Markovian) transition rules<sup>30,34</sup>. In our recordings, subsets of kinematically-related syllables co-occurred in bouts lasting up to a minute, suggesting that spontaneous behavior is structured over long timescales (Figure 1C). To test if this structure was non-Markovian, we compared observed syllable sequences to synthetic sequences drawn from a Markov model. In the synthetic sequences, mutual information between syllables (which captures the predictability of behavior over time) decayed to chance in just a few seconds (Figure 1D)<sup>53</sup>. In contrast, real syllable sequences remained predictable for tens of seconds (Figures 1D and S1G). This order-of-magnitude gap persisted across a variety of syllable timescales and resolutions<sup>54</sup> (Figures S1H–S1M). Thus, simple transition rules cannot explain the persistent patterns captured by MoSeq, implying that higher-order states may organize behavior on a seconds-to-minutes timescale.

Hierarchical hidden Markov models (HHMMs) offer one approach for identifying such states. In an HHMM, each state specifies the expected usage and transition frequencies between MoSeq syllables. Until now, hierarchical extensions of MoSeq have faltered on the large number of syllables and their strong autocorrelations<sup>32</sup> (Figures S2A–S2B). To overcome these challenges, we devised a new type of data-efficient HHMM called state-based hierarchical MoSeq (shMoSeq). ShMoSeq has three levels corresponding to different timescales in behavior: a chain of multi-second behavioral states, which bias the selection of sub-second syllables, which in turn govern the dynamics of pose (Figure 1E). Each behavioral state is defined by a unique matrix describing transitions between syllables, which shMoSeq constructs by combining a

baseline transition matrix (that is shared across states) with syllable usage biases that are unique to each state.

ShMoSeq effectively captures the range of timescales in behavior; synthetic syllable sequences generated by shMoSeq were virtually identical to real syllable sequences when assessed by mutual information, indicating that the dynamics instantiated by shMoSeq are sufficient to explain the temporal correlations observable in mouse behavior (Figure 1F). Furthermore, in contrast to syllables, shMoSeq-derived state sequences were well-approximated by a simple Markov chain, suggesting that additional hierarchical layers are not necessary to explain behavior within the one-hour timespan of our recordings (Figure 1G).

During our initial experiments (in which mice explored an open field and interacted with a C57 conspecific), shMoSeq identified five behavioral states including grooming, investigation (of either the mouse's immediate surroundings or the conspecific), and two forms of exploratory locomotion in which mice circumnavigated the arena in a clockwise or counterclockwise pattern (Figures 1H–1I, S2C–S2J and Video S3). Consistent with the premise that similar movements can serve different high-level goals<sup>30</sup>, the mapping between syllables and states was not one-to-one; except for grooming, most syllables were used in most states, and even grooming syllables were neither limited to — nor continuous during — the grooming state (Figure 1J). Consequently, there was often an interval of uncertainty as one state transitioned to the next, and up to several seconds of behavior were needed to classify the current state (Figures S2K–S2M). Nevertheless, the output of shMoSeq was largely consistent across model fits, suggesting that states are readily identifiable (Figure S2G). Together, these findings demonstrate that exploratory behavior is organized at three hierarchically nested timescales (millisecond poses, sub-second syllables, and seconds-long states) that are captured by shMoSeq.

### Affordances sculpt the distribution of behavioral states

Animal behavior is shaped by affordances, which offer opportunities for interaction and serve as substrates for goal-oriented behaviors<sup>35</sup>. Engagement with different affordances might therefore structure the behavioral states captured by shMoSeq. In an empty arena, mice primarily interact with the walls (Figure 2A). These interactions formed the basis for two behavioral states in which mice explored the circular arena clockwise (keeping the wall on their left) or counterclockwise (keeping the wall on their right) (“exploratory locomotion”, Figures 2B and 2C). When a male conspecific was added to the arena — expanding the space of affordances — a new social state emerged, which included stationary investigation, investigation amidst

pursuit, and outright pursuit that sometimes tipped into aggression (Figures 2D–2F). Statistically, the social state was distinguished by proximity to the conspecific and high mutual information between the subject’s syllables and those of the conspecific, which was driven in part by their tendency to express similar behaviors at the same time (Figures 2G and S3A–S3B).

These data suggest that behavioral states reflect nearby affordances [although proximity alone does not determine which state will be expressed (Figure S3C and S3D)]. To further test this idea, we performed additional experiments in which five unfamiliar objects were added to the arena (Figure 2H). To capture fine-scale movements, we tracked 3D keypoints using an array of high-speed cameras; this setup allowed us to quantify head and limb positioning during object investigation and to capture nose oscillations, which serve as a proxy for sniffing<sup>49,55</sup>. Despite these changes in the framerate and recording modality, the five behavioral states identified by shMoSeq had similar durations as those derived from depth cameras (Figures S3E and S3F). The states included two forms of investigation, one directed toward the air (“sniffing”) and the other directed toward the ground (“local investigation”) (Figures S3G and S3H). Mice used these states at different frequencies depending on context. For example, novel objects elicited a 3–4 fold increase in the local investigation state, which was further characterized by proximity to objects and deployment of object-oriented syllables such as “hunched investigation” and “slow approach” (Figures 2I–2L and S3I–S3J).

Although mice preferred recently added objects in the minutes after they first appeared, instances of the investigation state were distributed throughout each session and therefore were mostly self-initiated (Figure S3K). A separate 3D keypoint dataset comparing social and object investigation revealed subtle differences in posture and syllable expression between object- and conspecific-associated behavior states, reinforcing the notion that objects and conspecifics function as distinct affordances that drive unique patterns of behavioral engagement (Figures S3L–S3O). Taken together, these experiments demonstrate that spontaneous behavior is composed of behavioral states in which mice flexibly interact with a particular affordance. Mice remain in each state for up to a minute, and transitions between states capture the mouse disengaging from one affordance and engaging with another.

### **Behavioral states structure neural activity in dmPFC**

The observation that spontaneous behavior is composed of states — each associated with distinct affordances and patterns of movement — raises the possibility that these states correspond to self-motivated tasks. One strong prediction is that if

states indeed correspond to tasks, their identity should be apparent in areas like dmPFC, where neural activity is known to reconfigure as animals engage in different experimenter-defined tasks<sup>4,6,7,37</sup>.

In simultaneous recordings of behavior and neural activity in dmPFC, up to 20% of neurons were activated or inhibited during each behavioral state, with up to 60% of neurons responding to at least one state (Figures 3A and 3B). Although stringent thresholding suggested that most neurons were tuned to a single state, lifetime sparseness calculations identified many with broad tuning (Figures 3C and S4A–S4B)<sup>56</sup>. These correlations were reliable enough to decode behavioral states from dmPFC activity (Figures 3D and 3E), with narrowly and broadly tuned neurons yielding similar performance (Figures S4C and S4D). Indeed, state-related neural activity appeared to be the dominant source of structure in dmPFC, as distinct behavioral states consistently segregated to different regions of a two-dimensional (2D) embedding of neural activity (Figures 3F–3G and S4E–S4F); furthermore, the axes of neural activity associated with each behavioral state tended to align with the top handful of neural principal components (Figures S4G–S4I). Consistent with recent reports that PFC activity evolves continuously as mice progress through structured tasks<sup>57,58</sup>, we observed continuous turnover in neural activity during each state, with a transient increase in turnover when one state transitioned to another (Figure S4J). However, unlike in these recent reports, there was no detectable stereotypy in state-associated neural trajectories (Figure S4K).

The accuracy with which states could be decoded from neural activity was higher for shMoSeq models with greater heldout likelihoods, and peaked when state durations respected our original (behavior-only) criterion for parameter selection (Figures S4L and S4M). Thus, models that did a better job explaining the hierarchical structure of behavior were also better aligned to neural activity. In contrast, there was no clear peak in decoding accuracy when different numbers of behavioral states were tested, suggesting that the neural data are compatible with several different levels of behavioral coarse-graining (Figure S4N).

### Prefrontal representations privilege states over instantaneous movements

State-related activity in dmPFC could reflect a succession naturalistic tasks or be a trivial correlate of ongoing movements, which are broadcast throughout rodent neocortex<sup>59–64</sup>. However, we find little evidence for this kinematics-centered alternative. In 2D maps of neural activity, syllable representations scattered more broadly than state representations, suggesting that syllables play a smaller role in structuring dmPFC activity (Figure S4E and S5A). Decoding accuracy was also lower for syllables than for

states (Figure S5B). Furthermore, when we trained encoders to predict neural activity from syllables, kinematics, and (optionally) states, the inclusion of states improved accuracy for almost every neuron; this result replicated in an empty arena, with a conspecific, and in recordings with novel objects (Figures 3H–3I and S5C–S5E). The inclusion of states also improved accuracy when encoding models had access to world-centric variables such as distance and angle to nearby boundaries and conspecifics, demonstrating that state-related activity does not simply encode spatial relationships to affordances (Figures S5F and S5G).

To test whether privileged encoding of behavioral states is unique to dmPFC, we performed similar recordings in dorsolateral striatum (DLS), which encodes syllables and influences their selection during spontaneous behavior<sup>48,65,66</sup>. Unlike in dmPFC, the inclusion of shMoSeq states did not impact DLS encoder performance, indicating that states provide no useful information about DLS activity beyond that already contained in syllables and kinematic variables (Figures 3I and S5E). Consistent with this finding, behavior state decoders were more accurate for dmPFC than DLS, whereas syllable- and kinematic-decoders were more accurate for DLS (Figures 3J and S5H–S5I). The timescale of neurobehavioral correlates also differed between regions. In dmPFC, correlations were strongest when kinematic variables were smoothed over several seconds, indicating that dmPFC activity tracks slow, state-level changes in behavior rather than instantaneous movements. In contrast, sub-second smoothing maximized correlations between DLS activity and behavior (Figure S5J). Fluctuation rates of neural activity (analyzed independently of behavior) also differed between DLS and dmPFC, with dmPFC neurons remaining autocorrelated ten times longer on average than those in DLS (Figures S5K and S5L). This separation of neurobehavioral timescales reifies the hierarchical division of behavior into sub-second syllables and multi-second states.

### Affordance-related variables are selectively emphasized during specific behavioral states

In conventional experiments with explicitly-defined tasks, dmPFC not only encodes the identity of the current task (e.g. that reward is contingent on stimulus A and not stimulus B) but also information about task-relevant variables (e.g., the current value of stimulus A)<sup>6,8</sup>. If spontaneous behavior is analogously composed of self-initiated tasks that involve interacting with specific affordances, then affordance-related variables should similarly be emphasized during the states in which they are relevant. Consistent with this possibility, we found that diverse spatial and affordance-related variables were represented in dmPFC at the single neuron level, and neurons tuned to these variables overlapped with those modulated by specific states (Figures 4A and S6A). For example, neurons encoding distance and direction to the nearest wall were preferentially active

during the exploratory locomotion states, which are reminiscent of a behavioral strategy called thigmotaxis, in which walls are used as a guide for navigation and source of safety (Figures 2B–2C, 4A–4B, and S6B–S6C). This state-specific modulation remained even when we controlled for the mouse’s distance, angle, and movement relative to the wall (Figures 4C and S6D–S6E). As a consequence, wall position could be decoded with greatest accuracy during the thigmotactic states – i.e. during the putative task in which wall position is most relevant (Figure 4D).

Rather than specifically supporting thigmotaxis, wall-related activity in dmPFC could alternatively play a more general role in spatial navigation. Indeed, such a role has been attributed to egocentric boundary vector (EBV) cells in areas such as retrosplenial cortex (RSC)<sup>67,68</sup>, which similarly represent the distance and relative direction of nearby walls. However, comparing our dmPFC data to RSC recordings from a previous study<sup>68</sup> revealed important coding differences between the two brain areas. Whereas EBV cells in RSC tiled a range of preferred distances to the boundary, most wall-tuned neurons in dmPFC preferred close boundaries and monotonically increased their activity as boundaries grew closer (Figures 4E and S6F). Neurons in RSC were also more strongly modulated by wall angle than those in dmPFC (Figure S6F). Furthermore, whereas RSC neurons exhibited a spectrum of angle preferences that tiled the full 0–360° range, neurons in dmPFC formed two discrete clusters tuned to walls on the left or right of the animal respectively (Figures 4G and S6F). Thus, dmPFC representations appear specialized for wall-guided exploration rather than generic spatial coding, consistent with dmPFC being enriched for neurons encoding specific aspects of the task at hand.

State-dependent coding of specific affordances was also evident during social engagement and object investigation. In recordings with a conspecific, up to six percent of dmPFC neurons were tuned to social proximity, and these responses were much more pronounced during the social engagement state — an effect that persisted after excluding bouts of aggression and controlling for the relative position and angle of the conspecific (Figures 4H–4J). This enhancement may reflect the slow timescale of dmPFC activity, which required longer and more frequent social encounters to fully develop (Figures S6G – S6L). Population decoding of social proximity was also more accurate during intervals that encompassed the social engagement state, even though timepoints outside these intervals spanned the full distribution of conspecific distances (Figures 4K and S6M). Similarly, in experiments with novel objects, decoding of object proximity and identity were most accurate during local investigation, even when mice were a full body-length away from the object itself (Figures 4L–4O). The activity of object-responsive neurons was also highest during the local investigation state, even after controlling for distance and angle to the nearest object (Figures S4N and S4O).

Thus, representations of walls, objects, and conspecifics are each enhanced during the behavioral states in which they are most relevant.

### **Changes in task relevance alter representations of affordance-related variables**

If behavioral states correspond to self-initiated tasks, then making a task especially salient should enhance the strength of task-related representations in dmPFC. To test this hypothesis, we subjected mice to attack by a conspecific, which prompted them to use the wall as a defensive affordance. Subject mice were recorded across 5-minute blocks with an aggressive conspecific (belonging to the CD1 strain) who attacked frequently, a non-aggressive CD1 conspecific who never attacked, or no partner mouse (Figures 5A and S6P). During attacks, mice entered a shMoSeq-identified state that consisted of flights, submissive postures, and periods of immobility near the wall. This defensive state – which did not occur in our earlier experiments – often continued after attacks were over and even persisted during subsequent interactions with the benign conspecific, albeit at a lower rate (Figures 5B–5C and S6Q–S6S). During the defensive state, mice hugged the wall and dmPFC neurons tuned to wall direction or proximity became more active (Figures 5D–5E and S6T). Consequently, wall-tuned neurons were more active during aggression blocks (even after controlling for the position of the wall), and relative wall position could be decoded with greater accuracy during aggression blocks than during blocks alone or with the benign conspecific (Figures 5F–5I and S6U–S6V).

In the aggression experiments, information related to a particular affordance (the wall) was encoded more prominently in a relevant context (conspecific attack). Conversely, might affordance representations diminish in less relevant contexts? Indeed, during our original social interaction experiments (with a neutral C57 conspecific) dmPFC responses to social proximity declined over time — as did the accuracy of social distance decoding — suggesting that one goal of social engagement is to gain familiarity, and that conspecific representations become de-emphasized as familiarity is gained (Figures S6W–S6Y). Similarly, during novel object exploration, there was a progressive decrease in the activity of object-responsive neurons at the timepoints of object encounter (Figure S6Z); this effect was especially pronounced when we introduced five identical objects instead of five unique objects.

Thus, environmental affordances — including walls, objects, and conspecifics — drive the expression of specific, long-lasting behavioral states in which affordance-related variables — such as distance to a wall or the identity of an object — are represented more prominently in dmPFC. Transitions between states occur both spontaneously and in response to perturbations that render certain affordances more

relevant (such as walls during aggression). This pattern is consistent with the idea that behavioral states capture affordance-related tasks that arise naturally during self-directed behavior.

### **Neural dynamics are a lagging indicator of behavior**

To understand the causal processes that underly state- and affordance-coding in dmPFC, we asked whether changes in neural activity occurred before or after changes in behavior, reasoning that if dmPFC were driving behavior — as predicted by some models of hierarchical action planning<sup>70-72</sup> — then neural activity should precede behavior initiation. We instead observed a systematic delay in neural activity, with state-tuned neurons becoming active (or inactive) after state onset (or offset) (Figures 6A and S7A). A similar lag was evident when decoding behavioral states from population-wide activity (Figures S7B and S7C) and was undiminished in look-ahead decoders that were trained to predict future states from current neural activity (Figure S7D).

The delay in social-state encoding is somewhat surprising since dmPFC stimulation can encourage social approach<sup>41</sup>. However, some delay might be expected if state onsets are frequently triggered by actions of the conspecific. To distinguish these possibilities, we reanalyzed published dmPFC recordings from the ‘tube test’ of social dominance, in which pairs of mice enter a tube from opposite ends and compete over who can move forward and who must back out (Figure 6B); prior examination of these data found that dmPFC represents self- and other-behavior but did not report the relative timing of these signals<sup>40</sup>. Our reanalysis revealed delays common to both subject and opponent behaviors. For example, neural activity associated with self-approach was virtually absent before behavior onset and did not peak until ~2 seconds after onset (Figure 6C); this manifested as a 1 second delay in the cross-correlation between true and decoded behavior (Figure S7E). Cross-correlation peaked at similar offsets for other annotated behaviors, with no statistical difference between self and other actions (Figures 6D and S7E). Thus dmPFC representations of social interactions appear to lag behavior in multiple contexts.

The apparent lag between dmPFC and ongoing behavior could be exaggerated by our use of calcium indicators to measure of neural activity. We therefore recorded from dmPFC using chronically-implanted neuropixels probes as mice explored an open field arena<sup>69</sup>. As observed earlier, behavioral states could be decoded from population activity and occupied distinct territories in 2D maps derived from the spiking data (Figures S7F and S7G). To assess the relative timing of dmPFC activity and behavior, we constructed peristimulus time histograms (PSTHs) aligned to state transitions. Most PSTHs showed increases in activity after state onset (Figures 6E and S7H). There were

instances where the rise started earlier, but these may have arisen from uncertainty in the timing of state transitions. Indeed, when we focused on transitions with more definite timing (see Methods), we no longer observed pre-transition increases in neural activity (Figures 6F and S7I-S7J). Neurobehavioral cross-correlations were similarly negative (i.e. neural activity lagging behavior) or not significantly different from zero when measured with respect to state transitions (Figure 6G). Although changes in dmPFC activity led changes in some non-state behavioral variables (such as height and wall direction), they lagged behind the derivatives of those variables, suggesting that the changes in activity may reflect immediately preceding movements (such as rearing up or turning), rather triggering those movements per se (Figure 6I). Together, these data suggest that shifts in dmPFC activity generally lag changes in behavioral state, arguing against a model in which dmPFC exerts continual, moment-by-moment control over self-directed behavior.

### **dmPFC is required for appropriate selection and sequencing of behavioral states**

What other roles might state- and affordance-coding play in spontaneous exploratory behavior? The canonical function of PFC is to exert top-down, context-dependent control over behavior, which during spontaneous behavior could involve biasing the selection, duration, or content of behavioral states. To explore these possibilities, we lesioned dmPFC (including the prelimbic area and the margins of the infralimbic, anterior cingulate, and medial orbital areas) and recorded open field exploration and novel object interaction (Figures 7A and S7K). Lesioned and control mice were indistinguishable in raw behavioral videos and had nearly identical distributions of wall-distances and object-distances respectively (Video S4 and Figure S7L). Lesions also had no effect on the duration or usage of syllables within each behavioral state (Figures S7L and S7M). However, lesions did systematically contract the timescales over which behavior was organized. Mutual information between syllables decayed faster in lesioned mice than in controls, reaching chance levels in less than half the time (Figures 7B and 7C). The difference was especially stark during open field recordings without novel objects (Figure 7D). Similar effects were evident in a second cohort of animals in which the gap between surgery and recording was shortened to reduce compensatory effects (Figure S7N).

Consistent with the possibility that dmPFC influences the higher-order structure of behavior, dmPFC lesions altered behavioral state usage, reducing the expression of less commonly-used states like grooming and local investigation (Figures 7E and S7O–S7S); in contrast, the overall distribution of state durations was unaltered (Figure 7F). These shifts in state usage likely explain the contraction of behavioral timescales. Time spent in the grooming and local investigation states correlated session-by-session with

the timescale over which behavior remained predictable (Figure 7G), likely because these specific states are themselves especially well-structured over time, and hence contribute disproportionately to the sustained mutual information between syllables that we observe during spontaneous behavior (Figure S7T). These results argue that dmPFC contributes to the higher-order structure of behavior by differentially influencing the usage of behavioral states.

The observation that dmPFC lesions promote common states like exploratory locomotion<sup>70</sup> at the expense of less common states like grooming and local investigation (Figure 7E) could be explained either as a generic increase in the most common types of behavior or as a more specific loss of context-specific behavioral control. Consistent with the latter idea, loss of the investigation state was most pronounced in recordings with novel objects (Figure 7H). Furthermore, whereas control mice groomed more in the vicinity of novel objects, this effect almost disappeared in lesioned mice (Figure 7I). Together, these lesion results suggest that dmPFC imparts hierarchical structure to behavior by facilitating the expression of a broader set of behavioral states — especially rare states that depend on context — without substantively altering their granular (syllabic) contents; this stands in contrast to DLS lesions, which perturb both syllable usage and sequencing<sup>48</sup> but have no effect on the overall timescale at which behavior is organized (Figure 7J).

## DISCUSSION

Animals act toward many ends — to gain information, escape danger, find food, water, shelter and much else<sup>29,71,72</sup>. Sometimes one need predominates, as when researchers deprive animals of food and then offer food rewards during a task. However, in less structured contexts, the purpose of behavior is often mysterious; animals may have competing needs that they pursue at different times, each corresponding to a kind of self-motivated task<sup>73</sup>. In principle these emergent tasks could have profound effects on behavior and neural dynamics<sup>36,74</sup>, yet the hypothesis that self-paced exploratory behavior entails a set of tasks has not been explicitly tested in the lab. Here we define criteria that such task-related states should fulfill: they should organize low-level movements, correspond to recognizable behaviors, reflect available affordances, and be visible in neural activity, especially in areas (such as dmPFC) that are important for goal-directed behavior. Given these criteria, we find that spontaneous behavior is structured as a succession of self-directed tasks. These observations required the development and validation of shMoSeq, a hierarchical model that bridges the gap between microscopic behaviors identified at the level of poses and syllables, and the macroscopic behaviors that enable animals to solve problems and reach goals.

Comparisons between real and simulated data show that the hierarchy instantiated by shMoSeq — millisecond poses, sub-second syllables and multi-second states — can fully explain the spectrum of timescales apparent in a one-hour recording of exploratory behavior. Of course, this does not preclude the existence of structure at other timescales; circadian rhythms and internal states such as hunger and thirst (which are distinct from the seconds-long “behavioral states” we describe here) would likely become evident in longer recordings<sup>75-77</sup>, and substates that are intermediate between states and syllables — though not strictly necessary to explain the statistics of our data — might be practically useful for segmenting behavior (e.g., by splitting the social state into separate components corresponding to investigation, pursuit and aggression)<sup>78</sup>. That said, it is notable that state decoding accuracy peaks at the seconds-to-minutes timescale nominated by shMoSeq, consistent with the role of states in scaffolding neural activity.

The correspondence between behavioral states and self-initiated tasks is supported by their representation in dmPFC, which in several ways resembles that of experimenter-defined tasks. When experimenter-defined tasks involve shifting between distinct contexts, the representation of each context is typically stable over time and abstracted from moment-to-moment action selection<sup>4,7,9,13,57,79</sup>. Similarly, during spontaneous behavior, dmPFC encodes long-lasting behavioral states while abstracting

over low-level movements. When the rules of experimenter-defined tasks change over time, PFC preferentially encodes stimuli that are salient given the current rules<sup>6,8,79-82</sup>. We found that representations of environmental affordances (walls, conspecifics, or novel objects) became more prominent during the behavioral states in which they were relevant. These changes suggest that behavioral state transitions correspond to shifts in attention<sup>83</sup>, and hint at the animal's underlying goals. For example, the fact that object identity can be decoded better during the "local investigation" state suggests that this state is associated with the goal of seeking information about objects.

What causal role does dmPFC play in structuring behavior? Animals rely on PFC to adjust their behavior in a context-specific manner, especially when there are conflicts between lower-level systems of behavioral control<sup>4</sup>. Indeed at focal moments of conflict, dmPFC can participate directly in action initiation; examples include time-locked activity prior to active avoidance during a shuttle-crossing task<sup>84</sup>, and instantaneous induction of winning by optogenetic activation during social competition<sup>85</sup>. It is unclear if this instructive role for dmPFC persists across time or during self-directed exploratory behavior. One influential hypothesis places PFC at the apex of a network charged with action planning and execution<sup>86-88</sup>. In the strongest of these proposals, high-level behavioral states correspond to metastable attractors in PFC, and behavioral transitions arise from stochastic switching between attractors<sup>89</sup>. However, our findings suggest a more permissive role during spontaneous behavior; rather than actively initiating new states, dmPFC may construct abstract representations of ongoing behavior (e.g., by passively monitoring incoming sensory and motor information) that help specify the distribution of states in a given context. This mode of regulation could reflect the influence of dmPFC on brain-wide neural dynamics<sup>90,91</sup>, and is compatible with a wide range of brain areas instigating behavioral state transitions.

The attribution of goals to living organisms has a fraught history<sup>92</sup>. "Goal" can refer broadly to any process of control resulting from natural selection (i.e., "teleonomy"<sup>93</sup>) or more narrowly to directed programs of behavior that are under cognitive control<sup>94</sup>. Here we use "goal" and "task" in the latter sense. Whereas goal-directedness in traditional neuroscience is typically tested through the manipulation of task rewards (as during devaluation<sup>95</sup>), other approaches are required for naturalistic behavior, which often lacks explicit rewards under experimental control<sup>65</sup>. Here we addressed this problem by articulating a set of observational criteria for goal-directedness involving both behavior and neural activity. We note that much of spontaneous behavior remains intact after dmPFC lesions, suggesting either redundancy in the systems that support the expression of task-related states during unstructured exploration, or a more prominent role for sensorimotor habits. Longer

timescale behavioral recordings among a wider variety of affordances may provide a clearer view.

To identify behavioral states, we designed a data-efficient HHMM (shMoSeq). Unlike approaches that cluster syllables into mutually-exclusive groups<sup>34,54</sup>, shMoSeq does not enforce a strict mapping between syllables and states. Rather, states are defined by probabilistic changes in syllable usage, meaning syllables can be reused across states in a context-specific manner. This framework effectively captures the long-timescale (i.e. non-Markovian) structure of syllable sequences and was ultimately ratified by its close correspondence to neural dynamics in dmPFC. Several other methods for inferring behavioral hierarchy have also been reported; these employ methods like compression<sup>96</sup>, repeated sequence search<sup>97</sup> or context-free-grammars<sup>98</sup>, and as such are more geared toward short repeated action sequences than the prolonged behavioral states highlighted here. Future elaborations of shMoSeq that take advantage of self-supervised behavioral embeddings might reveal states with greater richness or specificity<sup>99,100</sup>. ShMoSeq analysis of uninstructed movements during experimenter-defined tasks could also be useful for uncovering changes in task engagement or strategy<sup>101</sup>.

We have released shMoSeq as a python package with documentation and tutorials (<https://state-moseq.readthedocs.io/en/latest/>). Information about runtime, compute requirements, parameter selection, and the types of data for which shMoSeq may be useful are available in the Methods. It is important to note that shMoSeq affords some choice in the total number of identified states. Although we heuristically chose this number based on the stability of model outputs, more than one level of coarse-graining may be reasonable for a given dataset, and the relevant level will doubtless vary based on the total amount of data and complexity of behavior therein.

Whatever form they take, hierarchical behavior models will be an important future tool for exploring cognition during unstructured behavior. For decades, researchers have had to constrain animals' movements and narrow their motivations in order to isolate specific cognitive processes and unspool their neural causes. The result has been to minimize the agency and spontaneity that characterize most behavior outside the lab<sup>1,31,102</sup>. Ultimately, we would like the best of both worlds — probing well-defined computations using normative models, even as animals set their own goals and pursue them freely<sup>103,104</sup>. Here we have taken a small step in that direction by identifying the behavioral states that emerge during unstructured exploration in mice. By bracketing intervals of relative stability in the animal's behavior, these states expose the possible succession of tasks that structure cognition over time — a hypothesis that is borne out in our analysis of dmPFC activity. This connection — if it proves durable — would allow

researchers to avail in naturalistic behavior the same normative frameworks that they typically seek through experimental reductionism.

## RESOURCE AVAILABILITY

### Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Sandeep Robert Datta ([srdatta@hms.harvard.edu](mailto:srdatta@hms.harvard.edu))

### Materials availability

This study did not generate new materials.

### Data and code availability

- Original data have been deposited at Zenodo at 10.5281/zenodo.17488068 and are publicly available as of the date of publication.
- All original code has been deposited at Zenodo at 10.5281/zenodo.17488068 and is publicly available as of the date of publication.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## ACKNOWLEDGEMENTS

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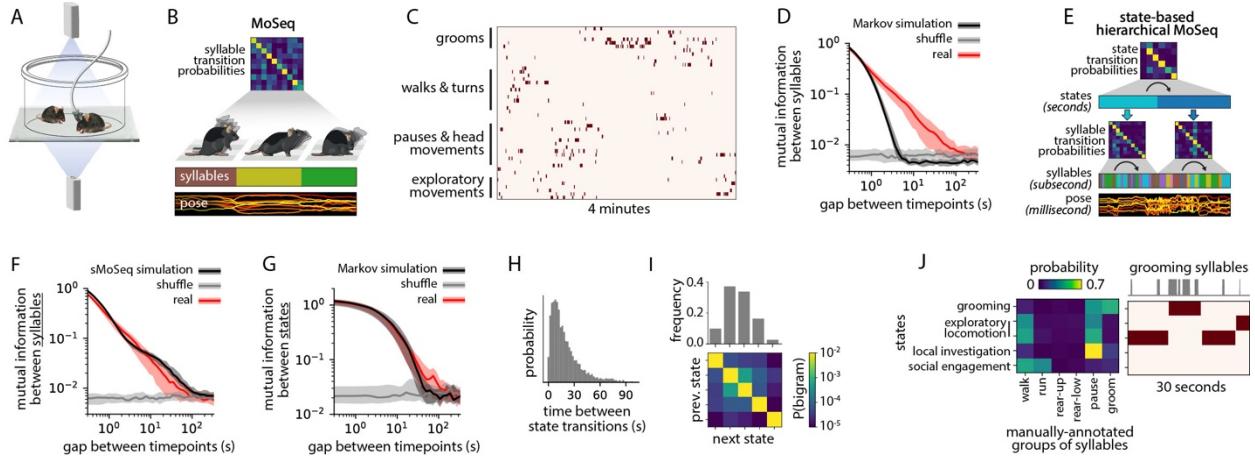
also thank M. Hasselmo and A. Alexander for sharing raw data related to egocentric boundary vector tuning in retrosplenial cortex.

## **AUTHOR CONTRIBUTIONS**

C.W. and S.R.D. conceived the project and designed the experiments. C.W. and S.W.L. designed the mathematical model. C.W. and J.E.P. wrote the pre-processing pipeline for depth videos. C.W., W.F.G., and T.S. collected data with assistance from L.T.K., A.N.B., A.P., and S.M. C.W. performed analysis with assistance from M.A.M.O. C.W. and S.R.D. wrote the manuscript with input from all authors. S.R.D. supervised the project.

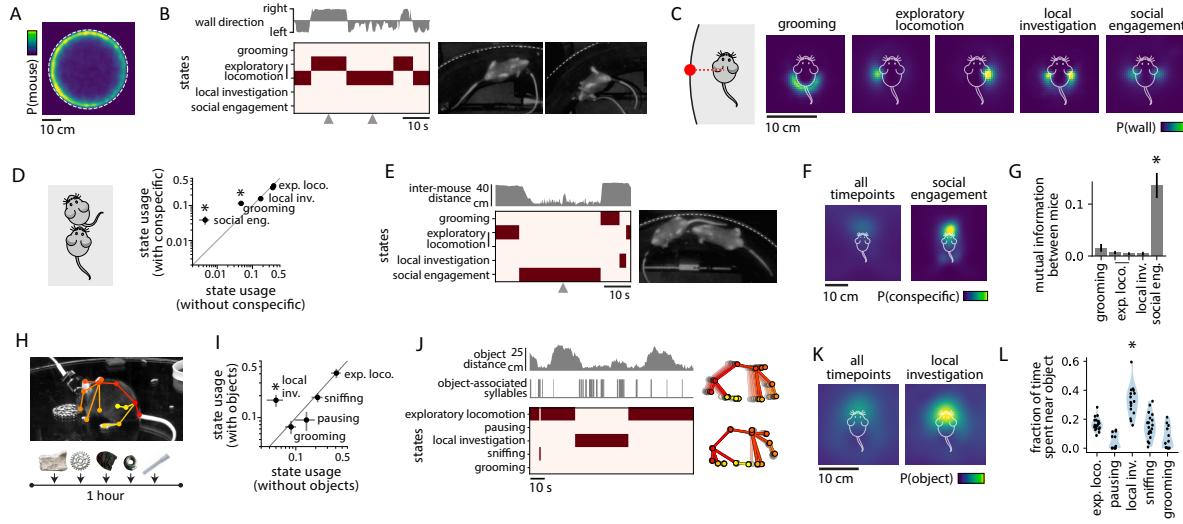
## **DECLARATION OF INTERESTS**

S.R.D. and C.W. have patent registrations and applications related to the use of Motion Sequencing (MoSeq) and State-based Hierarchical MoSeq (shMoSeq) for analysis of animal behavior.



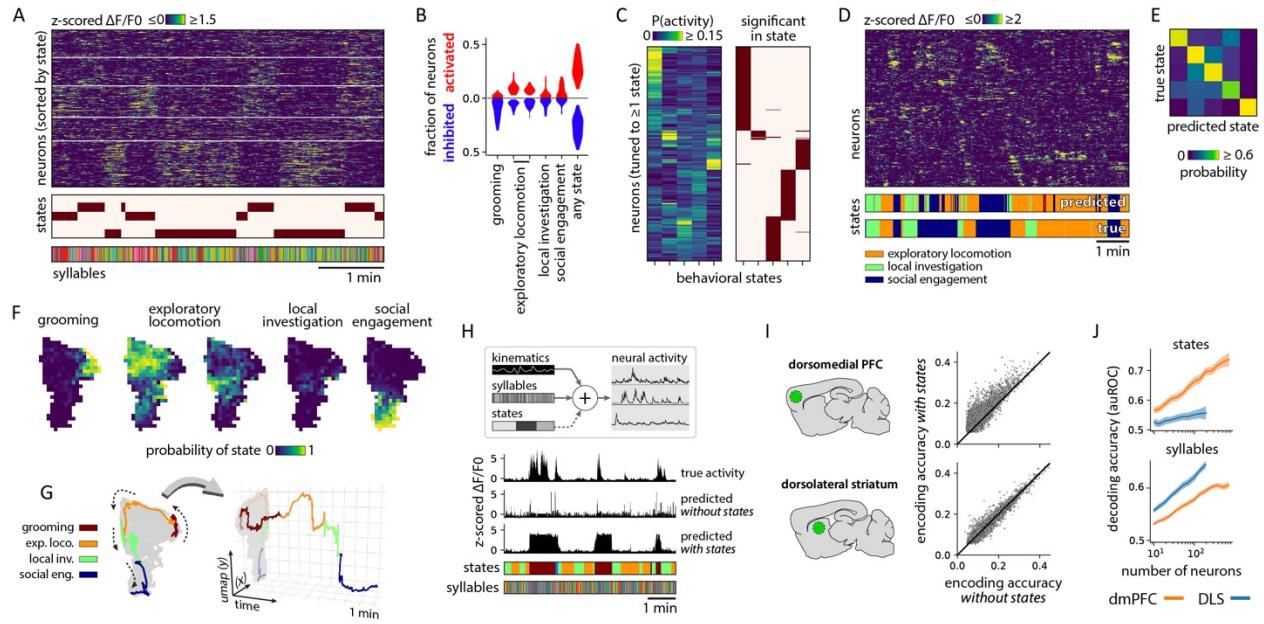
**Figure 1: Behavioral states capture the higher-order structure in exploratory behavior**

- (A) Arena for behavioral recordings.
- (B) Schematic for MoSeq. Pose trajectories (bottom) are determined by syllables (middle) which transition with fixed probabilities (top).
- (C) Example syllable sequence. The heatmap shows which syllable (row) is expressed at each timepoint (column).
- (D) Mutual information (MI) between syllables at a range of temporal lags, shown for real data (red), Markov model simulations (black), and shuffled data (gray). Line and shading indicate median and interquartile interval across recordings ( $N=31$ ). Syllables were kinematically clustered for MI computation to prevent sparsity-related artifacts.
- (E) Schematic for shMoSeq. A chain of behavioral states (top) specifies transition probabilities between syllables (middle), which govern pose (bottom).
- (F) As (D), here comparing real data (red) to shMoSeq simulations (black).
- (G) MI between behavioral states at a range of temporal lags, plotted as in (D). States were derived from real data using shMoSeq (red) or simulated from a Markov model (black).
- (H) Distribution of state durations.
- (I) Top: usage of each behavioral state. Bottom: bigram frequencies, capturing transitions between states.
- (J) Left: overlap between states and manually-annotated categories of syllables. Right: occurrence of grooming syllables (gray ticks) alongside behavioral states (heatmap) from an example interval.



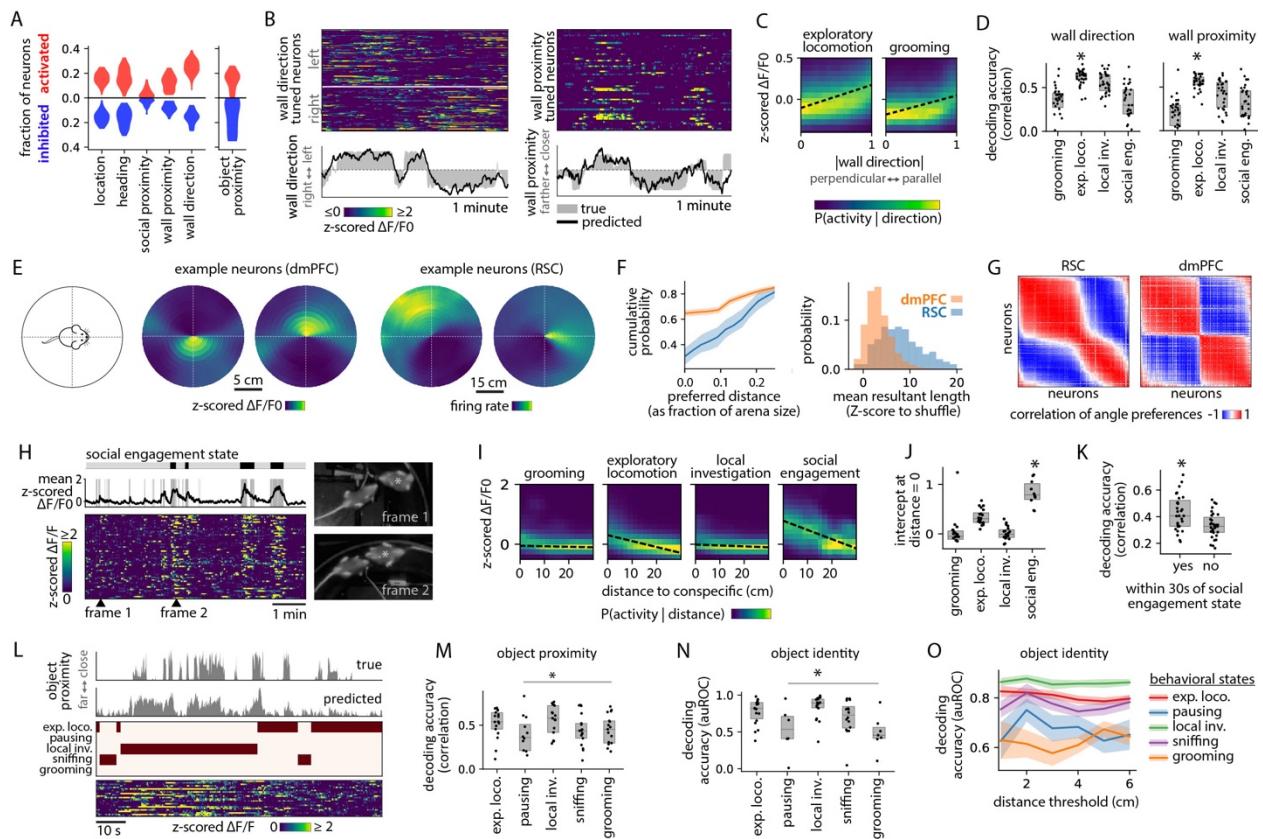
**Figure 2: Affordances sculpt the distribution of behavioral states**

- (A) Distribution of mouse locations across all recordings. Dashed line shows the arena boundary.
- (B) Example interval showing relative wall direction (top) and behavioral states (bottom). Video frames correspond to the two timepoints marked with triangles.
- (C) Distribution (for each behavioral state) of the nearest wall location in egocentric coordinates.
- (D) Changes in state usage when a (C57) conspecific is present, showing mean and standard error across N=31 recordings ( $P<1.5e-4$ ).
- (E) As (B), here showing distance between mice.
- (F) As (C), here showing location of the conspecific.
- (G) Mutual information between subject and conspecific syllables during each state. Error bars show bootstrap 95% confidence across N=31 recordings ( $P<0.005$ ). Syllables were kinematically clustered to minimize sparsity-related artifacts.
- (H) Design of the novel object experiment. One object was added every 10 minutes.
- (I) Changes in state usage when novel objects are present, plotted as in (D) ( $P=0.001$ , N=21).
- (J) Left: example interval showing distance to the nearest object (top), occurrence of object-associated syllables (middle) and behavioral states (bottom). Right: keypoint trajectories for two object-associated syllables.
- (K) As (C), here showing location of novel objects.
- (L) Fraction of time spent near objects during each state. Dots correspond to recordings (N=21,  $P<6e-5$ ).



**Figure 3: Behavioral states structure neural activity in dmPFC**

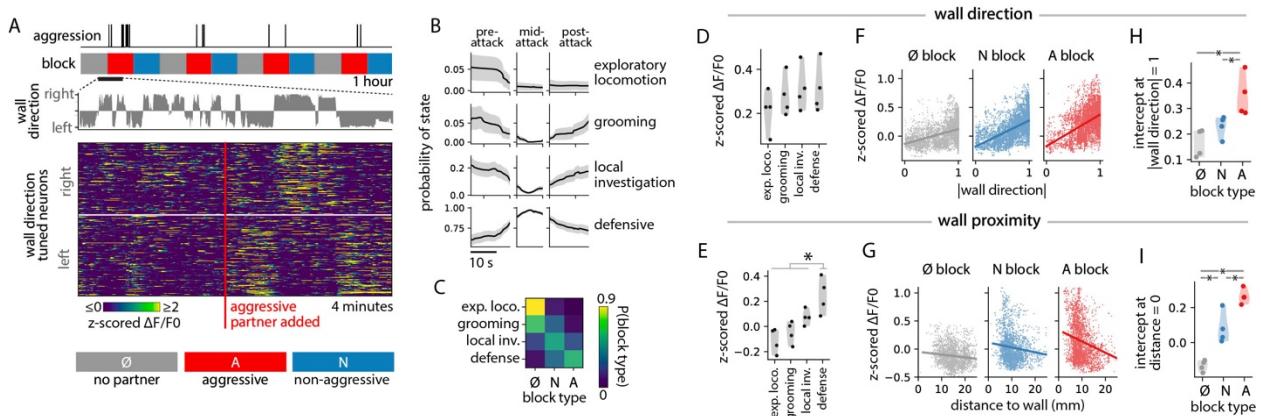
- (A) Example of state-related shifts in dmPFC activity.
- (B) Fraction of state-responsive neurons in each recording [N=31, false discovery rate (FDR)<5%].
- (C) Left: activity of state-responsive neurons across each behavioral state (shown for one recording, “active” defined as z-scored  $\Delta F/F_0 \geq 2$ ). Right: whether tuning is statistically significant (FDR<5%).
- (D) Behavioral state decoding for an example interval.
- (E) Agreement between true and decoded states.
- (F) Usage of each behavioral state across a 2D projection of neural activity from one recording.
- (G) Example trajectory from the neural activity map in (F), colored by the current behavioral state.
- (H) Top: design of encoding model. Bottom: predictions for an example neuron, shown for encoders with and without access to behavioral state information.
- (I) Encoding accuracy with versus without access to behavioral state information, shown for all recorded neurons in dmPFC (top) and DLS (bottom).
- (J) Decoding accuracy for states (top) and syllables (bottom) using activity from dmPFC versus DLS. Neural population sizes were matched via down-sampling. Line and shading show mean and 95% CI across recordings (11≤N≤31).



**Figure 4: Encoding of affordance-related variables depends on behavioral state**

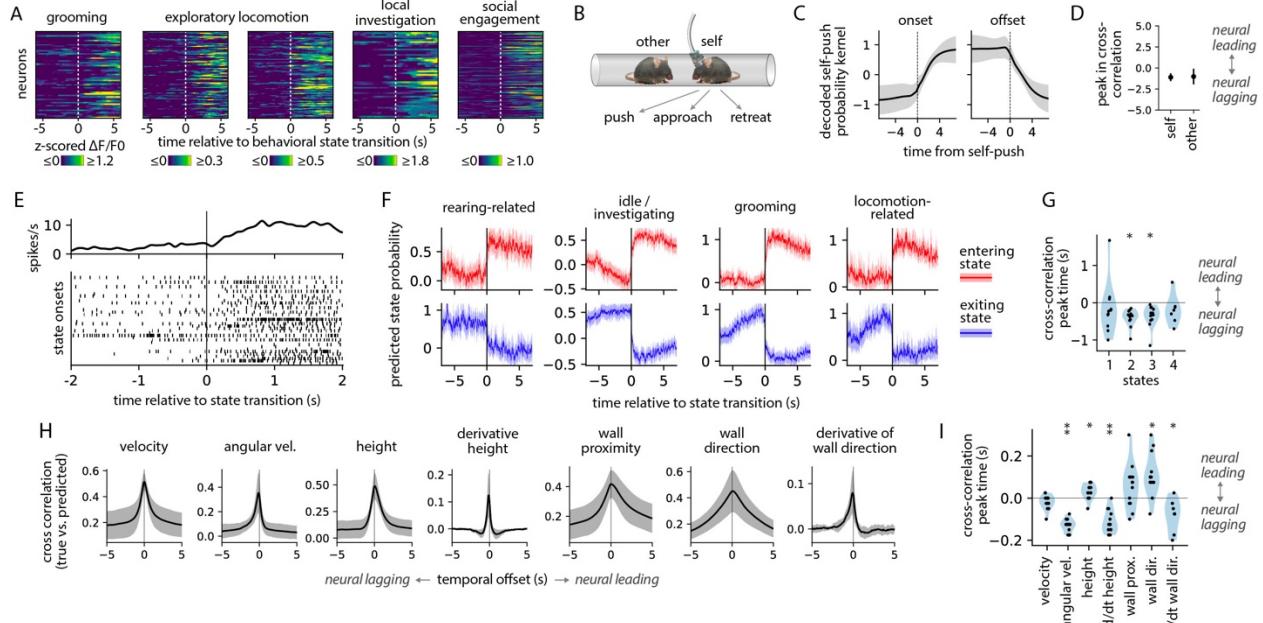
- (A) Fraction of neurons modulated by a range of spatial- and affordance-related variables, showing distributions across recordings (N=31, FDR<5%).
- (B) Top: activity of dmPFC neurons modulated by wall direction (left) or proximity (right). Bottom: true and decoded values of each variable over an example interval.
- (C) Activity of wall direction-tuned neurons as a function of wall direction, stratified by behavioral state.
- (D) Decoding accuracy for wall direction (left) and proximity (right) during each behavioral state (N=31 recordings, left: P<0.03, right: P<6e-4).
- (E) Wall-related activity for example neurons in dmPFC (left) and retrosplenial cortex (RSC, right). Heatmaps show average activity when boundaries are present at a given distance and angle relative to the mouse.
- (F) Left: cumulative distribution of preferred wall distances for neurons in dmPFC versus RSC. Arena diameters were respectively 125 cm (RSC) and 40 cm (dmPFC). Line and shading show mean and bootstrap 95% CI. Right: strength of wall angle-tuning (Z-scored mean resultant length) for neurons in dmPFC and RSC respectively.
- (G) Pairwise correlation of angle preferences for neurons in dmPFC versus RSC (neural population sizes were matched via down-sampling). The two blocks for dmPFC correspond to left-wall and right-wall-preferring neurons respectively.
- (H) State-dependent responses to social proximity. Top-left: instances of the social engagement state. Middle-left: intervals when mice are close together (gray, <5 cm) and mean activity of social proximity-tuned neurons (black). Bottom-left: activity of individual neurons tuned to social proximity. Right: video frames at the two marked timepoints.
- (I) Activity of social proximity-tuned neurons as a function of social distance, stratified by behavioral state.

- (J) Intercepts of the best-fit lines shown in (I), here calculated for each recording with  $\geq 10$  social proximity-tuned neurons ( $N=20$ ,  $P<3e-7$ ).
- (K) Decoding accuracy for social proximity, comparing timepoints near or far from the social engagement state ( $N=31$  recordings,  $P=0.003$ ).
- (L) Example of state-dependent responses to nearby objects, showing true versus decoded proximity (top), behavioral states (middle), and activity of object-proximity-tuned neurons (bottom).
- (M) Accuracy of object proximity decoding across behavioral states. Each dot represents one recording in which the state occurred at least once ( $11 \leq N \leq 18$ ). Correlations are highest during the local investigation state; differences are significant for all states except exploratory locomotion ( $P<0.02$ ).
- (N) Accuracy of object identity decoding [quantified as area under the receiver operating characteristic curve (auROC)]. Analysis was restricted to timepoints when the mouse's nose was within 1 cm of exactly one object and the mouse was facing the object ( $6 \leq N \leq 18$ ,  $P<0.05$ ).
- (O) Median accuracy of object identity decoding for a range of nose-object distances. Shading shows the standard deviation across 20 random train/test splits. Accuracy is highest during the local investigation state; this difference is significant with respect to train/test splits ( $P<7e-7$ ).



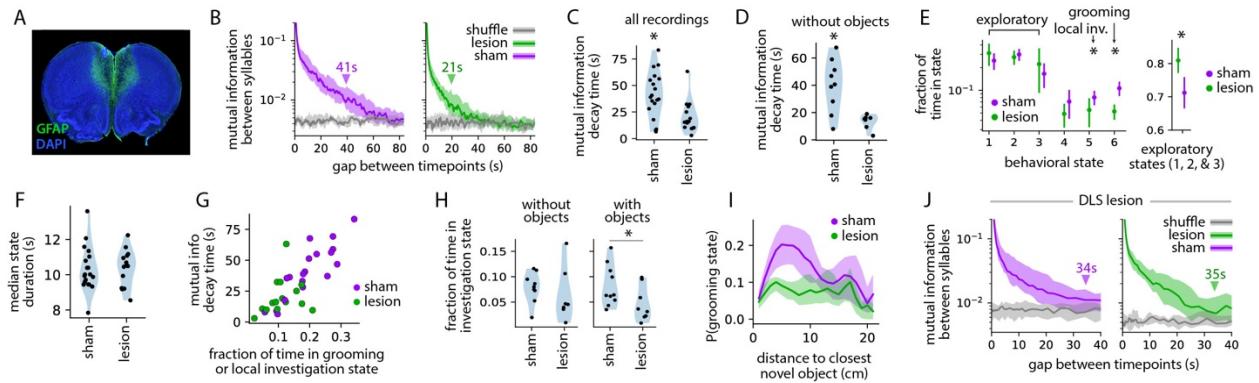
**Figure 5: Changes in task relevance alter the encoding of affordance-related variables**

- (A) Design of the conspecific aggression experiment. Zoom-in shows sudden intensification of wall encoding during the aggression block.
- (B) Behavioral state probabilities aligned to aggression bouts, showing mean and standard error across aggressive episodes (N=48).
- (C) Overlap between behavioral states and experimental blocks.
- (D) Average activity of wall direction-tuned neurons when mice are close and parallel to the wall (N=4 recordings).
- (E) As (D), here showing activity of wall-proximity-tuned neurons ( $P<0.057$ ).
- (F) Activity of wall direction-tuned neurons as a function of wall angle, stratified by block type. Dots represent timepoints from one example recording.
- (G) Activity of wall proximity-tuned neurons versus distance to the wall, plotted as in (F).
- (H) Intercept at  $x=1$  (parallel to the wall) of the best fit lines shown in (F), here shown for all recordings (N=4,  $P=0.028$ ).
- (I) Intercept at distance=0 of the best fit lines shown in (G), here shown for all recordings (N=4,  $P=0.028$ ).



**Figure 6: Neural dynamics lag behavioral transitions**

- (A) Average onset-aligned activity of state-tuned neurons from an example recording.
- (B) Illustration of tube test assay.
- (C) Decoded “self-push” probability at onsets and offsets of self-push bouts (estimated via kernel regression), showing mean and 95% CI across N=10 recordings.
- (D) Timing of peak cross-correlation between true and decoded annotations of self- versus other-behaviors. Dots and lines represent mean and 95% CI across recordings and behaviors ( $15 \leq N \leq 20$ ,  $P=0.22$ ).
- (E) Raster plot for an example neuron aligned to onset of the grooming state.
- (F) State probabilities predicted from spiking activity, aligned to state onsets (top) and offsets (bottom). Transitions were excluded from analysis if uncertainty in their timing was  $>1$ s.
- (G) Timing of peak cross-correlation between true and decoded state probabilities, restricted to state/recording pairs for which the peak correlation exceeded the 95<sup>th</sup> percentile of a shuffle distribution ( $8 \leq N \leq 13$ ,  $P \leq 6e-4$ ).
- (H) Cross-correlation between true and decoded behavioral variables (mean and 95% CI,  $N=13$ )
- (I) Timing of peak cross-correlation between true and decoded behavioral variables ( $7 \leq N \leq 13$ ,  $*P \leq 0.03$ ,  $**P \leq 7e-6$ ).



**Figure 7: dmPFC influences the composition of behavioral states**

- (A) Coronal section from a lesioned mouse, stained with an astrocytic antibody (GFAP) that highlights the lesioned area.
- (B) Mutual information (MI) between syllables at a range of temporal lags (median and interquartile interval across recordings, N=19 sham, N=15 lesion). Pointers show the mean decay time, defined as time to reach the 99<sup>th</sup> percentile of the shuffle distribution.
- (C) MI decay times for each recording, defined as in (B) ( $P=0.006$ ).
- (D) As (C), here restricted to recordings without novel objects (N=9 sham, N=7 lesion,  $P=0.01$ ).
- (E) Left: behavioral state usage (mean and 95% CI) for lesion versus sham recordings (FDR<5%). Right: combined usage of the exploratory locomotion states.
- (F) Median duration of behavioral states for sham and lesion recordings ( $P = 0.66$ ).
- (G) MI decay time versus usage of the grooming and local investigation states (N=34 recordings,  $R=0.79$ ,  $P=2.5e-8$ ).
- (H) Usage of the investigation state in lesion versus sham recordings, either with (right) or without (left) novel objects ( $P=0.034$ ).
- (I) Probability of the grooming state as a function of distance to the closest novel object, showing mean and 95% CI across recordings with novel objects (N=10 sham, N=8 lesion).
- (J) As (B), here showing DLS lesion data from (Markowitz et. al., 2018). There is no significant difference in MI decay times ( $P=0.3$ , N=14 sham, N=34 lesion).

## STAR METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Chicken Anti-GFAP	abcam	Cat# ab4674, RRID:AB_304558
Alexa Fluor 488, Donkey Anti-Chicken IgY	Jackson Laboratory	Cat# 703-545-155; RRID_AB_2340375
Bacterial and virus strains		
AAV1.Syn.GCaMP6f.WPRE.SV40	Douglas Kim & GENIE Project (Chen et al., 2013) <sup>105</sup>	Addgene viral prep # 100837-AAV1
AAV1.Syn.Flex.GCaMP6f.WPRE.SV40	Penn Vector Core	Cat# AV-1-PV2819
AAV1.EF1a.DIO.GCaMP6s.P2A.nls.dTomato	Jonathan Ting	Addgene viral prep # 51082-AAV1
AAV9.CBA.DO(Fas).GCaMP6s	UNC Vector Core	N/A
Chemicals, peptides, and recombinant proteins		
N-Methyl-D-Aspartate	Sigma	Cat# M3262-25MG
Vybrant™ CM-Dil Cell-Labeling Solution	ThermoFisher	Cat# V22888
Deposited data		
Raw and processed behavior and neural activity	Zenodo	10.5281/zenodo.17488068
Experimental models: Organisms/strains		
Mouse: CD1	Charles River	Strain code: 022
Mouse: B6.FVB(Cg)-Tg(Drd1-cre)EY262Gsat/Mmucd	MMRRC-UCD	Stock# 030989-UCD
Mouse: B6.FVB(Cg)-Tg(Adora2a-cre)KG139Gsat/Mmucd	MMRRC-UCD	Stock# 036158-UCD
Mouse: BALB/cJ	Jackson Laboratory	Jax stock #000651
Mouse: C57BL/6J	Jackson Laboratory	Jax stock #000664
Software and algorithms		
Custom depth-MoSeq pipeline	Zenodo	10.5281/zenodo.17488068
Multi-camera calibration pipeline	Zenodo	10.5281/zenodo.17488068
Keypoint-MoSeq	Weinreb et al., 2024 <sup>49</sup>	<a href="https://github.com/dattalab/keypoint-moseq">https://github.com/dattalab/keypoint-moseq</a>
ShMoSeq	Zenodo	10.5281/zenodo.17488068
CalmAn	Giovannucci et al., 2019 <sup>106</sup>	<a href="https://github.com/flatironinstitute/CalmAn">https://github.com/flatironinstitute/CalmAn</a>
Open Ephys (v0.6)	Open Ephys	<a href="https://open-ephys.org">https://open-ephys.org</a>
SpiketInterface	Buccino et al., 2020 <sup>107</sup>	<a href="https://spikeinterface.readthedocs.io">https://spikeinterface.readthedocs.io</a>
Kilosort 4	Pachitariu et al., 2024 <sup>108</sup>	<a href="https://github.com/MouseLand/Kilosort">https://github.com/MouseLand/Kilosort</a>
Pinpoint	Birman et al., 2023 <sup>109</sup>	<a href="https://github.com/AllenInstitute/Point">https://github.com/AllenInstitute/Point</a>
HRNet	Sun et al., 2019 <sup>110</sup>	<a href="https://github.com/HRNet">https://github.com/HRNet</a>

UMAP	McInnes et al., 2018 <sup>111</sup>	<a href="https://umap-learn.readthedocs.io">https://umap-learn.readthedocs.io</a>
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## EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

All experiments were carried out in accordance with Harvard Medical School institutional animal care and use committee (IACUC) protocol number IS00000138. Subject mice included wildtype C57BL6/J mice (Jackson Laboratory stock no. 000664), C57BL6/J mice harboring either the Drd1a-Cre allele (B6.FVB(Cg)-Tg(Drd1-cre)EY262Gsat/Mmucd; MMRRC #030989-UCD) or the A2a-Cre allele (B6.FVB(Cg)-Tg(Adora2a-cre) KG139Gsat/Mmucd; MMRRC #036158-UCD)<sup>112</sup>, CD1 mice (Charles River, #022), and BALB/cJ mice (Jackson Laboratory stock no. 000651). All mice were recorded between 3 and 7 months of age and maintained in a 12 h:12h light/dark cycle with food and water ad libitum. Individual housing was used following surgery and group-housing otherwise.

We recorded dorsomedial prefrontal cortex (dmPFC) activity in 6 male mice across 31 sessions in the initial round of solitary and social open field recordings, 4 male mice across 4 sessions during the aggression experiments, 9 female mice across 18 sessions during the object investigation experiments with unique objects, and the same 9 female mice across 9 sessions during the object investigation experiments with identical objects. We recorded dorsolateral striatum (DLS) activity in 21 mice across 30 sessions. For the lesion experiments, we recorded 10 male mice (5 lesion, 5 sham) across 34 sessions (18 with novel objects, 16 without), followed by a second cohort of 8 male mice (4 lesion, 4 sham) across 16 sessions (without objects). We also tracked 3D keypoints in 5 additional male C57 mice interacting with objects (21 sessions) or with female BALB/cJ conspecifics (19 sessions). For neuropixels recordings in dmPFC, we recorded 3 male C57 mice across 13 sessions.

## METHOD DETAILS

### Behavior Assays

All mice were habituated to handling, head-fixation, and the recording arena for before experiments began. On recording days, mice were brought to the laboratory and habituated in darkness for at least 20 minutes before recording. All experiments were performed during the dark cycle under infrared illumination.

#### *Open field exploration*

During the initial set of social and solitary open field recordings with a C57 conspecific, subject mice were recorded for 80 minutes in alternating blocks: 10 minutes alone, 30 minutes with an unfamiliar conspecific, 10 minutes alone, and then 30 minutes with the same conspecific. Some conspecifics bore a commutated patch cord that was not used during the experiment.

### *Conspecific aggression*

For recordings of aggressive social interaction, subject mice were recorded in an open field arena for one hour in alternating blocks: 5 minutes alone, 5 minutes with an aggressive conspecific (CD1), 5 minutes with a non-aggressive conspecific (CD1), and so on (same pair of conspecifics each time). Prior to the initiation of experimental trials, CD1 mice were pre-screened to identify highly aggressive or non-aggressive individuals using the following procedure: CD1 were placed alone in an open field arena and allowed to habituate for 10 minutes. We then performed a sequence of four probe trials in which a C57 mouse was placed in the arena for 10 minutes or until the onset of aggression (as identified by a trained observer). CD1 mice with the shortest average latency to aggression were defined as highly-aggressive. CD1 mice who never engaged in aggressive behavior were defined as non-aggressive.

### *Novel object exploration*

For the novel object experiments, mice were recorded for one-hour sessions in the 3D keypoint tracking arena. A new object was added to the arena every ten minutes, resulting in five (unique) objects by the end of the recording; objects were randomly selected from the following set: 15 mL Falcon tube cap, pencil nub, rolled up piece of lab tape, small rubber band, crumpled twist tie, cube of packing foam, origami star, gear, syringe cap, tin foil ball, bubble wrap clipping, hexnut, glove clipping. We also recorded sessions with five identical objects, which in every case were hex nuts.

### *Lesion recordings*

Lesioned mice were recorded in the 3D keypoint tracking arena. In a subset of recordings, novel objects (hex nuts) were added to the arena at ten minute intervals as described above. In another set, mice were allowed to explore the arena for one hour without interruption. Among this full set of recordings, we identified a handful of extreme outlier recordings in which mice remained in one small part of the arena for almost the entire session. We therefore excluded from analysis all sessions where the mouse was immobile (< 1 mm/s centroid velocity) at least 75% of the time; a total of 4 out of 56 sessions were excluded.

### **Stereotactic surgery procedures**

For all stereotactic surgeries, mice were anaesthetized using 1–2% isoflurane in oxygen at a flow rate of 1 L/min and injected with bupivacaine (1.25 mg/kg) under the scalp. All coordinate axes were zeroed relative to bregma (including dorsal/ventral, which was zeroed relative to the skull surface), and coordinates are in units of mm. All injections were performed using a Nanoject II or Nanoject III (Drummond) at 60 nL/min. Incisions were closed using Vetbond (3M). Postoperative care included a subcutaneous injection of buprenorphine SR (1 mg/kg, given 1 hour prior to surgery start) and carprofen (5 mg/kg) administered through drinking water.

### *Calcium imaging in dorsomedial prefrontal cortex (dmPFC)*

To record neural activity in dmPFC, virus injection and gradient index (GRIN) lens implantation were performed across two different surgeries. In the first surgery, we injected AAV1.Syn.GCaMP6f.WPRE.SV40<sup>105</sup> (Addgene #100837, titer: 0.8e13 – 1.2e13) unilaterally into the left hemisphere at two different depths (2.0 mm AP, 0.38 mm ML, -1.7 & -1.9 mm DV, 350 nL each). Incisions were closed and mice were allowed 4-8 weeks for recovery and viral expression. In the second surgery, a circular craniotomy was opened to the left of the injection site (center: 2.0 mm AP, 0.75 mm ML, diameter: 1.4 mm). Brain tissue exposed by the craniotomy was aspirated to an approximate depth of 1 mm from the skull surface at the margin of the craniotomy. The head was then rotated 10 degrees clockwise and a GRIN lens (1 mm diameter, 4 mm length, Inscopix part 1050-004623) was lowered through the center of the craniotomy to an approximate depth of 1.7 mm from the skull surface at bregma. The GRIN lens and medical-grade titanium headbars were then anchored to the skull using biocompatible cyanoacrylate superglue (Loctite 454). After 2-4 weeks, a baseplate (Inscopix part 1050-004638) was added with additional superglue and exposed glue was light-sealed using black nail polish.

### *Calcium imaging in dorsolateral striatum (DLS)*

To record neural activity in DLS, virus injection and GRIN lens implantation were performed in a single surgery, which varied slightly depending on the population being imaged (direct pathway, indirect pathway, or both at once). For pathway-specific imaging, 500-600 nL of AAV1.Syn.Flex.GCaMP6f.WPRE.SV40 virus (Penn Vector Core) was injected into the right DLS (AP 0.5, ML 2.25, DV 2.4) of Drd1a-Cre (N=7) or A2a-Cre (N=7) mice respectively and a GRIN lens (1 mm diameter, 4 mm length; Inscopix part 130-000143) was implanted 200uM above the injection site immediately following virus injection. Pathway-independent imaging followed a similar procedure, except we injected Drd1a-Cre mice (N=5) with a 1:1 mixture containing AAV1.EF1a.DIO.GCaMP6s.P2A.nls.dTomato (Cre-On; Addgene #51082) and AAV9.CBA.DO(Fas).GCaMP6s (Cre-Off; UNC Vector Core). Data from all pathways were combined for analysis since the key results did not differ by pathway (Figure S5I).

### *Electrophysiological recordings in dmPFC*

To record spiking activity in dmPFC, we implanted Neuropixels 1.0 probes (IMEC) chronically into the right medial prefrontal cortex (AP +2.3 mm, ML +0.5 mm, DV 3.5–4.5 mm from the cortical surface). Prior to implantation, probes were coated with the lipophilic tracer Dil (Fisher Scientific, catalog #V22888) for histological verification.

### *dmPFC lesions*

To lesion the dmPFC, we injected male C57BL/6J mice (8 weeks old) with N-Methyl-D-Aspartate (NMDA; Sigma, M3262-25MG) dissolved in saline (10 mg/mL). Control

(sham) mice were injected with saline alone. Two injections of 120 nL were made in each hemisphere (i.e., four total injections per mouse) at coordinates AP 2.3, ML  $\pm$ 0.4, DV -2.3 and AP +1.7, ML  $\pm$ 0.4, DV -2.0. Mice were recorded 5-12 weeks after surgery.

#### *Histological verification*

Following completion of behavioral tests, a subset of mice was anaesthetized using 1–2% isoflurane in air and perfused with cold PBS followed by 4% paraformaldehyde. Coronal brain sections (60 $\mu$ m) were sliced on a Leica VT1000 vibratome. All slices were stained with DAPI, and slices from lesioned mice were additionally stained with antibodies for glial fibrillary acidic protein (GFAP; Abcam; ab4674, 1/1000 dilution). Slices were imaged with an Olympus VS120 Virtual Slide Microscope.

### **Recording setups**

#### *Open field arena*

Neural and behavioral recordings in DLS were performed as previously described<sup>48</sup>. Recordings in dmPFC (excluding the novel object experiments) used an updated open field arena with a transparent floor that allowed simultaneous depth and infrared (IR) acquisition from above and below the animal. A pair of Kinect Azure cameras acquired synchronous 30Hz video data while neural calcium transients were recorded at 15Hz using the nVista 3.0 platform from Inscopix. To synchronize the two recording modalities, the nVista trigger port was connected to an Arduino that updated an array of IR LEDs in tandem with the neural data acquisition.

#### *High-speed 3D keypoint tracking*

Electrophysiological recordings, novel object recordings, and lesion recordings were captured using an array of 6 Basler ace acA1300-200um Monochrome USB 3.0 Cameras (Edmund Optics 33-978) as previously described<sup>49</sup>. The cameras were triggered at 120Hz using an Arduino, and a copy of the trigger signal was sent to the Inscopix or neuropixels data acquisition system for synchronization. The arena was illuminated with 32 near-infrared high power LED stars (LEDSupply, CREEXPE-FRD-3). To avoid reflections and saturations effects, the bottom camera was triggered slightly out of phase with the top cameras and the LEDs were split into two groups: one group below the arena that turned on during the bottom camera's exposure, and one group above the arena that turned on during the top and side cameras' exposures.

## Preprocessing of neural data

### *Miniscope recordings*

Calcium-fluorescence videos were recorded at 15Hz using the nVista 3.0 platform from Inscopix. Videos were spatially down sampled by a factor of 4 and motion corrected using CalmAn<sup>106</sup>. We used the constrained non-negative matrix factorization for microendoscopic data (CNMF-e) algorithm<sup>106,113</sup> to extract regions of interest (ROIs) corresponding to putative cells and their corresponding activity traces. ROIs were filtered using quality metrics output by CalmAn (CNN prediction > 0.05; signal-to-noise ratio > 2.5). Recordings with fewer than 50 detected neurons were rejected. Raw CNMF-e traces were Z-scored before downstream analysis.

### *Electrophysiology recordings*

Neuropixels signals were recorded with Open Ephys (V0.6). Data was then preprocessed with SpikeInterface<sup>107</sup>; we applied a bandpass filter between 300 and 6000Hz, followed by a global common median reference. We then spikesorted each recording independently using Kilosort 4<sup>108</sup>. In Kilosort 4, we set three hyperparameters: ‘amplitude\_cutoff\_thresh’ was set to 0.1, isi\_violations\_ratio\_thresh was set to 1, and presence\_ratio\_thresh was set to 0.9; the remaining hyperparameters were left to default values. Spike times were aligned to video frames and then binned using a 100ms sliding window. Unit coordinates were determined based on the position of the Neuropixels channel with the maximum amplitude for that unit. That channel’s position within the brain was then estimated using the Allen Institute brain atlas using the Pinpoint library<sup>109</sup>. Only units mapping to the prelimbic cortex were used for downstream analysis.

## MoSeq analysis from top/bottom depth and infrared

To estimate syllables from top-down and bottom-up depth/infrared cameras in the presence of occluders (e.g., during social interaction), we developed a novel pipeline that included segmentation, multi-camera registration, missing data imputation, dimensionality reduction and MoSeq analysis (Videos S1 and S2). A version of this pipeline for single-animal recordings is available online (<https://github.com/calebweinreb/top-bottom-moseq>).

### *Segmentation*

We first used a neural network with a UNET++ architecture<sup>114</sup> to segment the two interacting mice as well as the miniscope and its attached cable. To reliably distinguish between mice, we marked the tail base of one with a black sharpie and performed multi-animal keypoint tracking using higherHRNET<sup>115</sup>. Gaussian activations corresponding to

tracked keypoints were included as additional channels when training and applying the segmentation pipeline (Figures S1A and S1B).

#### *Multi-camera registration*

Intrinsic parameters for the top-down and bottom-up depth cameras were extracted using the Kinect Azure API. Extrinsic parameters were calculated from videos of a checkerboard calibration object, as demonstrated in the “calibrate.ipynb” notebook from the top-bottom-moseq repository. After calibration, 3D point clouds of segmented mice were derived from each camera and then embedded in a common coordinate system. Outlier points were removed using a nearest neighbor algorithm from Open3D<sup>116</sup>. The embedded point clouds were then rendered as a pair of reconstructed depth-maps from virtual orthographic top-down and bottom-cameras. IR reflectance signals were propagated along with the point clouds. The resulting depth and IR images were similar to the original camera outputs, but now registered to a common coordinate system. Areas that were out of view from the original (non-orthographic) camera angles (e.g., along the far flank of the mouse) were registered as missing data (see Video S1 for an example). These steps can be reproduced using the “orthographic\_reprojection” function from the top-bottom-moseq repository.

#### *Missing data imputation*

Imputation of missing pixel values was performed in three steps: a forward pass, a backward pass, and a merging step (Figure S1C, Video S2). Each pass was performed using a convolutional neural net (CNN) with a U-net architecture. During the forward pass, images were imputed based on masked observations from the current timepoint and imputation outputs from the previous timepoint. The purpose of recurrence here was to propagate relevant pose information across frames and to ensure temporal continuity of the imputed videos. Depth and IR channels from both cameras were imputed simultaneously using a single network. The backward pass was the same except that frames were fed in the opposite order. Another neural net was used to merge the forward and backward pass outputs. The resulting consensus images for each frame thus incorporated relevant pose information from past and future timepoints. The above steps can be reproduced using the “inpaint\_session” function from the top-bottom-moseq repository.

#### *Dimensionality reduction and MoSeq*

After imputation, top-down and bottom-up depth/IR images were centered and aligned and then projected into low-dimensional pose space. Rather than using principal components analysis (PCA) for dimensionality reduction – as is done in the standard MoSeq pipeline<sup>52</sup> – we opted for a shallow convolutional autoencoder due to its greater representational capacity (Video S2). The autoencoder included a 10-dimensional bottleneck layer and activations in this layer were used as the final pose representation.

Pose trajectories were then modeled using the standard MoSeq pipeline<sup>52</sup>. Separate MoSeq models were fit for the initial set of open field dmPFC recordings (with a C57 conspecific), the DLS open field recordings, and the aggression recordings with a CD1 conspecific.

### **MoSeq analysis from 3D keypoints**

For the high-speed multi-camera setup, 3D keypoint tracking and syllable inference were performed as previously described<sup>49</sup>. Briefly, 2D keypoint detection from each of the six camera angles was performed using an HRNet<sup>110</sup>. We then performed 3D triangulation using a custom multi-camera calibration pipeline ([https://github.com/calebweinreb/multicam-calibration/tree/main/multicam\\_calibration](https://github.com/calebweinreb/multicam-calibration/tree/main/multicam_calibration)). Keypoint trajectories were refined using GIMBAL<sup>117</sup>, which is a model-based approach that leverages anatomical constraints and motion continuity. The refined keypoint estimates were then fed to keypoint-MoSeq<sup>49</sup> for syllable inference. For the latter step, we used a 6-dimensional latent space, set the maximum syllable count of 50, and targeted a median syllable duration of 400ms. Separate models were fit for the lesion dataset, the miniscope recordings with novel objects, and the neuropixels dataset.

### **Syllable coarse-graining**

#### *Clustering syllables based on kinematic similarity*

To cluster syllables by kinematic similarity, we calculated the pairwise distance between their mean pose trajectories and then performed hierarchical clustering using complete linkage. For syllables derived from depth-MoSeq, the pose trajectory for a single syllable instance was defined as the sequence of dimensionally reduced poses spanning 5 frames before to 15 frames after the syllable start time and the mean trajectory was calculated as by averaging 1000 instances. The similarity between mean trajectories was computed using Pearson correlation. Keypoint-derived syllables, mean trajectories were estimated using the “get\_typical\_trajectories” function from the keypoint-moseq python package (version 0.4.7), with parameters set as follows: pre=20, post=60, min\_duration=12, density\_sample=False. The similarity between mean trajectories was computed using cosine distance.

#### *Clustering syllables based on transition probabilities*

To cluster syllables based on transition probabilities (Figures S1J and S1K), we represented the transition matrix as a directed graph. We then applied the Paris algorithm for hierarchical graph clustering<sup>54,118</sup>, which outputs a dendrogram describing the successive merges of syllables into clusters. To select levels of the hierarchy at

which to partition the dendrogram, we examined the differences in merge distances across consecutive steps. Specifically, we calculated the first derivative of the dendrogram linkage values and identified prominent local maxima using a peak-finding algorithm (minimum threshold = 0.02, minimum spacing = 3 merges). Three maxima were identified corresponding to  $N = 13$ ,  $N = 9$ , and  $N = 4$  clusters respectively.

### **Analysis of behavioral timescales**

To assess the predictability of syllable sequences, we computed mutual information (MI) at a range of temporal lags. Given offset  $\Delta t$  and syllable sequence  $z_1, \dots, z_T$ , we defined:

$$\text{predictability}(\Delta t) = \text{MI}(\{z_t, z_{t+\Delta t} \mid \text{for all } t \text{ such that } 1 \leq t \leq T - \Delta t\})$$

For Markov model comparisons, we calculated an empirical transition matrix for each recording and then sampled a Markovian sequence with the same length as the real sequence. For comparisons to shMoSeq, we fit the parameters of a shMoSeq model using real syllable sequences and then sampled synthetic state and syllable sequences by conditioning on those parameters (see “Inferring behavioral states” for details of model fitting and simulation).

We used a shuffle procedure to estimate the null distribution of MIs corresponding to a complete lack of predictability in behavior. The shuffle MIs were calculated by pairing independent syllable sequences from separate recordings. Specifically, given offset  $\Delta t$ , syllable sequence  $z_1, \dots, z_T$ , and a second syllable sequence  $z'_1, \dots, z'_T$  from another recording, we defined:

$$\text{shuffle predictability}(\Delta t) = \text{MI}(\{z_t, z'_{t+\Delta t} \mid \text{for all } t \text{ such that } 1 \leq t \leq T - \Delta t\})$$

this procedure was repeated once for each syllable sequence  $z_1, \dots, z_T$ , with the second sequence  $z'_1, \dots, z'_T$  sampled uniformly at random from the dataset. To estimate the temporal horizon over which a syllable sequence remains predictable, we calculated minimum  $\Delta t$  required for its MI to reach a noise floor (“mutual information decay time”). The noise floor was defined as the 99<sup>th</sup> percentile of the shuffle MIs (calculated over all shuffled sequences and  $\Delta t$ ’s).

In figures 1D, 1F, 7B, 7J, S7N and S7T, we used coarse-graining to improve the accuracy of MI estimation. Our reasoning was that MI becomes unreliable when there are too many syllable categories, as some categories occur only rarely. With limited samples, these rare categories inflate apparent correlations simply by chance, leading to an overestimate of predictability. To mitigate this sampling noise, we grouped

syllables into 8 clusters based on kinematic similarity and relabeled them before computing MI (i.e. after the Markov and shMoSeq simulations mentioned above).

Coarse-graining was also applied in figures S1K and S1M, but here to test whether non-Markovian sequence statistics were due to over-fragmentation of behavior. Syllables in this case were grouped into  $4 \leq N \leq 13$  kinematic clusters (Figure S1K) or  $3 \leq N \leq 16$  transition-based clusters (Figure S1M) and relabeled before both MI computation and Markov simulation.

To examine predictability within individual states (Figure S7T), we calculated MI over pairs of timepoints that fell within contiguous instances of each state. Since MI calculations are sensitive to the total amount of data used, we sampled an equal number of instances of each state ( $N = 200$  instances) and then used these to calculate per-state MI curves. This procedure was repeated 100 times to generate a bootstrap distribution of MI values for each temporal offset.

## **ShMoSeq: generative model and fitting algorithm**

### *Model motivation*

We sought a model that could (i) identify behavior states corresponding to different patterns of syllable usage over time; (ii) efficiently use the data available in a typical experiment (i.e. avoid over-parameterization). To that end, we created a hierarchical hidden Markov model (HHMM) called state-based hierarchical MoSeq (shMoSeq). In the model, each behavioral state specifies a transition matrix over syllables. Crucially, shMoSeq parameterizes each state's transition matrix by combining a baseline transition matrix (that is shared across behavioral states) with a vector of syllable biases (that is specific to each state) (Figure S2A). The baseline matrix captures general first order statistics – such as self-transitions among syllables or the tendency of mice rear down after they rear up – whereas the bias term state-dependent shifts in overall syllable usage. By combining these two terms, shMoSeq can express a wide variety of sequence statistics while limiting the total number of parameters.

shMoSeq has several advantages over a standard HHMM. First, it allows the dominant feature of syllable transitions – their autocorrelation – to be represented non-redundantly in the baseline transition matrix, rather than being separately ‘relearned’ by each state. Second, it allows for robust fitting given limited data; whereas a standard HHMM would have roughly  $n_{\text{states}} * n_{\text{syllables}}^2$  total parameters, shMoSeq has on the order of  $n_{\text{states}} * n_{\text{syllables}} + n_{\text{syllables}}^2$  parameters. To illustrate why this is important, note that a typical experiment includes several hours of data with roughly 5000 syllable transitions per hour. If we assume that there are 50-100 unique syllables and 5-10

behavioral states, then the number of parameters in a standard HHMM ( $10^4 - 10^5$ ) would rival or exceed the total number of independent observations in the dataset. By contrast, the number of parameter in a shMoSeq model ( $10^3 - 10^4$ ) would be well below the number of observations.

To formally test this intuition, we simulated data from a shMoSeq model instantiated with random parameters and then performed model fitting using either shMoSeq or a standard HHMM. The simulated datasets had 250,000 frames (~2.5 hours at 30 frames per second) and 5 states with average durations of ~10 seconds. For the underlying syllable sequences, we scanned over a range of syllable counts (5, 10, 20, or 50) and durations (1 – 100 frames). We found that for small numbers of syllables and/or short syllable durations, shMoSeq and the standard HHMM performed equally well (Figure S2B). However, in the more realistic scenario of 50 syllables with durations of  $\geq 10$  frames, shMoSeq continued to perform well while the standard HHMM failed catastrophically.

We note that an alternative approach for reducing the parameter count is to use a categorical hidden Markov model (CatHMM), where each behavioral state specifies a categorical distribution over syllables. CatHMMs have the virtue of being parameter efficient, but they introduce a new problem by assuming that syllables are conditionally independent given states. Because of this false assumption, CatHMMs are liable to overestimate the certainty of state sequences. This is illustrated by the following scenario: imagine there is a “walk” syllable that typically lasts half a second (15 frames) and occurs with 30% probability in state A and 60% probability in state B. Whenever this syllable occurs, the CatHMM will treat it as ~15 independent observations. If states A and B are equally likely a priori, the posterior probability of state B would be:

$$P(B \mid 15 \text{ frames of "walk"}) = \frac{P(\text{walk} \mid B)^{15}}{P(\text{walk} \mid A)^{15} + P(\text{walk} \mid B)^{15}} = 0.99997$$

whereas the correct estimate would be closer to:

$$P(B \mid \text{one instance of "walk"}) = \frac{P(\text{walk} \mid B)}{P(\text{walk} \mid A) + P(\text{walk} \mid B)} = 0.67$$

Thus, compared to alternatives such as standard HHMMs or CatHMMs, the parameterization instantiated in shMoSeq is well-suited to the syllable counts, autocorrelations, and dataset sizes typical of MoSeq datasets.

### Model definition

The generative model implemented in shMoSeq is defined as follows, where  $w_t$  denotes the current behavioral state and  $z_t$  denotes the current syllable.

$$\begin{aligned} w_t &\sim \text{Cat } \pi_{w_{t-1}} && \text{(state sequence)} \\ z_t &\sim \text{Cat } T_{z_{t-1}}^{(w_t)} && \text{(syllable sequence)} \end{aligned}$$

To prevent rapid switching between states, the transition matrix  $\pi$  has a sticky Dirichlet prior (defined below), in which each row  $\pi_w$  is drawn from a Dirichlet distribution that is boosted in the  $w$ 'th position by a stickiness hyperparameter  $\kappa$ .

$$\pi_w \sim \text{Dirichlet}(\beta, \dots, \beta + \kappa, \dots, \beta)$$

The size of  $\kappa$  determines the bias toward self-transitions, hence the durations of behavioral states. The syllable transition matrices  $T^{(w)}$  are constructed using a generalized linear model that adds state-specific syllable biases  $B_w$  to a baseline matrix  $A$  that is shared between states.

$$T_z^{(w)} = \text{Softmax}(A_z + B_w)$$

Because softmax is invariant to constant shifts of its inputs, we require that the rows of  $A$  and  $B$  are centered. We also assume that the columns of  $B$  are centered, since any uniform shift in a column of  $B$  can be countered by an equal and opposite shift in the same column of  $A$  without affecting the sums  $\{A_z + B_w \mid \forall w, z\}$ . We therefore define

$$\begin{aligned} A &= A' C_n & A'_{i,j} &\sim \mathcal{N}(0, \sigma_A^2) \\ B &= C_m^\top B' C_n & B'_{i,j} &\sim \mathcal{N}(0, \sigma_B^2) \end{aligned}$$

where  $n$  is the total number of syllables,  $m$  is the total number of states, and  $C_n$  is an orthonormal matrix that embeds  $\mathbb{R}^{n-1}$  into the space of centered vectors in  $\mathbb{R}^n$ .

### Parameter inference

Syllable sequences were derived using MoSeq and treated as observed. The shMoSeq model parameters and behavioral state sequence were fit using Gibbs sampling. We used a Laplace approximation to sample  $(A', B')$ . The mode of the Laplace approximation was approximated by gradient descent and the Hessian was computed through auto differentiation. All the other variables were sampled exactly. The behavioral state sequence sampled during the final Gibbs step was carried forward for analysis. Marginal probabilities of behavioral states were computed exactly using the forward-backward algorithm.

## ShMoSeq: practical application

### *Software availability and compute requirements*

shMoSeq can be run on any operating system that supports JAX (<https://github.com/jax-ml/jax>). A CUDA-capable GPU substantially reduces runtime but is not required. For reference, a 90-hour dataset takes ~1 minute to fit on a GPU and requires ~17GB of memory. The same dataset takes ~6 minutes on a CPU. Memory and runtime scale approximately with dataset duration, the number of syllables, and the number of states. shMoSeq can in principle be applied to any sequence of behavioral labels, not just those derived from MoSeq. Examples include labels generated by other unsupervised algorithms (e.g., B-SOiD<sup>119</sup>, VAME<sup>120</sup>) or from supervised classifiers, provided the sequences exhibit non-Markovian structure. We provide an online tutorial for mutual information-based analysis of non-Markovian statistics (as in Figures 1F–G).

To understand how much data is needed for model fitting, we fit shMoSeq to randomly down-sampled copies of our initial open field dataset. The quality of each fit was assessed using (1) log probability of the model (when applied to the full dataset; Figure S2N); (2) similarity to the final model that was fit using the full dataset (Figure S2O); this latter metric tests whether the down-sampled dataset is functionally equivalent to the full dataset with respect to model outputs. Both metrics rose steeply from 0 - 10 hours of data and then plateaued (or rose gradually) beyond 20 hours. To test the generalizability of these results, we repeated the same experiment using a larger dataset [200 hours of open field exploration from a previous paper<sup>65</sup>] and a range of values for the number of states (Figures S2P and S2Q). This time model quality plateaued after 20-40 hours of data, although some improvement beyond this point was evident for higher state counts. Based on these results, we recommend that datasets be  $\geq 10$  hours at minimum and ideally  $\geq 40$  hours.

When applying shMoSeq to a new dataset, it is often necessary to scan over stickiness values, state counts, and random seeds. To reduce the burden of hyperparameter search, we analyzed how the optimal stickiness varies with dataset size and number of states. Specifically, we fit shMoSeq to down-sampled copies of a large open field dataset from a previous paper<sup>65</sup>; we down-sampled to a range of dataset sizes and fit models with a range of state numbers, stickiness parameters and random seeds. After determining the optimal stickiness for each state count and dataset size, we observed a power-law scaling relationship (i.e. linear scaling in log space), suggesting that users can extrapolate the optimal stickiness from a smaller set of test fits (Figures S2R–S2T). The scaling relationship can be expressed as follows; coefficients will vary by dataset.

$$\log(\text{optimal stickiness}) \approx \beta_0 + \beta_1 \log(\text{number of states}) + \beta_2 \log(\text{amount of data})$$

### *Fitting and hyperparameter selection*

We fit separate MoSeq models and thus separate shMoSeq models for each dataset in the paper (see Table 2 for a list of datasets). In each case, we set  $\sigma_A = \sigma_B = 1$ ,  $\beta = 1$  and performed 500 Gibbs iterations, at which point we always observed a plateau in the held out log likelihood. We note that while 500 Gibbs iterations is enough to obtain a reasonable point estimate of the model parameters, we do not observe full mixing, so the outcome of fitting should not be viewed as a representative sample from the posterior distribution of the model. To determine the stickiness parameter and the total number of states for each dataset, we performed a two-parameter grid scan and fit 5 to 10 models for each parameter combination. Models were fit using 75% of the data and held-out probabilities were computed using the remaining 25% of the data. Across datasets, we found that the held-out probability tended to peak for intermediate values of the stickiness parameter; in each case, we used this peak to choose a stickiness value for the final model.

### *Choosing the number of shMoSeq states*

When scanning over the number of states, held-out likelihood typically rose monotonically with state number and therefore could not be used to pick a final state count. This is similar to MoSeq, where likelihood increases monotonically with the number of syllables. We therefore turned to cluster stability – which measures the consistency of state assignments across independent model fits. Our reasoning was that under-counting states would force shMoSeq to arbitrarily merge different behaviors, and this might occur differently on different model runs. Similarly, over-counting states would force shMoSeq to arbitrarily split behaviors, which again might lead to instability across model runs. Stability was calculated as follows. For each candidate number of states, we fit 5 to 10 independent models at the optimal stickiness and then calculated pairwise similarity (adjusted rand index<sup>121</sup>) between the resulting state sequences. A consensus sequence was selected based on average similarity to other sequences in the ensemble, and its average similarity was reported as the “cluster stability.” We selected the consensus sequence with greatest cluster stability for downstream analysis (Figure S2F). After selecting a final model, we excluded states that occupied fewer than 1% of all timepoints. States were named *post hoc* based on inspection of videos and analysis of syllable usage.

It is important to note that for most datasets, there will be multiple valid choices for the number of states. Cluster stability provides one heuristic, but other considerations may be appropriate given the specific biological question. It may be a useful exercise to vary the number of states and observe how behaviors are split and merged as a result. For example, in our initial open field dataset (for which 5 was the optimal number of states),

a 4-state model merged social engagement with exploratory locomotion, whereas a 6-state model split social engagement into two states with different velocity distributions (Figures S2H and S2I). Importantly, however, the timing of state transitions was very consistent across a wide range of state counts (Figure S2J), which suggests that the underlying notion of long-lived behavioral states does not depend on any specific level of coarse-graining.

## Breadth of neural tuning

### *Lifetime sparseness*

Lifetime sparseness (Figure S4B), which measured the breadth of neural tuning<sup>56</sup> was calculated as follows, where  $r_{ij}$  denotes the average activity of neuron  $i$  during timepoints assigned to state  $j$  ( $1 \leq j \leq m$ ):

$$\text{sparseness(neuron } i) = 1 - \frac{\text{mean}(r_{i1}, \dots, r_{im})^2}{\text{mean}(r_{i1}^2, \dots, r_{im}^2)}$$

### *State decoding from top-N sparsest or least sparse neurons*

To test the relative contribution of sparsely tuned versus broadly tuned neurons to behavioral state decoding, we ranked neurons by sparseness and then computed decoding accuracy using only the top N most sparse neurons or bottom N least sparse neurons for varying N (Figures S4C and S4D). In each of these cases, neurons were re-ranked based on the specific training data from each step of k-fold cross-validation to prevent leakage between the training and testing datasets.

### *State decoding from top-N most informative neurons*

To test the relative contribution of highly informative versus weakly informative neurons to behavioral state decoding, we ranked neurons based on the MI between neural activity and behavioral state and then computed decoding accuracy using only the top N neurons for varying N (Figure S4D). To calculate MI for a given neuron, its activity was discretized into quartiles. As above, MI was recalculated based on the specific training data from each step of k-fold cross-validation to prevent leakage between the training and testing datasets.

## Decoding models

Decoding analyses were performed using either ridge regression (for continuous variables) or logistic regression (for discrete variables) with Z-scored neural activity traces as input. Models were implemented using scikit-learn version 1.2.2<sup>122</sup>.

Measurements of accuracy were performed using 5-fold cross validation. To prevent

leakage between neighboring frames, recordings were split into non-overlapping blocks of 1-5 minutes each and each block was randomly assigned to one of five groups; this yielded a 5-way partition of the recording that was used for cross-validation. For continuous variables, accuracy was quantified as the Pearson correlation between true and predicted values of the decoded variable. For discrete variables, accuracy was quantified using area under the receiver operating characteristic curve (auROC).

### **Analysis of single-neuron behavior associations**

Significant associations between individual neurons and behavior variables were computed using an auROC-based approach<sup>40,123</sup>. To compute auROC, we treated behavior as a binary “outcome” and neural activity as a continuous “predictor”. To determine statistical significance, the auROC was recalculated across 1000 cyclic permutations. P-values were computed relative to this simulated null distribution and then transformed using the Benjamini-Hochberg procedure to control the false discovery rate.

### **Neural encoding models**

To understand if neurons contain information about states that is independent of instantaneous kinematics, we constructed encoder models that predicted neural activity from kinematics, syllables, and states or just kinematics and syllables (Figures 3H–3I and S5C–S5E). Prediction was performed using ridge regression. The kinematic variables included height, centroid velocity, and angular velocity. Syllables were encoded one-hot. States were represented by their marginal probabilities. Thus the final design matrices had dimension (3 + num syllables) or (3 + num syllables + num states). In one case (Figures S5F and S5G), we also included four world-centric variables in both encoders (distance to wall, egocentric direction to wall, proximity of conspecific, egocentric angle to conspecific). Accuracy was assessed using the correlation between true and predicted neural activity. A neuron’s accuracy was considered significant when it exceeded the 95<sup>th</sup> percentile of a shuffle distribution generated via cyclic permutation. Neurons were only included in downstream plotting and analysis if predictions from both encoder models were significant. For the recording- and mouse-level comparisons in Figure S5E, we only included recordings where at least 10 neurons met this criterion.

### **Neural activity manifolds**

To generate low-dimensional representations of neural activity, we binned neural activity in non-overlapping half-second intervals, normalized with a rolling Z-score (using a 20 minute window) and then performed PCA to 5 dimensions. Projection into 2D was then performed using Uniform Manifold Approximation and Projection (UMAP)<sup>111</sup>. To visualize

behavioral features in the 2D map while minimizing the impact of crowding/plot-order, we superimposed a square lattice and generated a heatmap by averaging the values within each lattice cell. To quantify neural segregation of different behavioral partitions, we computed the modularity<sup>124</sup> of this partition with respect to the k-nearest-neighbor graph of neural activity 5D PCA space. Specifically, for each timepoint we counted the proportion of its 500 nearest neighbors that shared the same label and then computed the average of this proportion across all timepoints. We repeated this procedure for a shuffled partition and finally defined a “segregation index” as the difference between real and shuffled values. To minimize the effect of autocorrelation in the neural signal, we restricted analysis to neighbors that were far apart in time. Specifically, each recording was split into two subsets (“A” and “B”) consisting of alternating 10 min blocks separated by one-minute gaps. For each timepoint in “A”, we only considered nearest neighbors from “B” (and vice versa). This ensured that all edges linked timepoints separated by at least one minute.

### **Mapping behavioral states to neural principal components**

To determine whether behavioral states constitute the dominant axes of neural activity, we compared the neural activity vectors associated with each state to the top-ranking neural principal components (PCs) (Figures S4G–S4I). State-associated vectors were defined through least squares regression as follows. Let  $Z_{n,t}$  represent the activity of neuron  $n$  at timepoint  $t$ , and let  $P_{i,t}$  represent the marginal probability of state  $i$  and timepoint  $t$ . The vector associated with state  $i$  was defined by

$$\hat{v}_i = \operatorname{argmin}_v \sum_t \left( P_{i,t} - \sum_n Z_{n,t} v_n \right)^2$$

### **Analysis of stereotypy in neural trajectories**

To determine if neurons were preferentially active during a certain phase of a behavioral state (Figure S4K), we used the following time-warping procedure. For each neuron that was tuned to a particular state, we collected all instances of the state between 5 and 60 seconds in duration and placed them on a common timeline using linear time-warping. This yielded a matrix  $Z_{i,t}$  of neural activity across instances  $i = 1, \dots, N$  and warped timepoints  $t$ . If a neuron is preferentially active at particular times within each state instance, then the column-wise mean of this matrix should significantly deviate from a flat line. To assess this possibility, we computed the following test statistic for real data and for shuffle data in which the rows of  $Z_{i,t}$  were cyclically permuted.

$$\text{test statistic} = \sum_t \left| \sum_i Z_{i,t} \right|$$

This yielded a P-value for each neuron/state pair. The P-values were then transformed using the Benjamini-Hochberg procedure to control the false discovery rate.

### **Analysis of relative wall position during open field exploration**

#### *Definition of relative wall angle and direction*

At each timepoint, we calculated a “wall-vector”, defined as the vector that points toward the mouse’s centroid and originates at the nearest point on the wall. We then defined the “egocentric wall angle” as the angle between the wall-vector and mouse’s heading vector; this angle was  $0^\circ$  when the mouse pointed away from the wall,  $180^\circ$  when the mouse pointed toward the wall, and  $\pm 90^\circ$  when the mouse was parallel to the wall. Relative “wall direction” was then defined as the cosine of the egocentric wall angle.

#### *Wall distance and proximity*

To capture how close the mouse was to the wall, we defined two metrics: “wall distance” and “wall proximity”. Wall distance was defined as the distance from the mouse’s centroid to the nearest point on the wall. “Wall proximity” was a function of wall distance:

$$\text{wall proximity} = -\tanh \left( \frac{\text{wall distance} - d_0}{d_0} \right)$$

where  $d_0$  was 2.5 cm (Figure S6B); this function was designed to magnify variation near 0 and disregard fluctuations in wall distance when the mouse was far from the wall.

#### *Definition of direction-tuned and proximity-tuned neurons*

Neurons tuned to wall proximity or wall direction were identified using the auROC-based method described in “Analysis of single-neuron behavior associations”. This entailed defining binary variables and testing whether neurons were more or less active than chance during the on-times for each variable. Wall proximity was represented as a binary variable by thresholding wall distance at 5 cm. Wall direction was represented as a pair of binary variables by thresholding below -0.8 or above 0.8.

### **Analysis of aggression experiments**

#### *Annotation of aggressive episodes*

We used manual labeling to identify instances of aggression in behavioral videos. Labeling was performed on a frame-by-frame basis. Frames were included if they

contained any of the following behaviors. Aggression bouts were defined as continuous blocks, ignoring gaps of less than 500ms.

- **Chasing:** One mouse actively pursues another at high speed.
- **Pouncing:** One mouse lunges or leaps towards another.
- **Mounting:** One mouse climbs onto the back of another.
- **Tumbling:** Mice become entangled and roll or tumble together at high speed.
- **Boxing:** Mice rear and face each other, usually with one in a defensive posture.

#### *Visualization of pre- and post-aggression behavior state probabilities*

For visualization of pre-, mid-, and post-aggression behavioral state probabilities (Figure 5B), we included all aggression bouts where no aggression occurred in the preceding or succeeding 15 seconds respectively. Since aggression bouts have variable duration, mid-aggression state probabilities were linearly time-warped to a common duration of 10 s.

#### *Regressing neural activity against wall direction and proximity (aggression experiment)*

When assessing whether neural responses to wall direction or proximity were altered by the presence of an aggressor (Figures 5F–5I), we tried to minimize the effect of correlations between behavioral variables (e.g. mice tended to be close to the wall and parallel to the wall at the same times). Specifically, we isolated the effects of distance to the wall by restricting to timepoints when mice were already parallel to it ( $|\cos(\text{egocentric wall angle})| \geq 0.9$ ) and isolated the effects of angle to the wall by restricting to timepoints when mice were already close to it (within 2 cm). Furthermore, to emphasize state-dependent (rather than motor-dependent) responses, we excluded from analysis all timepoints when the subject mouse was actively being attacked.

#### *Wall-related activity in each behavior state (aggression experiment)*

To assess wall-related activity in each behavioral state (Figures 5D and 5E), we restricted to timepoints when mice were near the wall (within 2 cm) and parallel to it ( $(|\cos(\text{egocentric wall angle})| \geq 0.9)$ ) and excluded timepoints when mice were being actively attacked. Mean activity of wall-direction-tuned neurons was calculated using the subset of neurons that preferred the current wall direction at each timepoint.

## **Analysis of novel object experiments**

### *Object tracking*

To track object bounding boxes, we trained a yolov5 object detection neural network<sup>125</sup>. Post processing of object detections varied depending on the dataset type. For videos where every object was unique, we: (1) filtered out detections with confidence below 0.7; (2) denoised the remaining detections using a 4-second median filter; (3) performed

linear interpolation to fill in missing detections. For videos where all the objects were identical, we: (1) filtered out detections with confidence below 0.5; (2) filtered out frames where the number of detections differed from the true number of objects; (3) tracked object identities over time by maximizing pairwise intersection-over-union with the Hungarian algorithm; (4) performed linear interpolation to fill in missing detections. In both cases, we defined object locations by the centroids of the resulting bounding boxes.

#### *Definition of object-associated syllables*

To identify syllables that occurred with greater than chance frequency in the vicinity of objects (Figure S3I), we calculated – for each syllable – what fraction of instances occurred while the mouse’s nose was within 6 cm of the object centroid. Syllables were deemed significant if this fraction exceeded the 95<sup>th</sup> percentile of a shuffle distribution generated through cyclic permutation of the mouse’s tracking data. “Object-associated syllables” were defined as the set of 5 syllables exceeding this significance threshold, with the exclusion of one syllable that consisted entirely of grooming.

#### *Definition of object distance and proximity*

We defined distance to an object as the planar distance between the x/y coordinates of the mouse’s nose and the x/y coordinates of the object centroid. When multiple objects were present, we used the distance to the closest object. “Object proximity” was a function of object distance:

$$\text{object proximity} = -\tanh \left( \frac{\text{object distance} - d_0}{d_0} \right)$$

where  $d_0$  was 2.5 cm (Figure S5B); this function was designed to magnify variation near 0 and disregard fluctuations in object distance when the mouse was far from an object. Because tanh has an asymptote at -1, we set object proximity = -1 when no objects were present.

#### *Neural activity related to object proximity*

Neurons tuned to object proximity were identified using the auROC-based method described in “Analysis of single-neuron behavior associations”. As the target variable, we used a binary indicator that was 1 when any object was within 3 cm and 0 otherwise. When regressing neural activity against object proximity (Figures S6N and S6O), we excluded recordings with fewer than 5 object-tuned neurons and state/recording pairs that contained very few object interactions (i.e. where object distance fell below 6 cm in fewer than 10% of frames). The number of recordings that passed this filter varied from 8 to 16 depending on the behavioral state.

### *Decoding of object proximity*

When comparing the accuracy of object proximity decoding across states (Figure 4M), we only included recordings where object proximity could be decoded with above chance accuracy when assessed using all frames. Specifically, the correlation between true and predicted object proximity had to exceed the 95<sup>th</sup> percentile of a shuffle distribution (N=17 recordings satisfied this criterion).

### *Decoding of object identity*

When training and testing decoding models for object identity (Figures 4N and 4O), we restricted to timepoints when mice were within threshold distance to one and only object, and also facing that object. The distance threshold varied from 1 cm to 6 cm; Figure 4N shows results for 1 cm, and Figure 4O shows results across the full range of thresholds. We classified a mouse as ‘facing’ a target object if it deviated from the mouse’s heading angle by less than 30 degrees. Analysis was restricted to the first three objects added to the arena, since the last two objects were absent for most of the recording.

## **Analysis of social engagement state**

Our initial open field dmPFC recordings were 80 minutes long and contained two 30-minute blocks when a C57 conspecific was present. All the analyses below were restricted to these 60 minutes.

### *Mutual information between subject and conspecific syllables*

To characterize the degree of social interaction across behavioral states, we computed the MI between subject and conspecific behavior (Figure 2G). To mitigate the effects of sparsity, behavior was represented using coarse-grained syllable labels (N=8 clusters, defined as described in “Kinematic clustering of syllables”). We used bootstrap resampling to quantify uncertainty (N=200 bootstrap samples). For each resampling round, recordings were sampled with replacement, concatenated, and then filtered by each behavioral state respectively for calculation of MI. The same 8-way clustering of syllables was used to calculate the frequency with which mice performed kinematically similar behaviors at the same time (Figure S3B).

### *Social distance and proximity*

Because subject mice primarily interacted in a ‘nose-first’ manner, social distance was defined as the distance from the nose of the subject mouse to the centroid of the conspecific mouse. “Social proximity” was a function of social distance:

$$\text{social proximity} = -\tanh \left( \frac{\text{social distance} - d_0}{d_0} \right)$$

where  $d_0$  was 10 cm. Neurons tuned to social proximity were identified using the auROC-based method described in “Analysis of single-neuron behavior associations”. As the target variable, we used a binary indicator that was 1 when the social distance was  $\leq 5$  cm and 0 otherwise.

#### *Time-dependent changes in neural activity*

To capture temporal changes in the strength of activity elicited by social contact (Figure S6W), we divided each recording into 2-minute bins that tiled the first 10 minutes of each social interaction block. The bins were overlapping and spaced at 30s intervals. Within each bin, we calculated the average activity of social proximity-tuned neurons during “close” timepoints (social distance  $\leq 5$  cm) and “far” timepoints (social distance  $\geq 20$  cm). Bins with no “close” or “far” timepoints were counted as missing data. Recordings were only included if the number of social proximity-tuned neurons was  $\geq 10$ . Since some decline in activity is expected due to photobleaching, we also generated a baseline curve (Figure S6X) by calculating the 90<sup>th</sup> percentile of activity across neurons at each timepoint and then reporting its average within each 2-minute bin.

#### *Regressing neural activity against distance to conspecific*

When regressing the activity of social proximity-tuned neurons against distance to the conspecific (Figures 4I and 4J), we restricted analysis to recordings with at least 10 social proximity-tuned neurons. Additionally, for each behavioral state, we only included recordings where the state occurred for a total of 10s or more. To emphasize state-dependent (rather than trivial motor- or sensory-dependent) effects, we excluded bouts of aggression and restricted to timepoints when the subject mouse was facing the conspecific.

### **Tuning to allocentric spatial variables**

To determine whether neurons were tuned to absolute location (Figures 4A and S6A), we divided the circular open field into 8 sectors (like slices of a pie) and then identified neurons that were significantly active in one slice using the auROC-based method described in “Analysis of single-neuron behavior associations”. To determine whether neurons were tuned to absolute heading, we similarly discretized the mouse’s heading angle into 8 bins and then identified neurons that were significantly active in one bin.

### **Analysis of tube test dataset**

Neural activity and behavior annotations from (Kingsbury et al., 2019)<sup>40</sup> were obtained upon request to the authors. The dataset consisted of six sessions, each with multiple tube test trials per session. Each session included simultaneous behavior and neural

recordings from both mice. Three behaviors were annotated: “push”, “retreat”, and “approach”. We treated each mouse/session pair as an independent recording and labeled behavior as “self” and “other” accordingly. All analyses were limited to session/behavior pairs where the behavior decoding had above chance accuracy ( $P < 0.05$ ); this was assessed by computing an auROC for the true predictions and 100 cyclic permutations. Cross-correlations were between decoded behavior probabilities and binary indicator variables representing each behavior.

We used kernel regression to characterize neural activity associated with onset versus offset of the “self-push” behavior (Fig 6C). Decoding models were first trained to predict the probability of “self-push” from heldout neural activity. The decoded behavior probabilities were then Z-scored and modeled as follows

$$P_{\text{self-push}}(t) = \sum_{i,j} \alpha_j K_j(t - t_i^{\text{onset}}) + \sum_{i,j} \beta_j K_j(t - t_i^{\text{offset}}) + \text{error}$$

Where  $[t_i^{\text{onset}}, t_i^{\text{offset}}]$  represent on/off times for the  $i$ 'th “self-push” bout and  $\alpha_j / \beta_j$  are onset/offset coefficients for the  $j$ 'th kernel basis function. Basis functions were defined by  $K(t) = \tanh((t - t_0)\tau)$  for a range of shifts ( $-0.5 \leq t_0 \leq 0.5$ s) and slopes ( $-0.5 \leq \tau \leq 0.5$ ). Parameters were fit using Ridge regression.

### **Analysis of DLS lesion experiments**

Timescale analysis of DLS lesion data (Figure 7J) was performed using the procedure described in “Mutual information analysis of behavioral timescales”. Data (including syllable annotations) were obtained from<sup>48</sup>. We restricted analysis to recordings in an empty open arena, i.e. without a trimethylthiazoline (TMT) or control odor source.

### **Comparative analysis of egocentric boundary vector tuning**

We analyzed egocentric boundary representations in retrosplenial cortex (RSC) using data kindly provided by the authors of (Alexander, 2020)<sup>68</sup>, which included rat trajectories, headings, boundary coordinates, spike times, and unit annotations. The data included 117 recordings with 555 units total, of which 134 were classified as egocentric boundary vector (EBV) cells. Analysis was restricted wall-tuned neurons in dmPFC and EBV cells in RSC<sup>68</sup>.

We assessed neural tuning using egocentric boundary ratemaps (EBRs), which capture responses to boundaries at an array of locations in egocentric coordinates (Figure 4E). Each map was parameterized as a grid in polar coordinates, and the value at each grid location represented the expected activity of a neuron given that a boundary was

present at that particular distance and angle relative to the animal ( $0^\circ$  = in front,  $90^\circ$  = left,  $180^\circ$  = behind,  $270^\circ$  = right). The grids were spaced with angle increments of  $3^\circ$  and distance increments of either 2.5 cm for RSC or 1 cm dmPFC (finer spacing for dmPFC reflects the smaller size of mice relative to rats). We only considered boundary points within a threshold distance equal to one-quarter the width of the area (30 cm for the RSC dataset, 10 cm for the dmPFC dataset). EBRs were smoothed with a two-dimensional Gaussian kernel ( $\sigma = 5$  bins, truncation =  $0.5\sigma$ ) prior to downstream analysis.

Preferred boundary distance (Figure 4F) was defined as the distance with the highest average activity (i.e. the peak in the EBR). Angular tuning was quantified using the mean resultant length (MRL), computed as the magnitude of the vector sum across distance/angle bins weighted by their EBR values. Significance was assessed using a shuffle distribution of MRLs obtained by cyclically permuting neural activity; observed MRLs were Z-scored against this null distribution (Figure 4E).

## QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were conducted with Python using scikit-learn, statsmodels, and NumPy/SciPy. Statistical tests, P-values, measures of uncertainty, and sample sizes (N) for each panel are reported in the corresponding figure legends. Unless noted in the legends, the independent unit of analysis (N) is a recording/session. All hypothesis tests were two-sided with  $\alpha=0.05$  unless stated otherwise. Mann-Whitney U tests were used to compare the means of populations. Multiple comparisons were controlled using the Benjamini–Hochberg false discovery rate (FDR) procedure where families of tests are performed (e.g., per-neuron screening or per-state comparisons); the legend specifies when FDR is applied. In all boxplots, the box and center line correspond to the median and interquartile interval respectively.

## ADDITIONAL RESOURCES

ShMoSeq documentation page with installation instructions, example datasets, and step-by-step tutorials for fitting models and selecting hyperparameters: <https://statemoseq.readthedocs.io>

## SUPPLEMENTAL VIDEO TITLES AND LEGENDS

**Video S1: Segmentation and inpainting pipeline, related to Figure 1 and STAR Methods.** Example video illustrating the results of segmentation, registration and inpainting. The four columns show (1) top camera depth channel; (2) top camera IR channel; (3) bottom camera depth channel; (4) bottom camera IR channel. The top row shows the results of segmentation and top-bottom registration. Red and blue pixels represent missing data. Red pixels correspond to occlusions (conspecific mice, camera,

or cables). Blue pixels represent areas that were not visible from the original (non-orthographic) camera angle. The bottom row shows the results of inpainting.

**Video S2: Top/bottom depth and infrared dimensionality reduction, related to Figure 1 and STAR Methods.** Example video illustrating the autoencoder used to represent pose for downstream processing with MoSeq. The four columns are as in Video S1. The top row shows the output of inpainting. The second row shows the same output after centering and rotational alignment. These data are fed to the autoencoder, whose output (after encoding and decoding) is shown in the third row.

**Video S3: Illustration of behavioral states, related to Figure 1.** Example clips showing behavioral states derived from the initial dmPFC recordings with a C57 conspecific. Instances of each state were randomly sampled and then synchronized to state onset, which is marked by the appearance of a white dot. Disappearance of the dot marks the end of the state. Note that end-times are asynchronous since instances of a state can have variable duration.

**Video S4: Head-to-head comparison of control and dmPFC-lesioned mice, related to Figure 7.** Example clips illustrating no obvious behavioral differences between a control mouse (left) and one with lesioned dmPFC (right).

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