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Recording single neurons' action potentials from freely moving pigeons across three stages of learning

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Abstract:	While the subject of learning has attracted immense interest from both behavioral and neural scientists, only relatively few investigators have observed single-neuron activity while animals are acquiring an operantly conditioned response, or when that response is extinguished. But even in these cases, observation periods usually encompass only a single stage of learning, i.e. acquisition or extinction, but not both. However, acquisition and extinction entail different learning mechanisms and are therefore expected to be accompanied by different types and/or loci of neural plasticity. Accordingly, we developed a behavioral paradigm which institutes three stages of learning in a single behavioral session and which is well suited for the simultaneous recording of single neurons' action potentials. Animals are trained on a single-interval forced choice task which requires mapping each of two possible choice responses to the presentation of different novel visual stimuli (acquisition). After having reached a predefined performance criterion, one of the two choice responses is no longer reinforced (extinction). Following a certain decrement in performance level, correct responses are reinforced again (reacquisition). By using a new set of stimuli in every session, animals can undergo the acquisition-extinction-reacquisition process repeatedly. Because all three stages of learning occur in a single behavioral session, the paradigm is ideal for the simultaneous observation of the spiking output of multiple single neurons. We use pigeons as model systems, but the task can easily be adapted to any other species capable of conditioned discrimination learning.
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TITLE

Recording single neurons' action potentials from freely moving pigeons across three stages of learning

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SHORT ABSTRACT
Learning new stimulus-response associations engages a wide range of neural processes which are ultimately reflected in changing spike output of individual neurons. Here we describe a behavioral protocol allowing for the continuous registration of single-neuron activity while animals acquire, extinguish, and reacquire a conditioned response within a single experimental session.

LONG ABSTRACT
While the subject of learning has attracted immense interest from both behavioral and neural scientists, only relatively few investigators have observed single-neuron activity while animals are acquiring an operantly conditioned response, or when that response is extinguished. But even in these cases, observation periods usually encompass only a single stage of learning, i.e. acquisition or extinction, but not both (exceptions include protocols employing reversal learning; see [1] for an example). However, acquisition and extinction entail different learning mechanisms and are therefore expected to be accompanied by different types and/or loci of neural plasticity.

Accordingly, we developed a behavioral paradigm which institutes three stages of learning in a single behavioral session and which is well suited for the simultaneous recording of single neurons' action potentials. Animals are trained on a single-interval forced choice task which requires mapping each of two possible choice responses to the presentation of different novel visual stimuli (acquisition). After having reached a predefined performance criterion, one of the two choice responses is no longer reinforced (extinction). Following a certain decrement in performance level, correct responses are reinforced again (reacquisition). By using a new set of stimuli in every session, animals can undergo the acquisition-extinction-reacquisition process repeatedly. Because all three stages of learning occur in a single behavioral session, the paradigm is ideal for the simultaneous observation of the spiking output of multiple single neurons. We use pigeons as model systems, but the task can easily be adapted to any other species capable of conditioned discrimination learning.

INTRODUCTION

Learning new stimulus-response-outcome associations engages a wide range of neural plasticity processes. These processes are ultimately reflected in the changing spike output of individual neurons. Arguably, one of the most frequently employed learning paradigms is Pavlovian fear conditioning conducted with rodents. In this setting, the acquisition and extinction of a conditioned response take place within a few dozen trials[2]. The rapid development of conditioned fear can be advantageous because it allows running a large number of animals within a short time. Also, acquisition and extinction can be observed within a few tens of trials on a single day in naive animals[3,4] or spread across two to three days[2,5–8]. However, the insights gained about the changes of neural activity during learning in these experiments do not necessarily apply outside the domain of fear conditioning. For example, goal-directed behavior driven by positive reinforcement is more adequately modeled by operant rather than Pavlovian conditioning procedures, and may in part depend on different neural substrates[9,10]. Also, fear conditioning develops so rapidly that neural responses to the CS can only be observed for a few dozen trials, placing severe limits on the analysis of changes of neural activity during learning.

Unfortunately, the acquisition and extinction of operant responding usually takes many days. This is detrimental for neurophysiological investigations, because it is notoriously difficult to record the activity of single cells over more than a few hours. Due to the high similarity of the waveforms of extracellularly recorded action potentials, it is problematic to claim that spikes recorded on one day are generated from the same cell as spikes with similar waveforms recorded on the next[11,12], especially in areas with a high cell density such as the hippocampus.

To address these issues, we developed a novel behavioral paradigm utilizing three learning conditions within one experimental session on a single day. This requires that the experimental animal is willing to perform hundreds of trials under varying conditions on a thin schedule of reinforcement. Homing pigeons (*Columbia livia forma domestica*) are classic model organisms in experimental psychology[13–17]. These birds are able to perform complex visual discriminations[18], can flexibly adapt behavior to changing reinforcement contingencies[19,20], and are uniquely avid workers, performing 1,000 trials with minimal amount of reinforcement. These characteristics make them especially suitable for the experiments described below.

PROTOCOL

Ethics statement

All experiments were conducted in accordance with the German guidelines for the care and use of animals in science. Procedures were approved by a national ethics committee of the state of North Rhine-Westphalia, Germany.

System overview

Operant testing chamber

The operant chamber (Figure 1) measures 34 cm x 34 cm x 50 cm. Three translucent response keys (4 cm by 4 cm, located approximately 20 cm above floor level) are recessed into the back wall of the chamber. Stimuli are shown through an LCD flat screen mounted behind the response keys. Two 2-Watt light bulbs located at the side walls provide dim illumination. The chamber is housed in a sound-attenuating cubicle to mask extraneous sounds. Loudspeakers provide white noise at all times. Food (grain) is provided by a food hopper located below the center key. Experimental hardware is controlled by custom-written Matlab code[21]. Animals are constantly monitored through a digital camera attached to the front wall of the chamber.

Custom-built microdrives

Microdrives housing 16 electrode wires are custom-built in our laboratory; the design is based on work by Bilkey and colleagues[22,23], and the reader is referred to these articles for a detailed description. We modified their design to allow for a larger number of electrodes (16 instead of 8; 25 µm nichrome wires), and we connect the electrode wires via conductive silver glue to the headstage socket. Additionally, we use gold-plating of the electrode tips to reduce impedance and to achieve better signal-to-noise ratios (apply –3 V for ~3 s; impedances should drop to <100 kΩ).

Once the microdrive is assembled, electrodes are cut to the desired length, tips are cleaned in an ultrasonic bath (Tergazyme in distilled water) for 20 minutes and rinsed another 20 minutes in distilled water. Gold-plating of electrode tips should take place immediately before implantation. For grounding, we use a silver ball electrode placed above the lateral cerebellum. Specification of materials is provided in the Excel sheet which accompanies this article.

An important issue when working with freely moving animals is movement artifacts. We found that movement artifacts in our setups are largely due to a) high electrode impedances (>500 kΩ) and b) imperfect attachment of the contacts between the plug (implant) and the socket (headstage) while the animal is moving. A variety of commercially available microconnectors does not perform satisfactorily for recording from freely moving birds, because the mechanical contact between plug and socket rapidly deteriorates through vigorous movements of the pigeons (head-bobbing, key-pecking). The best mechanical connection between implant and headstage was achieved with headplug assemblies from Ginder Scientific. These plug-socket assemblies feature 18 contacts and are firmly affixed to each other by a ring nut.

Electrophysiological recording setup

The electrophysiology setup comprises the following components: 1) a custom-built headstage with unity gain (operational amplifier) 2) 15 differential amplifier modules housed in two rack mount units (DPA-2FS and EPMS-07, respectively; npi electronic GmbH, Germany), 3) a 16-channel analog-to-digital converter (power 1401 mark I). Raw signals are amplified 1,000x and band-pass filtered (500-5000 Hz, 1st order filter), digitized with a sampling rate of 16-20 kHz and stored with Spike2 Version 7.06a for offline processing. Event times (such as stimulus onset or individual key pecks of the animal) are captured via a laboratory-built parallel port IO box (see [21]) and forwarded to the AD converter for storage along with the neurophysiological data (see Figure 1). Offline processing is described further below.

[Place Figure 1 about here.]

Single-Interval-Forced-Choice (SIFC) discrimination task

For clarity, we will describe the final SIFC task here and then explain the steps needed to train animals on this task below.

The SIFC task is outlined in Figure 2. After the intertrial interval (ITI) has elapsed, the center key is transilluminated green for up to 5 s ('initialization phase'). Immediately following the third response of the animal within 5 s, one out of several sample stimuli is presented on the center key for 2 s ('sample phase'; example stimuli are shown in the inset to Figure 2). After 2 s, the center key is again transilluminated green, and the animal has to respond once more before the two side keys are transilluminated ('confirmation phase'). Depending on the identity of the stimulus shown in the sample phase, the animal is required to direct a single response to either the left or the right key ('choice phase'). If it chooses the correct destination, access to reward (grain) is granted for 2 s. Thus, the core of the task consists of responding to the left choice key after presentation of one particular stimulus on the center key, and responding to the right choice key after presentation of another stimulus. The reason that the sample phase is bracketed by an initialization and a confirmation phase is to keep the animals' head in front of the center key while the sample stimulus is presented.

Once the animal masters this task for a single pair of stimuli (henceforth, 'familiar' stimuli, FS), it is presented with a novel stimulus (NS) pair in every new session, and has to learn which of the two novel stimuli is to be followed by a response to the left or the right choice key. The FS pair continues to be presented during those experiments to serve as suitable control condition. Adequate performance on the final task hinges crucially on the animals' willingness to perform >1,000 trials at overall reinforcement probabilities <0.5. The following paragraphs describe a training procedure in which task complexity is gradually increased until the animal reaches the level of the SIFC; at the same time, reinforcement probability and the number of trials per session need to be increased to ensure consistently high performance on the final task.

1. Animal training

1.1. Food restriction

1.1.1. Weigh the animals after at least two weeks of free access to food. Take this weight as the free-feeding weight. Restrict food access over the next 1-2 weeks until animals reach 85% of their free-feeding weight.

1.1.2. Carefully monitor the animals' weight across the entire duration of behavioral training and testing. Supply additional food if necessary to prevent further weight loss.

1.2. Autoshaping

Autoshaping serves to habituate the animal to the experimental chamber and establish conditioned responding.

1.2.1. Present a 5-s visual stimulus (henceforth, initialization stimulus, IS) on the center key. Immediately upon termination of the IS or a single peck to the response key (whatever comes first), switch off key illumination and present food reward (2 s activation of the food hopper).

1.2.2. Keep the ITI considerably longer than the sample presentation time to facilitate learning[24]. Use e.g. values of 120 s for ITI and run 40 trials per day. Later reuse the IS as the initialization, confirmation, and choice key stimulus in the final task (see Figure 2). This phase of training will take the animals approximately one week.

1.2.3. Once the animal responds reliably (in >85% of trials), decrease the ITI stepwise down to 10 s and the sample presentation time down to 2 s. At the same time, increase the number of responses required for reinforcement to 3 (fixed ratio of 3, FR 3). Additionally, increase the total number of trials per day. Choose parameters such that the animal is trained every day for approximately one hour. This phase of training will last roughly two weeks.

1.2.4. Repeat steps 1.2.1-1.2.3 for the left and right response keys until subjects reliably respond to the IS on all three keys. Alternate trials with activation of the left, right and center key randomly.

1.2.5. Now present the IS first at the center and then, conditional on a response, at either side key (omit reinforcement for the center key response). Alternate activated side keys randomly from trial to trial. Terminate trials in which the subject does not respond to the center key after 5 s. Repeat until the animal performs reliably (~ three days)

1.2.6. Introduce two new stimuli which will later serve as FS in the final task (see Figure 2, inset, for examples). Repeat steps 1.2.1-1.2.3 with these stimuli. Responding will be established more rapidly than with the first stimulus, usually within four days.

1.3. Training of a Single-Interval Forced-Choice (SIFC) Task for familiar stimuli

1.3.1. Establish the full sequence of initialization, sample, confirmation, and choice: on each trial, present first the IS (FR 3), then either of the two FS (2 s fixed duration), then again the IS (FR 1). Use a prompted-choice design: in each trial, transilluminate only the choice key which is correct for the given FS. This phase of training should take approximately one week.

1.3.2. Once the subject performs reliably (>85% responses to the respective side key), introduce free-choice trials (both side keys transilluminated during choice phase). If the animal responds to the correct side, provide food access for 2 s. Incorrect responses are followed by time-out punishment (houselights off for 2 s). If no response is given within 3 s, terminate trial and re-start ITI. Animals usually learn these subcomponents of the task within two weeks.

1.3.3. Gradually increase the fraction of free-choice trials during subsequent sessions from 20% to 100%.

1.3.4. If the subject performs >90% correct in free-choice trials, decrease reward probability for correct responses to 0.5 while in parallel increasing the number of trials per session to 1,000. Do not change parameters every day/session but choose them flexibly depending on the performance level of the subject concerning initialization omissions and percentage of correct responses. This phase of training will last about four weeks.

1.3.5. Pigeons tend to refuse responding to unfamiliar stimuli. Therefore, once the animal reliably performs >1,000 trials, autoshape responses to a large set of visual stimuli (see section 1.2). However, do not preexpose the visual stimuli destined for later use as novel stimuli in the final paradigm but uniform color displays.

1.4. Final Single-Interval Forced-Choice Task with novel stimuli under different reinforcement conditions

1.4.1. Warm-up

Let the subject perform 50 trials with the FS only. Set reward probability for these stimuli to <1 (say, 0.5-0.8) during all phases to prevent premature satiation and, therefore, lack of motivation to respond.

1.4.2. Acquisition stage

Randomly alternate trials with presentation of FS and NS. Assign different response keys as correct for the two NS and reinforce every correct response. Compute percentage of correct responses as a running average over the last 120 trials. Acquisition is considered complete once performance for each of the NS exceeds 85%, but not before a minimum of 150 trials have been executed.

1.4.3. Extinction stage

Stop reinforcing correct and punishing incorrect responses to a random NS ('extinction stimulus'). Begin reacquisition phase when correct responding to the extinction stimulus drops below 60% and the animal has experienced at least 150 trials in this phase in total.

1.4.4. Reacquisition stage

Again reinforce correct and punish incorrect responses to the extinction stimulus, as during the acquisition stage. Terminate the session when performance for this stimulus exceeds 85% and the animals performed at least 150 trials in this phase in total.

2. Electrophysiology

2.1. Electrode implantation

Implantation surgery takes place after animals repeatedly (3-4x) completed the entire acquisition-extinction-reacquisition sequence and is described in more detail elsewhere[25].

2.1.1. Place five to six stainless steel microsrews on the skull for anchoring a dental cement head mount including the microdrive.

2.1.2. Perform a craniotomy just above the brain region of interest; then carefully dissect the dura and lower the electrodes to the desired position.

2.1.3. Before anchoring the microdrive to the head mount, apply Vaseline around the guide cannula; this will prevent dental cement from encasing the guide tube.

2.1.4. Use an insulated silver ball electrode placed underneath the skull overlying the cerebellum as ground.

2.2. Recordings while animals perform the task

2.2.1. Use a new pair of novel stimuli for each session and advance electrodes at least 125 µm (half a revolution of the drive screw) before starting. If no action potentials of sufficient signal-to-noise ratio are observed, abort the session, place the animal in the home cage and try again the next day.

2.2.2. Arrange the headstage cable such that it does not interfere with the animal's normal pecking and feeding behavior. This can be achieved by attaching the cable with several elastic straps to the top of the conditioning chamber and habituating the birds to the attached cable for some hours.

2.2.3. If available, make use of a commutator to provide extra freedom of movement for the birds.

2.3. Offline signal analyses

2.3.1. Band-pass-filter all channels from 500 to 5000 Hz with steep roll-offs offline using Spike2. Extract spikes with amplitude thresholds and sort them manually employing principal component analysis.

2.3.2. Examine sorting results with custom-written Matlab code (available at Matlab Central File Exchange, File ID #37339). A well-isolated single unit (Figure 3) should meet all of the following criteria: a) a clearly separated cluster in principal component space, b) no sign of multiple units when all recorded waveforms are overlaid and plotted as heat map (Figure 3A), c) symmetrically distributed peak waveform amplitudes (Figure 3B), d) stable recording throughout the session as evidenced by unchanging spike amplitude (Figure 3C), e) no or very few spike events that occur during the refractory period of the preceding spike (Figure 3D), and a signal-to-noise ratio (SNR) of at least 2 (SNR is here defined as the difference between the minimum and maximum of the averaged spike waveform, divided by the trimmed width of the noise band (2.5^{th} and 97.5^{th} percentiles of the distribution of values from the first bin of all waveforms)). SNR of the unit shown in Figure 3 is 3.9.

2.3.3. Inspect raw channels offline for movement-related artifacts. Discard channels when indicated.

2.3.4. Electrical artifacts occurring during key pecking can in rare cases be confused with proper spike waveforms. Test for the contamination of the recordings by examining the time histogram of spike counts relative to each registered key peck (peri-peck time histogram, PPTH, Figure 3E). Pecking-induced artifacts show up as a peak in the histogram close (± 5 ms) to time 0. As an extra check, plot the waveforms of all putative spike events registered within ± 20 ms of a key peck separately and compare it to spike waveforms detected outside this window (Figure 3F).

[Place Figure 3 about here.]

REPRESENTATIVE RESULTS:

Behavior

Figure 4A shows the behavioral performance of an animal in one example session. The performance level of the animal reaches criterion for NS 2 within 180 trials (45 stimulus presentations) and is close to 100% for the NS 1 from the beginning. This strategy – first responding to the same key for both new stimuli, and then adjusting responses for one of the stimuli – is about as often observed as initial random responding to both NS. In this session, the NS 2 was randomly chosen to undergo extinction, meaning that all choices following this stimulus remain inconsequential (transition between learning stages are indicated by vertical black dotted lines). During extinction, performance decreases for the extinction stimulus but stays high for the other NS. Criterion is reached in trial 370. Correct and incorrect responses are now reinforced and punished again (reacquisition) and performance level reaches criterion in trial 402. Performance level for FS is consistently high (>95%; data not shown). b) Mean number of trials needed to complete each stage of learning (averaged over 5 animals and 44 sessions in total). On average, animals needed ~700 trials to respond consistently respond correctly. Extinction took ~900 trials, and reacquisition merely about 60 trials, substantially less than the original acquisition (Figure 4B).

[Place Figure 4 about here.]

Neural data

Figure 5 shows the response pattern of two units in the nidopallium caudolaterale (NCL) recorded while an animal was performing the SIFC task. Response modulation during presentation of the NS is shown in Figure 5A. In the acquisition phase, the units responds strongly to NS 2 (designated for extinction), with responses declining towards the end of the acquisition phase and little change in firing during the other two stages of learning. There is little responding to NS 1 across the entire session. The response increase around 3-4 seconds after sample stimulus onset is due to reward delivery. Activity levels concerning familiar stimuli were not modulated (data not shown).

Figure 5B displays the response pattern of another NCL unit recorded during SIFC. This neuron responds during right- but not leftwards movements (upper left), suggestive of sensorimotor coding. However, response strength changed over the stages of learning: the two lowermost panels show spike density functions (SDFs) triggered to rightward choices for one familiar (left) and one novel stimulus (right), split up into successive quartiles to illustrate the development across the experimental session. Responses were lower for the familiar stimulus throughout the entire session, even though average movement times for the two stimulus conditions were highly similar (upper right). Moreover, responses during rightward choices after presentation of the novel but not the familiar stimulus decreased over the course of the experimental session (not paralleled by a decrease in baseline firing rate). Thus, both neurons decreased firing as a particular novel stimulus became increasingly familiar, with the neuron in Figure 5B coding for a specific movement in addition to the novelty of the stimulus preceding that movement.

[Place Figure 5 about here.]

FIGURE LEGENDS

Figure 1: System overview. Information flow is symbolized by colored arrows. Computer 1 controls hardware pertaining to behavioral output (stimulus display via the flat screen monitor, house light, food hopper, feeder light, response keys) and sends event timestamps to the AD converter. Computer 2 stores neurophysiological signals obtained from the A/D converter and event timestamps received from Computer 1. The photograph on the left shows the conditioning chamber inside the sound-attenuating cubicle. Its elements are: 1) Sound-attenuating shell, 2-4) response keys, 5) food hopper, 6) feeder light 7) house light, 8) observation camera.

Figure 2: Illustration of the behavioral paradigm. After an ITI of 5 s, the center key is transilluminated green for up to 5 s (initialization). If the animal responds three times within these 5 seconds, one out of the four sample stimuli is presented at the same position. After a fixed sample presentation time of 2 s during which the animal has to respond at least once, the central pecking key is transilluminated green again (confirmation). After another peck, the two side keys are transilluminated green. The subject indicates its choice by responding once to one of the side keys. During acquisition and reacquisition, correct responses are followed by 2 s food access accompanied by activation of the feeder light, or activation of the feeder light alone. If incorrect, house lights are turned off for 3 s. During extinction, both correct and incorrect responses to the extinction stimulus remain inconsequential. Inset shows example novel and familiar stimulus pairs.

Figure 3: Quality metrics for unit isolation. A) Heat map of all waveforms' time-voltage values. B) Distributions of maximum (red), minimum (green), and noise (blue) voltage values of all waveforms. The distributions are well separated, indicating excellent unit isolation. C) Spontaneous firing rate (red, calculated from 2-s segments in all intertrial intervals) and spike amplitudes (peak-to-peak) as a function of time in session. Both curves were smoothed with a boxcar function (width: 50 data points). D) Interspike-interval distribution for this unit. Bin width, 10 ms (inset: 1 ms). Very short intervals are nearly absent (<0.1% of intervals below 4 ms). E) PSTH triggered to key pecks. Event counts close to the key peck (±20 ms) are highlighted red. F) All 157 waveforms recorded within ± 20ms of key peck events. The waveforms compare favorably to overall waveform shape shown in panel A.

Figure 4: Example behavioral results. a) One bird's performance for the two novel stimuli across all three stages of learning. Curves depict percent correct choices (mean over the last 120 trials, corresponding to 30 presentations of the respective stimulus) as a function of the total number of trials, separately for novel stimulus 1, novel stimulus 2, and averaged across both stimuli. Performance for familiar stimuli was consistently above 95% correct (data not shown). b) Mean number of trials needed to achieve criterion performance in each of the three stages of learning; error bars, SEM.

Figure 5: Response patterns from two example units recorded during the SFIC task. A) Spike density functions triggered to onset of the two novel stimuli NS 1 and NS 2 (upper and lower row, respectively), split up for three learning phases (columns), with responses in each learning phase again split up in three equal parts (early, middle, late). NS 2 was designated for extinction. PSTHs (bin width 1 ms) were smoothed with an exponentially modified Gaussian kernel (σ=100 ms and τ=100 ms). B) SDFs from a putative motor neuron. Upper left panel shows SDFs (as in A, but σ and τ equaled 150 ms) triggered relative to left and right choices. Colored vertical dotted lines depict median leaving times for each choice. The two lowermost panels show SDFs for rightward choices following presentation of a familiar (left) or novel stimulus (right). SDFs are constructed separately for four equally sized subsets of the data, split up according to time in session. The panel in the upper right shows the distributions of movement times (rightward only), separately for each session quartile and preceding stimulus (familiar, F, novel, N).

DISCUSSION

This protocol describes a complex behavioral task suitable for concurrent single-unit recordings. We have described the SIFC task for pigeons, but it can be easily adapted to rodents e.g. by requiring nose pokes or lever pressing rather than key pecks, and substituting visual by olfactory, auditory, or tactile stimuli.

Perhaps the most critical steps during the training procedure are 1) gradual reduction of reward probability and 2) increase in trial number. Regarding intermittent reinforcement for the familiar stimuli, we decided on reward probabilities ranging from 0.5 to 0.8; these are high enough to produce stable performance but low enough to prevent premature satiation. That said, many birds are willing to perform well for reward probabilities down to 0.2.

The large number of trials per session (500-1,500) is necessary because the acquisition, extinction, and reacquisition of conditioned responding simply requires this many trials, and because the precise estimation of firing rates is difficult with less than, say, 25 trials, especially when recording from neurons with low firing rates (in the NCL, baseline firing rates are <1 Hz). Accordingly, we set the minimum number of trials necessary for completing a learning stage such that each stimulus is shown at least 35 times.

For a naïve animal, training on the SIFC task takes approximately 4 months, but the exact duration depends heavily on the individual. Due to the high demands of the task, it is quite likely that not all animals will end up performing well on the final paradigm. If an individual bird skips too many trials or produces high error rates during training, do not hesitate to replace this subject. In our experience, it is highly probable that this animal will never perform properly on the final task.

Most previous studies conducting single-unit recordings in freely moving pigeons failed to properly register motor output during recording. This complicates the interpretation of neuronal responses during critical periods of the trials, like sample presentation or delay phases[25]. This problem is e.g. inherent in go/no-go tasks in which the experimenter usually does not know what the subject is doing on no-go trials; the same caveat applies to working memory tasks incorporating a prolonged delay period. To achieve control over the movement of the animals without employing head-fixation, we designed a task in which animals have to perform the same action (key pecking) even though conditions (sample stimuli) change. In our SIFC paradigm, both visual input as well as motor output is well-controlled and constantly monitored. Since animals are required to peck at every sample stimulus throughout its presentation, we keep motor output constant while animals are viewing stimuli with different learning histories. We are currently exploring methods to achieve even better control of motor output, such as attaching an accelerometer to the headstage for the continuous registration of head movements. Also, we are developing methods for measuring the force of each key peck by means of a mechanoelectric transducer.

Our paradigm allows disentangling the contributions of sensory, motor, and cognitive variables to neural firing rates by identifying typical neural response patterns. For example, a premotor neuron for leftward responses would be expected to increase firing during the sample phase whenever the animal is going to make a leftward response, regardless of stimulus identity. Similarly, simple motor neurons would be expected to fire during key pecking, or left- or rightward motion. A neuron representing reward expectation, on the other hand, would fire during the sample phase, and more so for the FS than the NS during early acquisition (because subjective reward probability is higher on FS than NS trials before NS are learned), but this should reverse later when the NS are consistently classified correctly (because objective reward probability is higher on correct NS trials). Finally, neurons responsive to specific stimulus features are expected to fire consistently for one of the sample stimuli without any change across stages of learning.

Because extracellular unit recording is prone to record spikes from multiple units at a time [11,26], inspecting a range of quality metrics is important to properly classify spikes as originating from a single or from multiple neurons[27]. Using tetrodes instead of single electrodes would certainly yield an additional increase in sorting quality[11]. This should be considered when recordings in brain regions with a high cell density (for example hippocampus) or very high spontaneous activity (such as the entopallium) are intended. However, the microplugs we use are only available for up to 18 connections which for now constitute an upper limit on the overall number of recording channels.

In sum, we developed a task of high complexity for non-primate experimental animals. This task was tailored to enable the investigation of learning phenomena with single-neuron recordings, but at the same time is suitable to tackle subjects such as categorization, decision making, and reward coding.

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DISCLOSURES
The authors have nothing to disclose.

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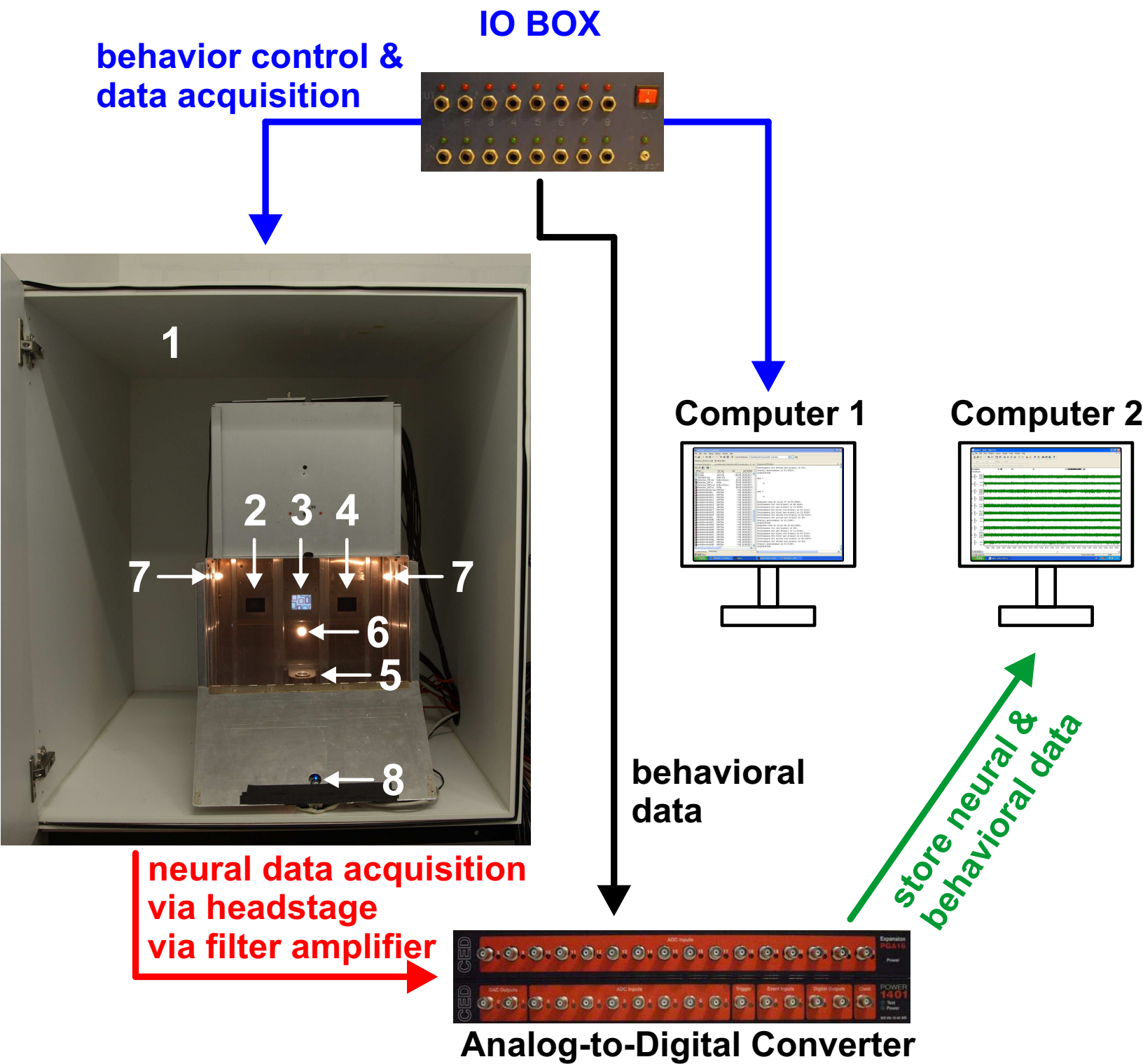

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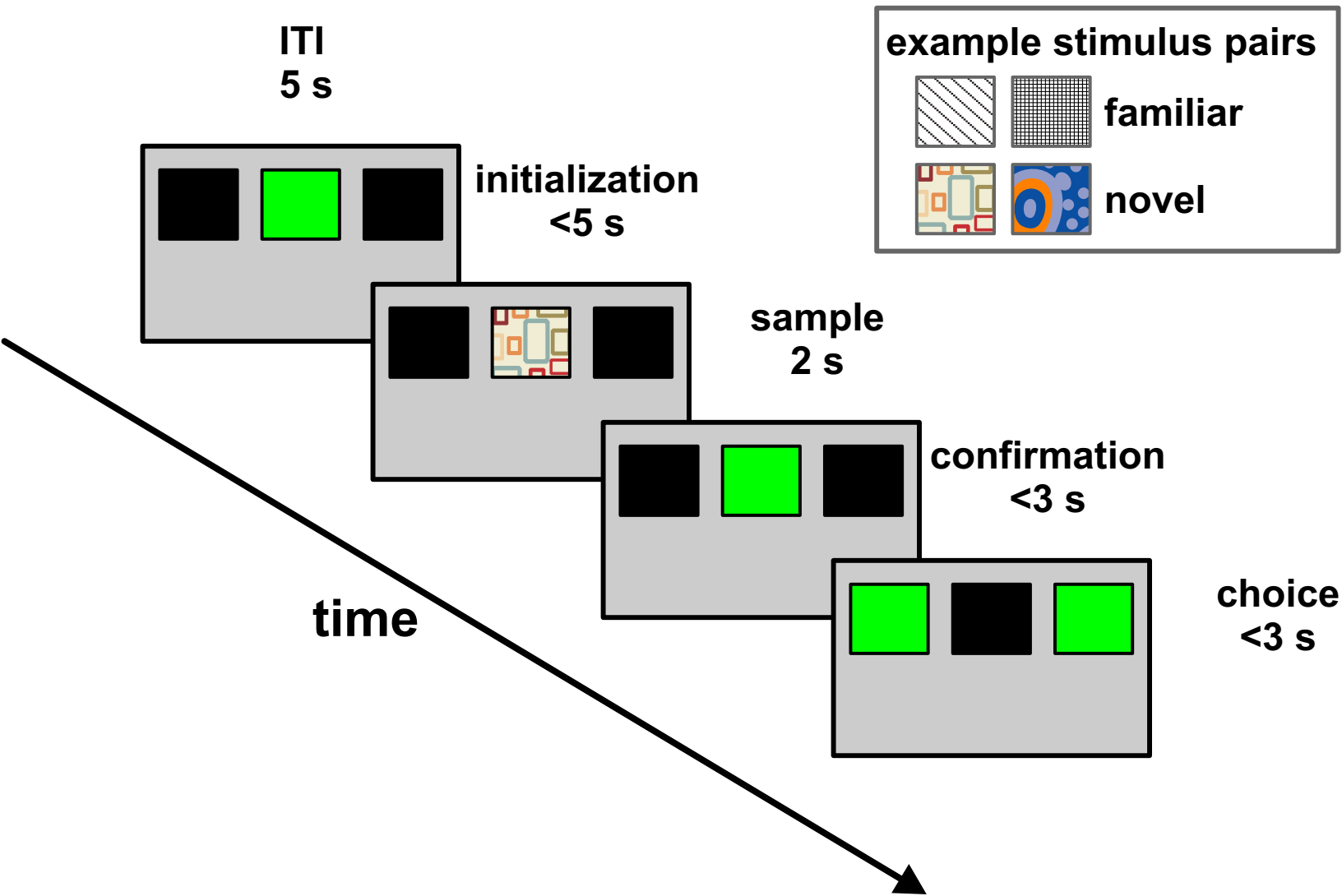

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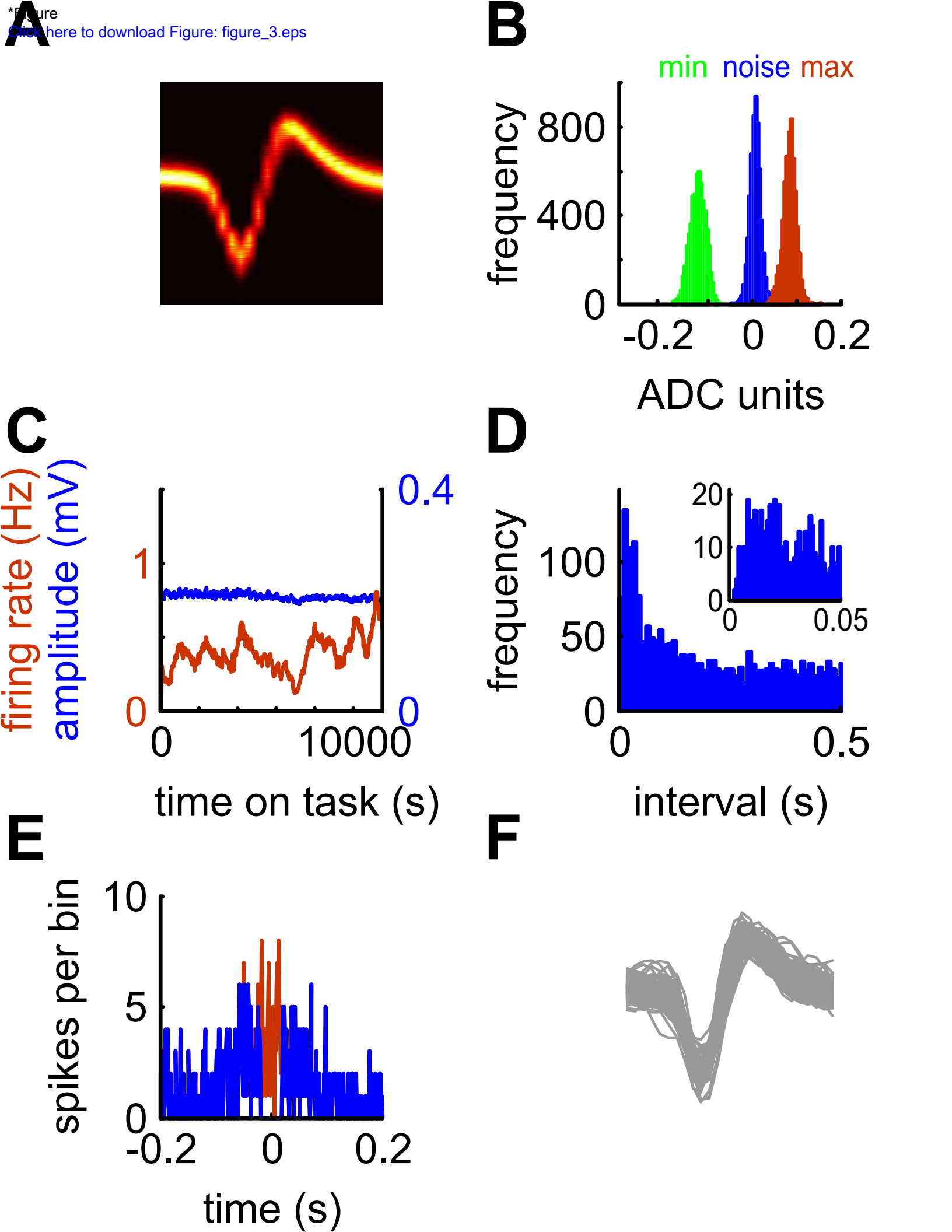

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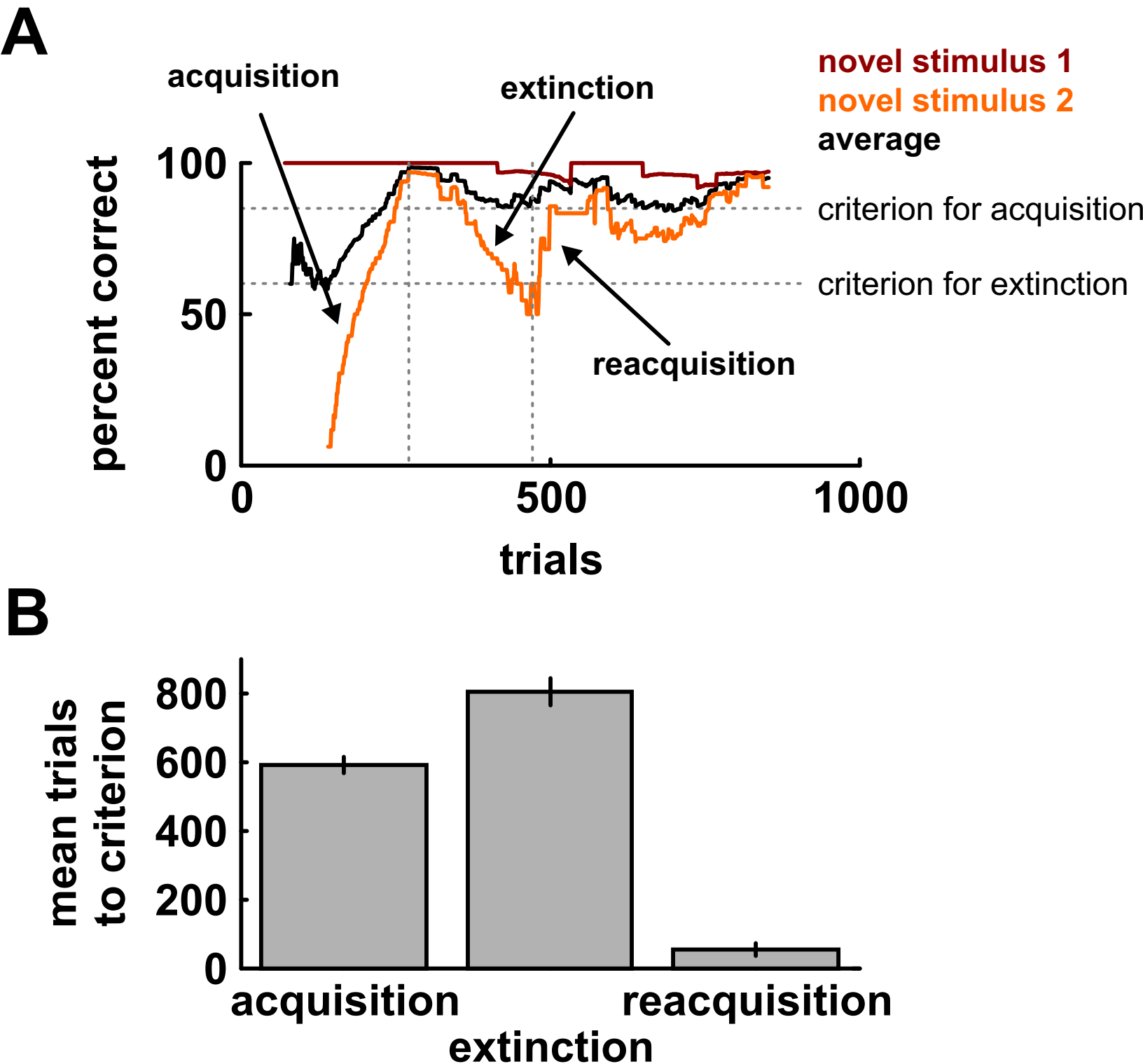

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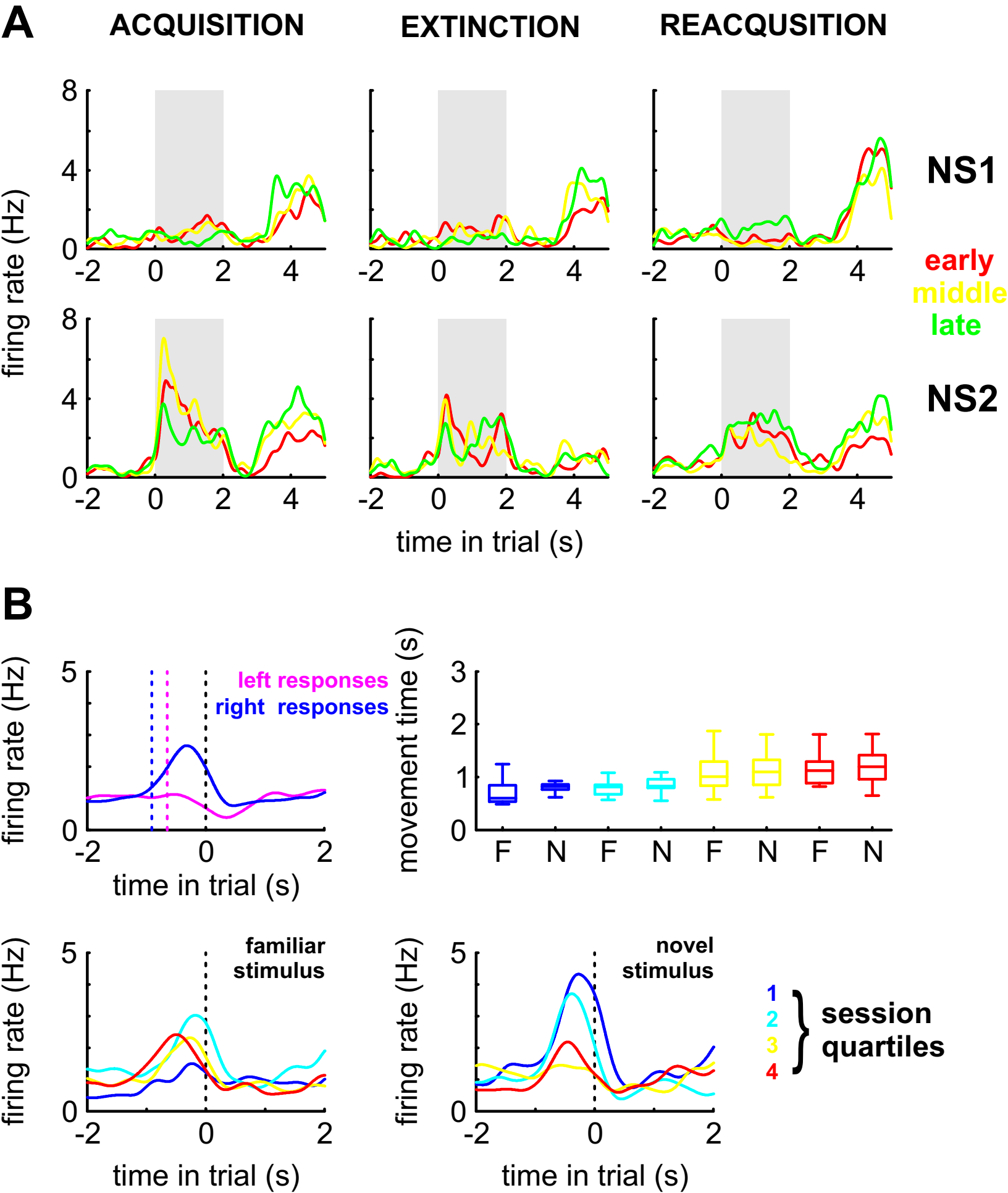

*Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company
Resistance wire (for use as electrodes)	California Fine Wire, Grover Beach (CA), USA
Microconnectors	Ginder Scientific, Nepean, Ontario, Canada
Cannulae	Henke Sass Wolf, Tuttlingen, Germany
Gold solution for plating	Neuralynx, Bozeman (MT), USA
Solution for ultrasonic bath	Alconox, Inc., New York, USA
Conductive glue	Henkel Loctite
Stainless steel screws	J.I. Morris, Southbridge (MA), USA
Light-curing dental cement	van der Ven Dental, Duisburg, Germany
Light-curing unit	van der Ven Dental, Duisburg, Germany
Filter amplifiers	npi electronic GmbH, Germany
A/D converter	Cambridge Electronic Design, Cambridge, UK
Spike2 software	Cambridge Electronic Design, Cambridge, UK
Matlab	The Mathworks, Natick (MA), USA

Catalog Number	Comments/Description
	Stablohm 675; formvar-coated nichrome wires (outer diameter 25 µm)
	GS18PLG-220 (plug) & GS18SKT-220 (socket to build headstage)
	0.4x20mm/ 27Gx3/4"
	SIFCO Process Gold Non-Cyanide, Code 5355
1304	Tergazyme
	LOCTITE 3888 Silver filled, conductive, adhesive
	F0CE125 self-tapping miniature screws, body length 1/8 inches
	Omniceram Evo Flow A2
	Jovident Excelled 215 Curing Light (wireless LED light curing unit)
DPA-2FS	
power 1401	
	Version 7.06a
	R2012a

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Author(s): Sarah Starosta, Maik C. Stüttgen, Onur Güntürkün

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Article Title: Recording single neurons' action potentionals from freely moving pigeons across three stages of learning

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Dear Ms. Jarzylo,

thank you very much for your work on our manuscript ”Recording single neurons’ action potentials from freely moving pigeons across three stages of learning”.

We addressed the reviewers’ comments concerning the protocol and feel that this resulted in a significant improvement of the protocol description. All changes are printed red in the revised manuscript.

We hope that you will now find the manuscript suitable for publication in the Journal of Visualized Experiments.

Yours sincerely,

Sarah Starosta

Reviewer 1

Their statement under "Long Abstract" that nobody has looked at single-unit responses in animals trained on both acquisition and extinction in the same session is incorrect. This was done by the below authors who should be cited:

Bingman, V. P., Gasser, B. A., & Colombo, M. (2008). Responses of pigeon wulst neurons during acquisition and reversal of a visual discrimination task. Behavioral Neuroscience, 122, 1139-1147.

The statement read that “observation periods usually encompass only a single stage of learning” and thus did not imply exclusiveness. Nonetheless, we followed the reviewer’s suggestion and modified the statement as follows:

“But even in these cases, observation periods usually encompass only a single stage of learning, i.e. acquisition or extinction, but not both (exceptions include protocols employing reversal learning; see [1] for an example)”.

Reviewer 2

Major Concerns:
1. It's not clear when the surgery in the animals occurred - before all training? after autoshaping and before training with stimuli? Also, were any sham animals run to ensure that the implant did not interfere with neuronal response in any way?

This information was indeed lacking from the manuscript. Surgery took place after animals repeatedly (3-4 times) completed the entire acquisition-extinction-reacquisition sequence. We added this note to the protocol.

Since there is no significant drop in performance after surgery, one can argue that surgery has no effect on behavioral performance. Whether the implant affects neuronal responses in any way cannot be tested, because the implant is a prerequisite for observing neuronal responses.

2. Will others be able to replicate the microdrive that is custom built without a picture/diagram? or is this something that is relatively standard among electrophysiology labs?

We agree that a picture of the microdrive would be very helpful. But since the microdrive was not invented by us and is not the focus of this paper, we refer to the work of Bilkey et al. (1999, 2003) where a detailed description of the microdrive including schematic drawings can be found.

3. The authors only tested this in one brain area - will this set up work in other areas that require the microdriver to advance through other regions before getting to the area of interest? perhaps note this in limitations.

In this paper, we only report recording from the nidopallium caudolaterale of the pigeon, a brain region which is extremely well suited for chronic electrode implantations due to its large extent in the dorsal-ventral direction. However, due to the very stable guide cannula of the implant, it is possible to reach deeper brain areas as well. For example, we recorded action potentials from the medial striatum of the pigeon. Other authors (Colombo et al., Brain Research 2001; Bingman et al., Behavioral Neuroscience 2008) used a highly similar implant to record from the entopallium and the visual Wulst of pigeons during behavior. Other recording sites are reported in the Bilkey papers describing the original microdrive. Thus, there is no need to include this issue in limitations.

4. Because you have to expose pigeons to all stimuli before training (to avoid neophobia), it would be better to refer to stimuli as "untrained" rather than "novel", since the birds had been exposed to them before. However, if the novel stimuli are actually novel, then make this clear in the procedure.

This issue is indeed confusing. Actually, the birds had not been exposed to the stimuli before. We expose them to a huge set of plain stimuli to avoid neophobia and use all kind of colored patterns as new stimuli in the final task. Therefore, we perceive the term "novel" as adequate, but refined the description of the stimuli in section 1.3.5.

5. Four months training on this task is a very long time to put in especially if some birds are never able to learn the task. Is this "cost effective" - time and ethics-wise considering the animals have invasive surgery? Could the method be refined to shorten this training time?

Due to the high complexity of the task, training time is definitely extensive compared to average training durations in small animals. However, in comparison to training procedure

of non-human primates, four months is relatively short. We believe that the complexity of the task and the resulting insights into neuronal mechanisms of learning and cognition are worth the effort. Also, animals which are not able to learn this complex task do not undergo surgery and can be allocated to other projects.

6. Is it realistic to expect that this could be applied to other animals (e.g. rats/mice) if you must require lots of responding for little reward? You state in your introduction that pigeons are "especially suitable" for this task.

We do not know whether rats or mice would be capable of performing this task, but we see no reason why they should not be. Reversal learning is certainly possible in these species (see e.g. Roesch et al., Nature Neuroscience 2007). However, pigeons are especially suitable for this task, because they are willing to work for extended periods of time at lean schedules of reinforcement and can be trained on a wide set of easy-to-produce visual stimuli.

Minor Concerns:
P6 step 1.2.3 - define "reliable"

We define reliable responding as >85% responses in average. We added this information to step 1.2.3.

P6 step 1.2.4 - when repeating steps for L and R response keys, do you include trials with previously trained keys?

Yes, we include trials with the trained middle key. We added this point to the description.

What happens on a trial when the pigeon pecks the centre key but then does not make a R or L choice?

This information was indeed lacking and is now included in the description of the behavioral paradigm (figure 2, step 1.3.2.). If an animal did not give a choice response within three seconds, the trial was aborted and the ITI started.

P7 step 1.4.1 - Let the subject perform 50 "SIFC" trials? How do you decide what reward probability to use?

We do not give a clear instruction what reward probability to choose, because it depends highly on the individual. However, this issue is addressed already in the discussion part. The reward probability should be chosen high enough to keep the animal motivated through the time course of the session and low enough to avoid premature saturation. Therefore, the optimal reward probability has to be found out for every animal

individually by trial and error. However, reward probabilities between 0.5 and 0.8 have been proven to work best.

Providing approximate or average number of sessions for each step may help others decide whether a pigeon is "getting it" or should be discarded.

This is a very useful suggestion. We added this information for some of the critical steps.

Provide full term Spike Density Function on first use in paper.

We now introduce the abbreviation in the section "representative results".

Can you advance the microdrive mid session if not getting any clear neuronal response?

We advance the electrodes before every session. If we do not get any clear neuronal response, we do not start the session but wait until the next day and advance the electrodes further. We expanded the paragraph describing this procedure to illustrate it more clearly.

Reviewer 3

1. Perhaps the authors could expand the introduction slightly to elaborate on why they developed this particular task. For example, some readers might wonder if a simpler task could works as well. For example, simple train a pigeon to peck the key to receive food, then extinguish it, and then retrain it. One could use a new stimulus each session. Is there a reason that a procedure like this was not adapted? This is not a criticism, because the new procedure is interesting in its own right, but I think the reader might benefit from a slightly more detailed explanation of why this task was developed.

The reviewer addresses an important issue. We discuss this issue in the 5th paragraph of the discussion section, describing why a Go/NoGo task (which is what the reviewer suggests) is suboptimal for concurrent single-unit recordings and how the developed task circumvents problems associated with such a simpler task.

2. My main comment/concern is with Figure 5A. In the text, the authors state that "response modulation during presentation of the NS designated for extinction is shown in Figure 5A". From my interpretation, Figure 5A shows both NS1 and NS2, and only one of these stimuli should subsequently undergo extinction (from my read of the methods, only 1 of the stimuli should subsequently undergo extinction). Is this correct? So, I think the authors can be a more clear about what is being shown in Figure 5A.

We added the information to the description of Figure 5 and changed the paragraph "Representative results; 2. Neural data" as follows:

"Response modulation during presentation of the NS ~~designated for extinction~~ is shown in Figure 5A. In the acquisition phase, the unit responds strongly to NS 2 (designated for extinction), with responses declining towards the end of the acquisition phase and little change in firing during the other two stages of learning.

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