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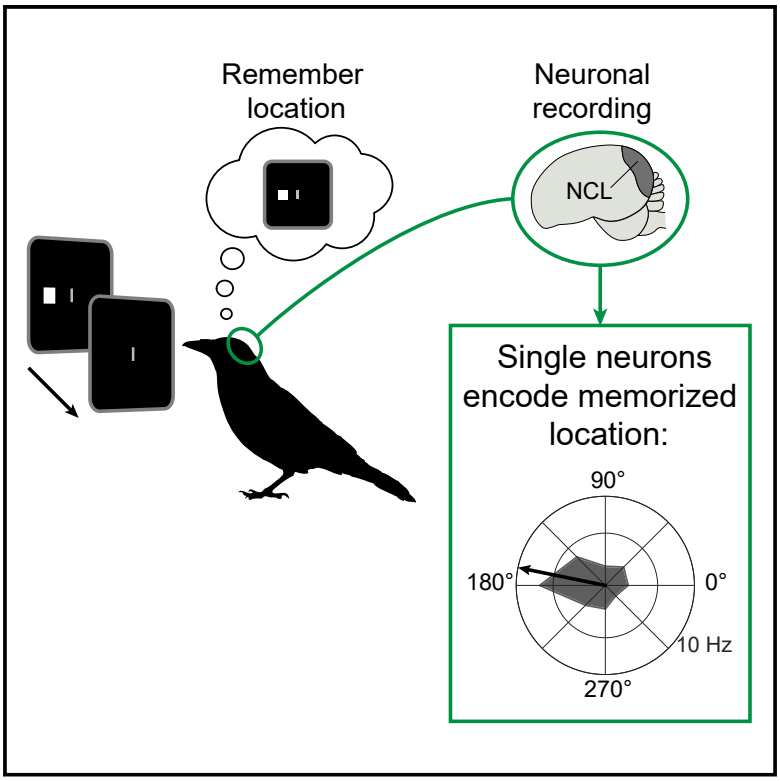
Neuronal Correlates of Spatial Working Memory in the Endbrain of Crows

Graphical Abstract



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In Brief

In crows trained to memorize the variable location of a visual item, Rinnert et al. show that neurons in the endbrain area *Nidopallium caudolaterale* are tuned in a behaviorally relevant way to individual preferred locations during working memory and are reminiscent of the convergently evolved primate prefrontal cortex.

Highlights

- Crows were trained to flexibly remember the variable location of a visual item
- NCL neurons were selectively tuned to spatial location in working memory
- Neurons stably maintained spatial information throughout the working memory period
- Spatially tuned neurons predicted the crows' future choices

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Neuronal Correlates of Spatial Working Memory in the Endbrain of Crows

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SUMMARY

Birds are renowned for their excellent spatial cognition. Corvid songbirds, in particular, rely on explicit representation of spatial cues in memory when caching food and retrieving caches for later consumption. However, the neuronal correlates of flexible spatial memory abilities are largely unknown in birds. We therefore trained carrion crows (*Corvus corone*) on a spatial delayed-response task in which they had to maintain the variable location of a visual item for a few seconds in working memory. After the crows performed this task with high precision, we recorded single-cell activity from the associative endbrain area *Nidopallium caudolaterale* (NCL) in the behaving crows. A large fraction of NCL neurons were tuned to individual preferred locations and selectively maintained the spatial location of items in working memory. A comparison of firing rates with reaction times suggested that the majority of delay-selective neurons represented stored location information rather than motor preparation. Almost 30% of all recorded neurons were tuned during both visual presentation and memory delay, and their spatial tuning was significantly correlated. The population of recorded neurons stably maintained spatial information over the course of the working memory period. Importantly, the neural responses of spatially tuned neurons were relevant for the crows' choices and allowed a statistical classifier to predict the subsequently chosen target location in free-choice trials. Our findings demonstrate the pivotal role of the avian NCL in spatial working memory that is reminiscent of the function of the convergently evolved primate prefrontal cortex in spatial working memory.

INTRODUCTION

Birds possess excellent spatial memory [1, 2]. Navigating birds, such as homing pigeons or migratory birds, travel hundreds of kilometers to reach precise target locations. Food-storing birds face similar challenges of spatial cognition when they create food caches during times of resource abundance for later retrieval during times of scarcity. Many corvid songbirds (jays and crows) cache food for later consumption and rely on precise spatial memory to retrieve their caches. In addition to their own caches, some corvid species also remember and later pilfer the caches of conspecifics [3, 4]. This led to the development of sophisticated cache protection strategies that again rely on flexible spatial cognition [5]. Corvids are also known to flexibly update their spatial memory: they switch from recovering perishable to non-perishable food after longer delays between caching and recovery [6], thus integrating information about the decay progress of caches [7]. To succeed in these situations, birds rely on the explicit representation, memorization, and manipulation of visual spatial cues in working memory, their visuo-spatial sketchpad. While the working memory capacity of pigeons and crows for object identity [8–10] and object categories [11–13] has been explored in some detail, their capacity to memorize the location of objects is largely unknown. Despite the importance of spatial working memory for birds, and corvids in particular, the neuronal correlates of this important type of working memory remain unknown.

We therefore explored the single-neuron mechanisms of visuo-spatial working memory in the telencephalic area known as *Nidopallium caudolaterale* (NCL) of behaving crows. The avian NCL is a high-level association area that receives input from all sensory modalities, interacts with long-term memory-related structures, and projects to premotor brain areas [14, 15]. As reflected by its anatomical connections, the corvid NCL plays an important role in a variety of cognitive functions [16–18]. Despite the independent and anatomically distinct evolution of avian and mammalian endbrains, the NCL is therefore considered to be the functional equivalent of the prefrontal cortex (PFC) [14, 15, 19], which enables working memory and cognitive control in primates [20–24]. Single-cell recordings showed that the NCL is involved in short-term memory representations: NCL neurons show selective delay activity in response to the identity of specific visual images and auditory events that serves to bridge temporal gaps in delayed-response tasks [25–27]. However, in ecologically relevant situations, such as the foraging situations described above, object location is another important feature that needs to be kept in mind in order to successfully solve tasks. To explore the neuronal mechanisms that allow birds to remember object locations, we recorded single-cell activity in crows performing a task that required visuo-spatial working memory.

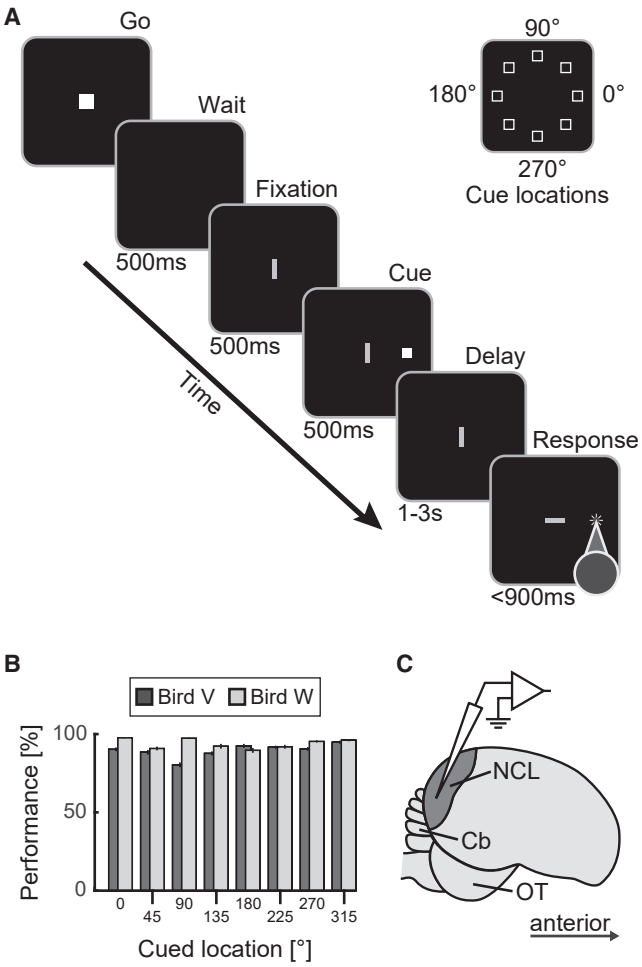

Figure 1. Spatial Delayed-Response Task and Behavioral Performance

(A) Behavioral task. The crow initiated a trial by positioning its head in front of the display in the go period. The go period was followed by a black screen (wait period, 500 ms). Next, a small vertical line appeared as fixation stimulus (fixation, 500 ms), which the crow was trained to observe and only respond when it changed its orientation. During the cue period, a peripheral cue (white square) was presented for 500 ms in one of eight possible locations (see inset "cue locations"). The bird had to memorize the location of the cue over the following variable delay period (duration between 1 and 3 s). Once the fixation line changed its orientation, the crow was required to respond as quickly as possible (within 900 ms) by pecking at the remembered location.

(B) Average behavioral performance for the eight cue locations over all recording sessions for bird V (black bars) and bird W (gray bars). Error bars indicate the standard error of the mean (SEM).

(C) Lateral view of a crow brain with the Nidopallium caudolaterale (NCL, shaded) located inside the telencephalon. Cb, cerebellum; OT, optic tectum.

RESULTS

We trained two hand-raised carrion crows [28] on a spatial delayed-response task in which they had to memorize for a few seconds the variable location of visual items displayed on a touchscreen (Figure 1A). In every trial, one of eight circularly arranged spatial locations was briefly cued by a gray square. After the cue had disappeared, the crows were required to remember the spatial location for an unpredictable time delay ranging from 1 to 3 s. After this delay, they were instructed to peck at this location on the all-black screen. To ensure that the crows maintained a stable visual field throughout the trial, they were additionally trained to fixate a central fixation target. The change of the fixation target after the variable delay of 1 to 3 s instructed the crows to peck at the previously cued location within 900 ms. Responses later than 900 ms were not rewarded. This forced the crow to look at the center of the screen throughout the trial in order not to miss the changing of the fixation target. Both birds performed above 80% for all eight cued locations over all recording days (Figure 1B).

Single Neurons Encode Cued Location in the Cue and Delay Period

While the crows performed this task, we recorded the activity of 291 single neurons in the right NCL [29] (Figure 1C). The cued location selectively modulated the activity of single neurons during the cue and delay period. An example neuron is shown in Figure 2A. This cell selectively increases its firing rate after onset of the bottom-left cue (location 225°) and the cues adjacent to this preferred location. After the cue vanishes, the firing rate returns to baseline for all locations. For each of the eight locations, an individual vector with a length corresponding to the average firing rate to a given location was calculated. The preferred direction of a neuron was defined as the angle of the average vector resulting from vector addition of all eight vectors [30] (Figure 2A, right panel). Neurons like this encode the location of the cue during its visual presentation and will be called cue-selective neurons. Other neurons modulated their firing rates only while the crows memorized the cued location in the delay period. Figure 2B shows such a delay-selective neuron that signals the right location (0°) only during the delay when the visual information had already disappeared. A third group of neurons, finally, responded selectively both during the cue and the subsequent delay period. The neuron in Figure 2C increases its firing rate for cues at locations 225°–315° (bottom locations) already during the end of the cue period. The selective responses are then maintained over the entire delay period. Such cue-and-delay-selective neurons bridge the temporal gap between cue presentation and the required pecking response.

A Large Fraction of NCL Neurons Encodes Spatial Information

We used analyses of variance (one-factorial ANOVA; $p < 0.01$) to test if the activity of a single neuron encoded the cued location. During the cue period, 55% (161/291) of all neurons were significantly tuned to the cued location (Figure 2D, example in Figure 2A). The preferred directions of these spatially tuned cue-selective neurons, as defined by the averaged firing rate over all locations, were equally distributed across all possible locations (Figure 2E). An equal number of those neurons preferred stimuli on the left and right side of the screen, respectively (55% versus 45%; binomial test, $p > 0.05$).

During the delay period, 41% (120/291) of all neurons were significantly encoding the memorized cue location (Figure 2D, examples in Figures 2B and 2C). The preferred directions of delay-selective neurons were distributed about equally to all

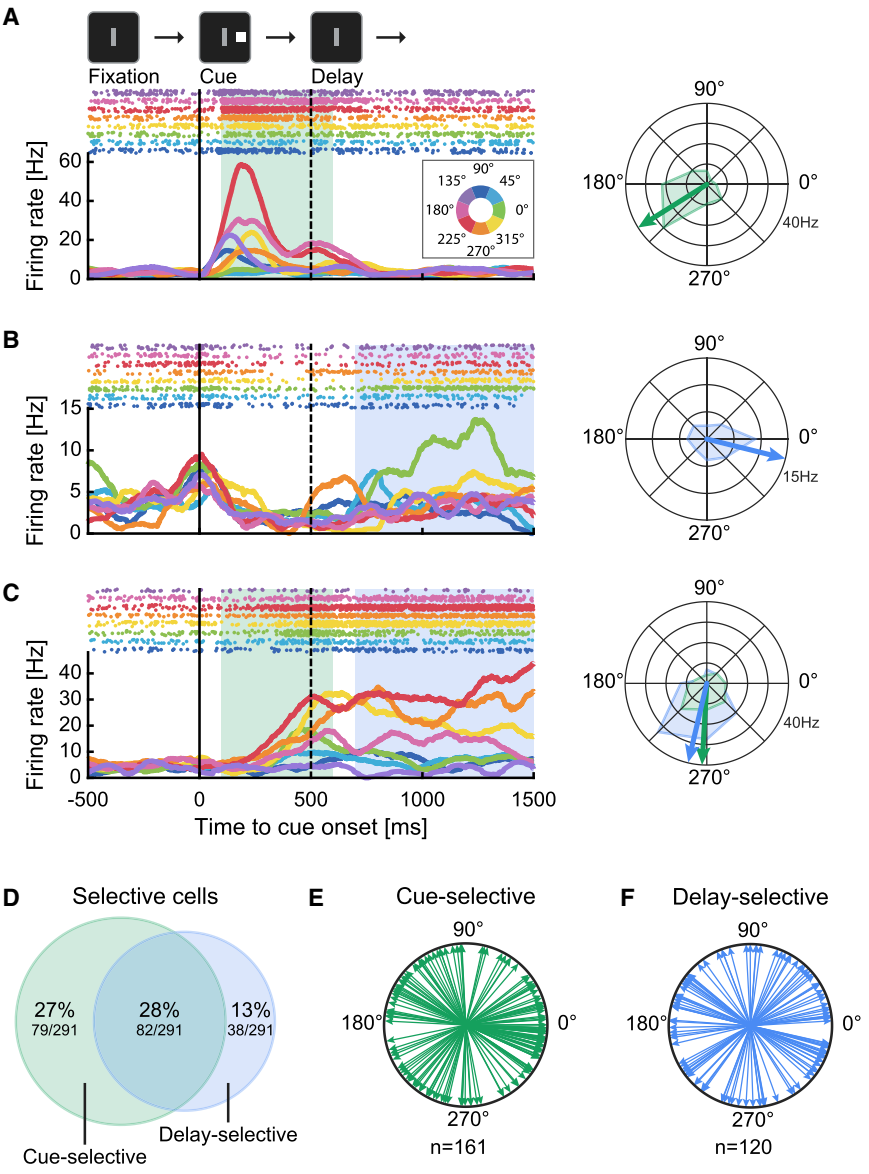

Figure 2. Single Neuron Responses to Spatial Locations

(A–C) Example neurons with cue-selective (A), delay-selective (B), and cue-and-delay-selective (C) activity. The left side shows dot-raster histograms (top) with corresponding spike-density functions (bottom). In dot-raster histograms, each dot represents an action potential, and each line represents a trial. Trials are sorted and color coded according to the cued location (see inset in A). Spike-density functions show the smoothed average firing rate over all trials for a cued location. Solid vertical lines represent the beginning of the cue period. Dashed vertical lines represent the beginning of the delay period. The right side shows polar plots with the firing rate for each cued location. The filled area in polar plots represents the average firing rate for the respective time window shaded in color in the respective spike-density histograms on the left side (green for cue period, blue for delay period). The colored arrow represents the preferred direction of a neuron.

(A) Example neuron tuned to location 225° (bottom-left) during the cue period.

(B) Example neuron tuned to location 0° (right) during the delay period.

(C) Example neuron tuned to cue location 270° (bottom) during late cue and the entire delay period.

(D) Venn diagram showing percentage of cue-selective neurons (green), delay-selective neurons (blue), and cue-and-delay-selective neurons (overlap).

(E and F) Preferred direction of all cue- and delay-selective neurons.

(E) Polar plot with arrows representing the preferred direction of each cue-selective neuron (n = 161).

(F) Same as in (E), but with preferred directions of all delay-selective neurons (n = 120).

possible locations (Figure 2F). Also, the proportion of delay-selective neurons preferring the left (47%) or right side (53%) of the screen was about equal (binomial test, $p > 0.05$).

Most of the location-selective neurons, i.e., 28% (82/291) of the whole population of neurons, were significantly tuned in both the cue and delay periods (Figure 2D). We calculated a circular-circular correlation between the preferred direction in the cue and delay periods. The preferred direction of the cue and delay period in individual neurons was significantly correlated (circular-circular correlation, $\rho_{cc} = 0.52$, $p < 0.01$).

The Majority of Delay-Selective Neurons Are Storage Rather Than Response Units

The spatial delayed-response task requires the crows to store location information in working memory but may also allow them to prepare a response during the delay period. Our task shares this problem of a potential combination of working memory and response preparation in the delay period with the classical oculomotor delayed-response task (ODR task) used for decades to study spatial working memory in monkeys [31]. We therefore investigated whether and to what extent persistent activity in NCL is composed of separable memory storage and response preparation activities. We reasoned that the memory storage and response preparation activities toward the end of the delay should differ in their relationship to the crows' pecking reaction time (RT). More precisely, we expected the discharges of neurons that encode motor preparation responses to co-vary with future RT. In contrast, this effect is not expected in memory storage units because only response preparation processes influence the timing of behavior after the delay. This rationale had previously been applied to differentiate storage and response modes in single PFC neurons of monkeys [32].

We therefore analyzed the population of delay-selective neurons for an increase in firing rate toward the end of the delay period (last 500 ms) as a function of RT. For any given neuron, we compared the firing rates occurring in the 50% fastest trials with those elicited in the 50% slowest trials. A total of 112 cells

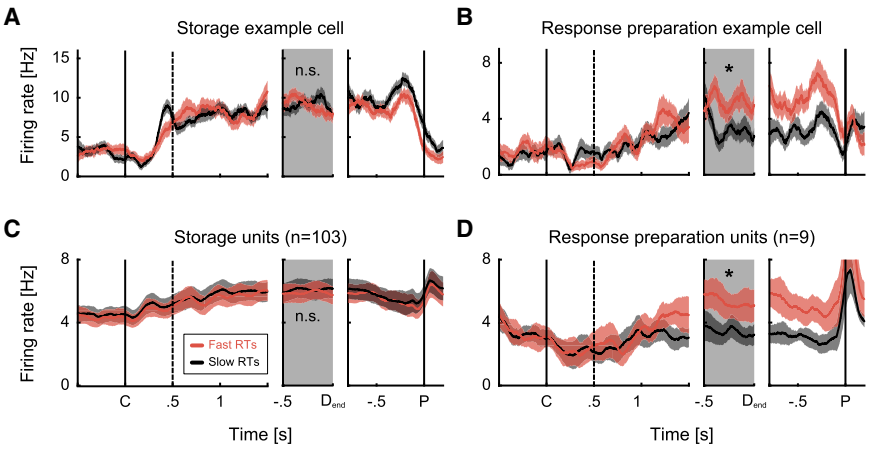

Figure 3. Delay-Selective Neurons Can Be Divided in Storage and Response Units

(A and B) Examples for a storage neuron (A) and response neuron (B). Spike-density histogram of neuronal activity is averaged for trials with fast reaction time (red) and slow reaction time (black). The left panel presents data aligned to the cue onset, showing activity during fixation, cue, and first second of the delay period. The middle panel presents data aligned to end of the delay period (D_{end}). The right panel presents data aligned to the peck on the screen (P). The gray area marks the analysis window.

(A) The firing rate of a storage neuron at the end of the delay is not affected by reaction time.

(B) A response-preparation neuron shows significantly increased activity for trials with fast reaction time.

(C and D) Activity of the population of storage units (C) and response preparation units (D). The shaded area in the graphs represents the standard error of the mean (SEM). The Asterix marks a significant difference in firing rates ($p < 0.05$) between fast and slow reaction-time trials.

(of the original 120 delay-selective cells) with at least 20 trials for both fast and slow RTs were included. Only firing rates to the preferred and the two neighboring locations were analyzed. We found that only 8% (9/112) of delay-selective neurons showed an increased firing rate for trials with a fast RT (Mann-Whitney-U test; $p < 0.05$) (Figure 3). Thus, only 8% of the delay-selective neurons were identified as "response-preparation" units, whereas 92% are considered as "storage" units.

Population Activity Encodes Cued Location

The activity of the entire population of recorded neurons, irrespective of their tuning behavior, encoded the cued location throughout cue and delay periods. To explore if an ideal observer could predict the cued location throughout the trial based on the activity of the neurons, we used a k-nearest neighbor classifier. We created a pseudo-population from all recorded neurons that were recorded for at least 20 correct trials for each location and performed a 5-fold-crossvalidation analysis in a sliding window over the trial ($n = 186$, $k = 5$, 100 ms window size, 20 ms steps). To compare the resulting decoding performance to chance level, we subsequently permuted the labels of the classifier 50 times and performed the same 5-fold-crossvalidation. The whole procedure was repeated 20 times to account for differences in trial selection. Decoding performance was defined as the average decoding performance across the true label cross-validations (20 repetitions). The decoding performance was defined as being significantly above chance if the decoding performance was above the 95th percentile of the permuted data (1,000 or 50 × 20 repetitions). As expected, the classifier performance was at chance level (12.5% for eight locations) during the fixation period (Figure 4). However, shortly after the cue was presented, the decoding performance increased significantly above chance level and peaked around 35% accuracy. For the time of cue presentation and 200 ms into the delay, the performance remained at a similar level around 30%. Over the rest of the delay period, decoding performance slowly decreased to around 22% but stayed significantly above chance level. Around the time of movement execution—and shortly before the peck on the screen was registered—decoding performance increased again to a level around 35%. This analysis shows that the neuronal population is able to continuously maintain the spatial information based on overall firing rates throughout the course of a trial from cue presentation and delay period until a movement had to be executed.

Stable Population Code across the Delay Period

Population codes may change from stimulus presentation to working memory periods, and they may even change dynamically within the ongoing delay period [33, 34]. We therefore explored the consistency versus dynamics of population coding across the different task period and within the delay period. To that aim, we segregated the task phase into six 500-ms time windows (fixation, sample, first, second, and last 500 ms of delay windows, and respond window), trained a k-nearest neighbor classifier in each specific time window, and then tested the classifier's performance in the different time windows of the task. A high classifier performance would suggest coding consistency across the different periods of time during the task, whereas low performance would suggest a change in population code from one time segment to the next. As expected from the previous analysis (Figure 4), decoding performance was significantly above chance whenever the classifier was trained and tested within the same time window (gray bars in Figures 5B–5F). Also as expected, and as a control analysis, the decoding performance was at chance level when the classifier was trained in the fixation period and tested within the same and subsequent periods (Figure 5A).

When training the classifier on neuronal data during the cue period, decoding performance was close to chance level for subsequent time windows in the delay and response periods (Figure 5B). The exception was the first 500 ms of the delay period immediately after sample offset, which was probably due to a spill-over of neuronal activity at the transition between sample and delay periods. This suggests that the population codes between the sensory cueing period and the working memory period differ. Conversely, the cue period also could not be decoded above chance when the classifier was trained

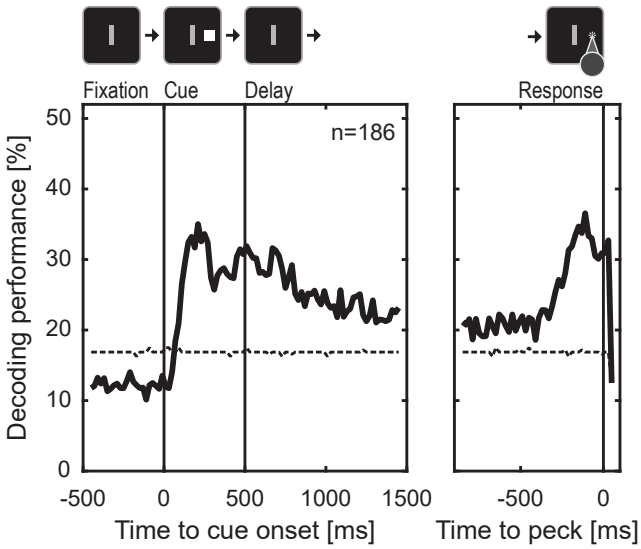

Figure 4. Neuronal Population Activity Encodes Cued Location throughout the Whole Task

Solid line represents cross-validation decoding performance of a k-nearest neighbor classifier. The dashed line represents the chance level (95th percentile of cross-validation with permuted labels). The left graph displays the decoding performance from fixation period until the first 1,000 ms of the delay. The right graph shows the decoding performance of data aligned to the peck on the screen (0 ms) during the response period.

in any of the delay and response windows (again, with the exception of the first 500 ms of the delay period).

Interestingly, however, the classifier trained in each of the three delay windows showed a significant decoding performance in all delay intervals (Figures 5C–5E). This indicates that the population code was stable across the delay period. Finally, a classifier trained on data of the time interval just before the peck also showed high decoding performance in the preceding delay periods (Figure 5F), arguably because of a spill-over of working memory information into the response period. In summary, this analysis suggests differing population codes between the sensory sample period and the subsequent working memory period. Within the delay period, however, the working memory code remained stable throughout the time-variable working memory period.

Activity Predicts Selections in Free-Choice Trials

In order to explore the significance of the NCL neuron firing rates for the crow's behavior, we introduced two-alternative free-choice trials. In such ambiguous trials, two opposite locations were cued simultaneously (either left and right, i.e., 180° and 0°, or top and bottom locations, i.e., 90° and 270°) (Figure 6A). The crows could pick either of these locations in the response period, and they were rewarded for either choice. We reasoned that if the activity of NCL neurons was behaviorally relevant, then the tuning of the neurons during the trial should allow a classifier to predict the crows' subsequent location choices. Both crows developed a response bias and tended to always chose the same of the two-alternative location. We trained a k-nearest neighbor classifier on the neuronal data of standard (i.e., non-ambiguous) trials in the cue, and the first, second, and last 500 ms of the delay period. After that, the classifier was used to predict the choice based on the neuronal data of ambiguous two-alternative choice trials. If the average performance based on the real data exceeded the 2.5 and 97.5 percentiles of randomly shuffled data, this performance was defined as significantly predicting the crow's choice. Figure 6B shows the data for ambiguous two-alternative choice trials in which the crows could choose between the top and bottom location (n = 25). The classifier was able to predict the later-chosen location significantly above chance in the cue period, the first, second, and last 500 ms of the delay period. Decoding performance was highest in the cue period and decreased across the delay but stayed above chance until the end of the delay period. Figure 6C displays the decoding performance in ambiguous two-alternative choice, in which the left and right locations were cued simultaneously (n = 31). The classifier was able to predict the crows' choice above chance level in the explored delay phases (first, second, and last 500 ms of the delay period) but failed to do so during the cue period. Decoding performance was highest in the first 500 ms of the delay period. Our data suggest different time courses for the two conditions. While the decision between top and bottom location was encoded both during the cue and delay periods, the decision between left and right was encoded only in the delay period. Overall, this analysis shows that the delay activity of NCL neurons reliably encoded the location the crow chose later in the trial across the whole delay period.

To further explore the functional role of NCL neurons in maintaining target location and predicting spatial choices, we compared population activity using a state space decoding analysis. In this analysis, the population activity over time is presented by a trajectory in three-dimensional space by the first three principal components. State space was calculated for the same populations used in the above analysis (n = 31 and n = 25). While the absolute positions of the trajectories in space are meaningless, spatial differences between the trajectories indicate coding differences. If the crow adopted the same behavioral strategy and the NCL population used the same code to represent the underlying memory content irrespective of whether regular or free-choice trials were presented, then the trajectories representing the same memory contents are expected to be very similar.

The trajectories resulting from this state space analysis are shown in Figure 7. Figure 7A shows the trajectories of population activity during regular trials with top (solid blue line) versus bottom (solid green orange) locations chosen, together with the trajectory during free-choice trials in which the crow chose the top target (dotted blue line). Figure 7B shows the trajectories of population activity during regular trials with left (solid magenta line) or right (solid green line) locations chosen, together with the trajectory during free-choice trials in which the crow memorized and later chose the right target (dotted green line). As expected for the crow's diametrically opposed memorized locations, population activity in regular trials diverged dramatically from cue period and throughout the entire delay period (solid blue versus orange in Figure 7A and magenta versus green trajectories in Figure 7B).

Interestingly, the trajectories derived for free-choice trials closely followed the trajectory of regular trials for identical memorized and later-chosen locations: when the crow

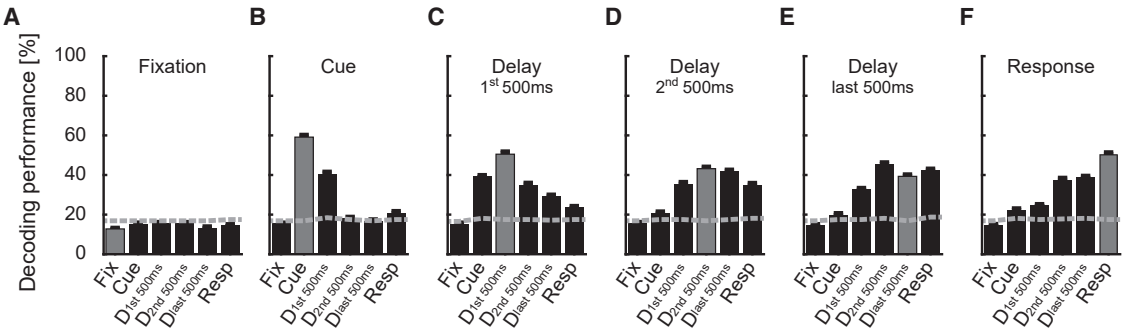

Figure 5. Exploration of Population Code across the Trial Periods

A k-nearest neighbor classifier was trained on data in a specific interval (gray bars in each diagram) and used to predict data in other epochs of the task. The classifier was trained on data during fixation (A), cue (B), first, second, and last 500 ms of the delay (C–E), and 500 ms before the peck (F). The gray bar indicates the cross-validation performance of a classifier tested within the trained interval (measure of cue information present in epoch). Black bars represent the prediction performance of the same classifier for other task epochs (measure of population code similarity between epochs). The dotted line represents the 95th percentile of classifications with permuted labels (chance level). Error bars represent the standard error of the mean (SEM).

memorized and finally pecked at the top location in free-choice trials (dotted blue line), the trajectory followed the one preceding top choices in regular trials (solid blue line) (Figure 7A). Similarly, when the crow memorized the right location in free-choice trials (dotted green line), the trajectory followed the one preceding right choices in regular trials (solid green line) (Figure 7B). This finding argues twofold: first, neuronal signals in free-choice trials and regular trials were virtually identical for the same behavioral representation by the crow, and second, the different memorized locations are reflected throughout the delay period to support the crow's future choice.

DISCUSSION

Over the past years, several functional similarities between the avian NCL and the primate PFC have been discovered [15, 19]. NCL neurons encode sensory [18, 35, 36] and cognitive [16, 17, 27, 37] variables and also participate in the execution of visually guided motor behavior [38]. While some studies have investigated neuronal correlates of working memory for objects in birds [26, 27, 39, 40], so far, none have addressed spatial working memory representations. We report that a large proportion of neurons signals and maintains visuo-spatial information, suggesting that the corvid NCL plays an important role in spatial working memory. Forty-one percent of all recorded neurons significantly encoded the memorized cue location. Almost 30% of all neurons were selective in both the cue and delay period, and their spatial tuning was significantly correlated. As evidenced by a classifier analysis, the population of all recorded NCL neurons maintained the spatial information of stimuli throughout the trial, thus bridging the temporal gap until a response was required.

NCL Activity Predicts Prospective Spatial Choice

The NCL neurons' activity is relevant for the crows' spatial behavior because in free-choice trials, the crows' prospectively chosen location could be predicted based on the tuned neurons' activity (Figure 6). This conclusion is also corroborated by the population state space decoding analysis. It showed that the population code throughout the delay period in free-choice trials and regular trials was virtually identical for the same choices made by the crow (Figure 7). This suggests that NCL neurons do not simply represent and store any visual information; rather, the NCL selectively represents information necessary to guide and control the crows' future behavior in space.

Such a selective storing of information that can also be witnessed in the primate prefrontal cortex (PFC) [41, 42] might be an important adaptation to the limited capacity of working memory [43]. Our results complement earlier findings in other cognitive domains that point to the corvid NCL as a pivotal brain center for cognitive control functions [16, 17, 19, 26, 27].

Balanced Representation of the Visuospatial Space in NCL versus PFC

In primates, spatial working memory has been studied extensively in the PFC with the oculomotor delayed-response (ODR) task, in which monkeys have to make a saccade to a briefly remembered spatial location [31, 44, 45]. Except for the responses required from the animals (eye movement in monkeys versus pecking in crows), the ODR is equivalent to the task we used in crows. Neurons in PFC readily encode spatial locations and maintain this information over delay periods [20, 31, 41, 44, 45]. In the PFC, more neurons are tuned to the visual hemifield contralateral to the recorded hemisphere [41, 44]. This contrasts our findings in the corvid NCL, in which an equal amount of neurons was tuned to both the left and right visual hemifields in both cue and delay periods (Figures 2E and 2F). This balanced representation of the visuo-spatial space is all the more surprising given that carrion crows have panoramic vision with only limited binocular overlap of maximally 37° of visual angle of the monocular visual fields [46]. In addition, and like all birds, they possess a complete decussation of the optic nerve, a purely contralateral projection of early visual information [47], and they lack a corpus callosum that connects the left and right endbrain hemispheres in mammals. This indicates major anatomical differences in the wiring of the main visual pathways between primates and birds [48], ultimately leading to a seemingly balanced representation of both visual fields in the NCL.

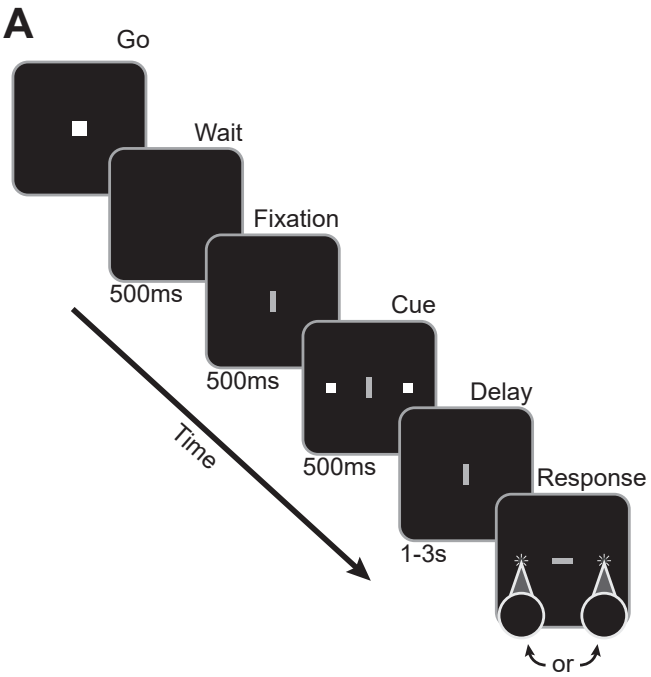

Figure 6. Task Layout and Classifier Decoding Performance for the Two-Alternative Free-Choice Trials

(A) Two-alternative free-choice protocol. The same protocol as main task (Figure 1A) but with two locations cued simultaneously during the cue period. The cued locations were either left and right (180° and 0°) or top and bottom (90° and 270°). During the response period, the bird was rewarded for choosing either of the cued locations.

(B and C) Choice prediction performance of a k-nearest neighbor classifier based on the responses of all neurons for the crow's top-bottom (B) and left-right (C) choices. The classifier was trained on neuronal data in the cue period and the first and second 500 ms of the delay period. The dashed line represents the chance level (2.5th and 97.5th percentile of prediction with randomly labeled trials).

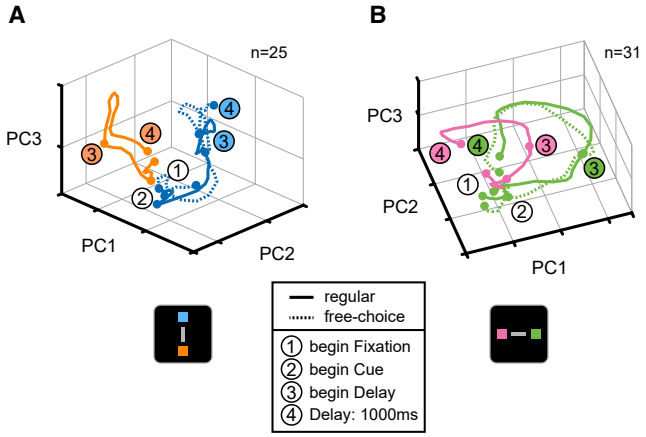

Figure 7. State Space Analysis Displays Similarity of Population Responses in Regular and Free-Choice Trials with the Same Goal

Development of population activity during free-choice and regular trials for cue locations top and bottom (A) and left and right (B). Solid lines represent the population activity trajectory during regular trials. The dotted line represents the activity during free-choice trials. The color of lines indicates the cued or chosen location (blue, orange, magenta, green for top, bottom, left, and right). For both the left-right and the top-bottom comparisons, the population activity of regular trials diverges after the beginning of the cue period and over the first second of the delay period. The population activity of free-choice trials resembles the activity of regular trials when chosen locations are identical.

The current study also highlights differences in working memory components found in the NCL and the primate PFC. Based on their firing rate increases with fast pecking reaction times, only a small proportion (8%) of the delay selective NCL cells were identified as motor preparation units; the vast majority of selective cells could be classified as storage units related to working memory. This is in stark contrast to findings in the monkey PFC, in which a majority of 55% of the cells could be linked to response preparation based on a very similar analysis [32]. It therefore seems likely that the avian NCL is less related to response selection processes than the primate PFC.

Sustained versus Dynamic Memory Code

NCL neurons showing sustained activity during memory delays are ideally suited to bridge the temporal gap between spatial cueing and response execution. Sustained activity has been reported as a neuronal correlate of WM since the early 70s [49, 50] and since then has been encountered in virtually all associative regions of the mammalian cerebral cortex, most notably, the prefrontal cortex (PFC) [31, 41, 51–53]. Sustained activity seems to be an evolutionarily conserved neuronal signature of working memory. It also exists in the endbrain of birds that do not have the sophisticated circuitry of a six-layered neocortex [16, 17, 25–27, 35, 39]. However, more recent studies in primates have emphasized that an additional code could be at work: many neurons show patterns of neural activity that are selective for only short periods of time during longer memory delay periods [54]. Analyses of neuronal population activity also revealed dynamic population coding phenomena associated with working memory tasks that were different from sustained activity [33, 34].

Our cross-temporal classifier analysis (Figure 3) suggests that the neuronal code for visuospatial working memory in the NCL is stable throughout the delay period because neuronal information extracted in one specific delay window enabled the classifier to predict performance in any other delay window. A likely explanation for this stability is the sustained firing of many

memory-selective neurons such as the one displayed in Figure 2C. Of course, this does not preclude other codes from play a part; future research is needed to explore whether dynamic codes might complement the stable code to represent working memory contents in the avian endbrain.

In sum, the current findings together with previous insights [15, 19, 55] highlight the NCL as the corvid brain's central executive. Intelligence in birds is realized with an endbrain design that is radically different from the mammalian neocortex and developed independently via convergent evolution [56–59]. Comparative neurophysiological data in corvids and primates will help to decipher the general principles and evolutionary constraints for the design of clever vertebrate brains [60].

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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AUTHOR CONTRIBUTIONS

P.R. and A.N. designed the experiment. P.R. and M.E.K. conducted the experiments. P.R. analyzed the data. P.R. and A.N. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
Corvus corone	University of Tübingen, Institute of Neurobiology	bird V, bird W
Software and Algorithms		
NIMH Cortex	National Institute of Mental Health	c598; https://www.nimh.nih.gov/research/research-conducted-at-nimh/research-areas/clinics-and-labs/ln/shn/software-projects.shtml
MAP Data Acquisition System	Plexon	https://plexon.com/
MATLAB R2017a	MathWorks	https://www.mathworks.com
Other		
Dental Cement	Heraeus	Paladur, ISO 20795, CE 0197
Microdrives	Animal Physiology Unit	Custom fabrication
Electrodes	Alpha Omega LTD	Cat.#: 366-130620-00

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Andreas Nieder (andreas.nieder@uni-tuebingen.de).

This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

We used one 2- and one 4-year-old male carrion crows (*Corvus corone*) (bird V and bird W respectively) from the institute's breeding facility for the experiment. They were hand-raised and housed in social groups in indoor aviaries [28]. The crows were on a controlled feeding protocol during the training and recording period. Body weight was measured daily. The daily amount of food was given as reward during, or if necessary, after the sessions. Water was *ad libitum* available in the aviaries and during the experiments. All procedures were carried out according to the guidelines for animal experimentation and approved by the responsible national authorities, the Regierungspräsidium Tübingen, Germany.

METHOD DETAILS

Experimental setup

The experiment was conducted in a darkened operant conditioning chamber. The birds were perched in front of a touchscreen monitor (ART development MT1599-BS) that was used for stimulus presentation and to collect behavioral responses. Reward was delivered by an automated feeder below the touchscreen. The food reward consisted of food pellets (*Beo Special*, Vitakraft, Bremen or *NutriBird Beo komplett*, Versele Laga, Belgium) and mealworms (*Tenebrio molitor* larvae). Additional visual feedback was provided by a lamp on top of the feeder and auditory feedback by speakers (Lasmex S-03) located behind the touchscreen. An infrared light barrier controlled by a reflector attached to the bird's head ensured a stable head position in front of the screen throughout the trial. We used the CORTEX system (National Institute of Mental Health) to carry out the experiment and collect behavioral data. Neuronal data was recorded using a PLEXON system (Plexon Inc., Dallas, Texas).

Behavioral protocol

The birds were trained on a spatial delayed-response task including visual fixation (Figure 1A). The crow initiated a trial by positioning its head facing the monitor whenever a go-stimulus (white square, 2x2° visual angle) was shown, thus closing an infrared light barrier, and maintaining this position throughout the trial. To indicate that the light barrier had been entered, the bird heard a click sound and the go-stimulus vanished. Whenever a crow made premature head movements and thereby left the light barrier during an ongoing trial, this trial was terminated and discarded.

The main protocol started with a black screen for 500 ms (wait period). In the following fixation period (500 ms duration), a small vertical bar (0.33 × 0.65 deg visual angle) was presented as a fixation stimulus in the center of the screen. The size of the fixation

target was close to the crows' visual acuity that is estimated to be 0.12 deg visual angle (or 8.4 cycles/degree) at a high luminance of 300 cd/m2 [61]. The crows were trained to observe this fixation stimulus throughout the following task periods and only respond when it changed orientation after an unpredictable time period. The fixation period was followed by the cue period (500 ms duration) in which one of eight different locations was cued by a white square (3.2x3.2° visual angle). These locations were arranged circular around the center of the screen, equidistant to the center (distance to center: 24° visual angle) and evenly distributed in angles of 45 degrees along the circular path (0°, 45°, 90°, 135°, 180°, 225°, 270°, 315°). The distance from one cue to the next was 18.4° visual angle. After the end of the cue period, the spatial cue disappeared and the delay period started with only the fixation target on an all-black background. The length of the delay period was varied pseudo-randomly between 1,000 ms and 3,000 ms (in steps of 500 ms). The end of the delay and beginning of the response period was indicated by a change in orientation of the central fixation bar from vertical to horizontal. After the fixation bar changed its orientation, the crow had to peck as fast as possible and no later than 900 ms after the change of the fixation bar at the previously cued location on the touchscreen. Trials with a reaction time longer than 900 ms were aborted without a reward and followed by a timeout of 1.5 s in which the beginning of a new trial was delayed as "punishment." Thus, the crows were discouraged from making head or eye movements and forced to closely pay attention to the fixation bar during the memory period by different factors: The small fixation bar close to the crows' perceptual threshold, the variable delay period, and response pecks under time pressure. The crows' overall correct performance of close to 100% (Figure 1B) further argue that they were fixating the fixation target throughout the delay period.

Pecks within ± 6° of visual angle (vertical and horizontal) around the center of the cued location were counted as correct responses and were rewarded by food. All pecks with a larger distance from the center of the cued location were counted as errors and were not rewarded. In addition, pecking errors were followed by timeouts. Pecking location errors between ± 6° and ± 8° of visual angle resulted in a shorter timeout of 1.5 s. Errors further away than ± 8°of visual angle from the target location resulted in 3 s timeouts. Both the cued location and the delay lengths were shuffled pseudo-randomly on a trial by trial basis by the computer running the task.

To explore the behavioral significance of spatial delay activity during this main task, we additionally presented crow W with two-alternative free-choice trials (Figure 4A). In these two-alternative choice trials, not one but two locations were cued simultaneously during the cue period. Either the locations left and right (180° and 0°) or the top and bottom locations (90° and 270°) were cued simultaneously. The crow could memorize and chose either of these locations to receive a reward in the response period. All other parameters were identical to the main task. Two-alternative free-choice trials were interleaved with the main task and occurred at a frequency of 10%.

Surgery and Recordings

The surgery was performed while the animal was under general anesthesia with a mixture of ketamine (50mg/kg) and Rompun (5mg/kg xylazine). The animal was placed in a stereotaxic holder. We targeted the in the medial part of NCL (*Nidopallium caudolaterale*) [29] by performing a craniotomy at 5mm anteriorposterior and 13mm mediolateral on the right hemisphere. This part of NCL, termed mNCL [29], is known to contain highly associative neurons [16–18, 26, 27]. Two manual micro drives containing four electrodes each (2MΩ, Alpha Omega Co.) were implanted at the craniotomy. In addition, a miniature connector for the headstage and a small holder for attaching the reflector were implanted. Each recording session started with adjusting the electrodes until a proper neuronal signal was detected on at least one channel. The neurons were never pre-selected for any involvement in the task. Single-cell separation was done offline (Plexon Offline Sorter, version 2.6.2). No obvious anatomical organization of location preferences was detected.

QUANTIFICATION AND STATISTICAL ANALYSIS

All analyses were performed in MATLAB. Values in main text and figures represent the mean ± SEM (standard error of the mean), if not stated otherwise. SEM was calculated as the standard deviation divided by the square root of number of samples.

Behavioral analysis

To measure the performance for normal trials we calculated the percentage of correct trials for each cued location on each recording day. For free-choice trials we calculated the percentage of choices to the top location (top-bottom-choice trials) or right location (left-right-choice trials). This measure allows to check for a bias toward always choosing the same location.

Neuronal location selectivity analysis

All cells that had at least 1Hz average firing rate and were recorded for at least 10 correct trials for each cued location were analyzed for this study. To analyze if a neuron selectively responded to different cued locations, we performed one-factorial analyses of variance (ANOVA; $p < 0.01$) with main factor "cued location" for the neuronal data on correct trials. For the cue period, selectivity was evaluated in a 500 ms window starting 100 ms after cue onset, to account for visual response latency of crow's NCL neurons [38]. For the delay period, selectivity was calculated over a 900 ms window starting 200 ms after the beginning of the delay period and reaching 100 ms into the choice period, again to account for the visual response latency of NCL neurons.

Neuronal preferred direction analysis

We calculated the preferred direction for each neuron in the cue and delay period. To that aim, we calculated the average firing rate across correct trials for each cued location. For each of the eight locations, an individual vector with a length corresponding to the

average firing rate to this location was created. The preferred direction of a neuron was defined as the angle of the average vector resulting from vector addition of all eight vectors. To evaluate correlation between spatial preferences in the cue and delay periods, we calculated a circular-circular correlation between the preferred directions using the CircStat toolbox for MATLAB [30].

Differentiating storage and response neurons

We analyzed whether delay-selective neurons at the end of the delay showed neuronal activity related to the crows' reaction time [32]. For each recording session, we split all trials into fast reaction time and slow reaction time trials (50% each). Fast reaction times were defined as reaction times faster than the median of the session (median split). We then tested if the firing rate of a neuron was significantly increased during trials with fast reaction times compared to slow reaction times. Only trials for the preferred location and the neighboring locations of a neuron were analyzed. The preferred location was defined as the location closest to the calculated preferred direction of the neuron. As an example, if a neurons preferred direction was at 85°, the preferred location would be defined as 90° (top), and trials from locations 45°, 90°, and 135° would enter the analysis. All delay-selective neurons that were recorded for at least 20 fast and 20 slow reaction time trials were analyzed (112/120). We analyzed the last 500 ms of the delay period. A Mann-Whitney-U test ($\alpha = 0.05$) was calculated over the average firing rates of the analysis window to identify neurons with a significantly increased firing rate for trials with fast reaction times.

Population analysis for decoding cued location

To investigate if the location chosen by the crows could be predicted based on neuronal population activity, we used a k-nearest neighbor classifier and performed a 5-fold cross-validation. The k-nearest neighbor classifier creates a matrix of all neurons of a population and trials of different conditions. This matrix is used to create a *n*-dimensional space with *t* data points in it, with *n* being the number of neurons in the population and *t* being trials. Each of these *t* data points has a position in the *n*-dimensional space, according to the normalized firing rate of each neuron in the trial, and a label that is the cued location in the trial. The construction of the *n*-dimensional space is also referred to as training of the classifier. Using the *n*-dimensional space, the classifier can predict the label of a data point by assigning it the label of the *k* data points that have the shortest Euclidean distance. If these *k* data points have different labels, the most frequent label is used. In the 5-fold cross-validation, all data points are split up into five equal sized groups. Four of these groups are used to create the classifier matrix and predict the label of the remaining group. This process is repeated until all groups were predicted once. In doing so cross-validation prevents that a data point is used in the prediction of its own label and thereby the overestimation of classification power. The cross-validation performance is equal to the percentage of correctly predicted labels.

We used a sliding window approach (window of 100 ms and step size of 20 ms) to evaluate the cross-validation performance across the trial. Cells had to reach the general criterion described above and additionally had to be recorded for at least 20 correct trials in each location. To create the classifier matrix in an analysis window, we randomly chose 20 correct trials for each cued location of each neuron. These trials were combined to a n x 160 matrix (n = 186), representing a population of pseudo-simultaneously recorded neurons. A 5-fold cross-validation was performed to calculate the decoding performance of the classifier (k = 5; 20 trials for each direction).

In order to be able to compare our results to chance level, we permuted the labels of the classifier 50 times and calculated the cross-validation performance for each permutation. This process was repeated 20 times, yielding 20 cross-validation performances for true labels and 1000 performances for the permuted labels. We averaged across the 20 true label performances to account for differences in trial selection. The average decoding performance was defined to be above chance level, if it was above the 95th percentile of the permuted data.

Stability of population code analysis

In order to analyze the consistency of selective encoding across time, we used a k-nearest neighbor classification while varying training and test time intervals. A classifier was trained over a 500 ms interval, and then used to calculate the inherent cue information in this interval using a 5-fold cross-validation. In addition, it was used to predict the cued location of data from other 500 ms intervals. The latter prediction was used to see if the code present in the training interval was similar to or different from other times during the protocol. The analysis of the data is similar to the k-nearest neighbor population analysis above (n = 186). Thus, only differences to the above analysis are described here. We compared neuronal data of fixation, cue, the first, second, and last 500 ms of the delay, and 500 ms before the peck in this analysis. The analysis windows of Cue period, the first, and second 500 ms of the delay were shifted by 100 ms to account for visual latency. For decoding performance inside the trained interval, we calculated a 5-fold cross-validation. As a chance level for this cross-validation we defined the 95th percentile of cross-validation performances with permuted labels. Qualitatively similar performance was obtained by leave-one-out classification. To calculate the prediction performance across intervals, we used the classifier to predict the labels of 20 randomly selected trials for each of the eight locations. As a chance level for this prediction analysis, we defined the 95th percentile of prediction performances using a classifier with permuted labels. To account for variability between trials, cross-validation and prediction was repeated 20 times, and permutation analysis 1000 times.

Prediction of choice in free-choice trials

We used a k-nearest neighbor classifier in two-alternative free-choice trials to determine whether neuronal activity encoded the spatial working memory of the crows' chosen location. Choice trials were only used and recorded in a subset of recording sessions

with bird W. The animal developed a strong bias to always choose the top location (90°) in the top-bottom and the right location (0°) in left-right choice trials. We therefore only used choice trials with top and right choices for the following analysis in order to have a sufficient number of trials for this analysis. All cells in the analysis had to be recorded for at least 10 correct choice trials toward the respective location and 20 normal trials for the respective and opposite location each (n = 25 for top-bottom; n = 31 for left-right choice trials). We constructed a classifier based on the neuronal data of normal trials (k = 5; 20 normal trials for each direction). Then 100 choice trials were constructed by randomly drawing a choice trial from each cell. We used the classifier to predict the labels for all choice trials. Decoding performance was defined as the percentage of correct predicted choice labels.

In order to compare our results to chance level, we subsequently assigned random labels (choice or opposite location) to the choice trials and calculated the percentage of correct labeled trials. This process was repeated 1000 times, yielding 1000 decoding performances for correct labels and 1000 decoding performances for randomly assigned labels. We defined decoding performance to be above chance level, if the average decoding performance was outside the 2.5 and 97.5 percentile of the performances of randomly assigned labels. We analyzed the cue period in a 500 ms window aligned to cue onset as well as the first and second 500 ms of the delay period. All windows were shifted by 100 ms to account for visual latency.

State Space analysis

We performed a state space analysis on the neuronal population activity to see if free-choice trial activity was different from normal trial activity. All neurons that entered the analysis for prediction of choice in free-choice trials entered the principal component analysis (PCA). The PCA performs a realignment of the dimensional axes in the n-dimensional space that is created by the population activity of n neurons. The new dimensional axes are chosen to explain the maximum variance within the data. The variance explained by the first principal component is highest, followed by the second, and subsequent principal components (for a more detailed explanation see [62]). For left and right regular and free-choice trials the first three principal components captured 61.7% of the total variance, for top and bottom they explained 44.8% of the variance. Neuronal activity of all trials was smoothed with a 300 ms Gaussian kernel and activity was averaged over time bins of 50 ms.

DATA AND CODE AVAILABILITY

The datasets and code supporting the current study have not been deposited in a public repository because of further analyses, but are available by request to the Lead Contact, Andreas Nieder (andreas.nieder@uni-tuebingen.de).