Evaluating Sequential Response Learning in the Rodent Operant Touchscreen System

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Sequential and cue-directed response learning in rodents have been previously shown to depend on intact striatal signaling. In particular, these behaviors rely on striatal dopamine and acetylcholine release, with an impairment of sequential response learning evident in animal models with alterations in the two systems. Here we provide a protocol for testing sequential response/response chain learning using the rodent touchscreen system. Specifically, the present protocol is designed to implement the heterogeneous sequence task, adapted from Keeler et al. (2014), in the rodent touchscreen apparatus. This task has been used previously to assess complex motor learning and response selection in mice. In the following protocol, the task is performed in touchscreen-based automated chambers with five response locations using food reinforcers to maintain performance. The sequence task requires the subject to make five nose pokes to white square stimuli appearing in five different locations sequentially from left to right. © 2021 Wiley Periodicals LLC.

Basic Protocol: Implementation of the heterogeneous sequence task
Support Protocol: Creation of the heterogeneous sequence task ABET II touchscreen schedule

Keywords: mouse • response chain • sequential learning • touchscreen

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INTRODUCTION

The present protocol can be used to evaluate the ability of mice to perceive and respond to a light cue that appears sequentially in five different locations and indicate the correct location in the sequence (Keeler, Pretsell, & Robbins, 2014; Turner, Svegborn, Langguth, McKenzie, & Robbins, 2021). Although there are other commonly used

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touchscreen-based protocols that allow for the testing of relatively simple operant learning, this protocol requires animals to respond sequentially and stereotypically to a long and heterogeneous sequence of cues. The increased complexity of the task allows for detection of more subtle changes in animal models, including impairment of cue-directed response chain learning, that cannot be recognized by common fixed-ratio and progressive-ratio protocols because responses in those tasks are emitted to a single (homogeneous) location. The heterogeneous sequence task also allows researchers to dissociate the animal's response to different cue locations based on their proximity to the reward. An impairment of cue-responding and sequential response learning can be detected as an increase in the time necessary for the completion of a predefined number of sequences and/or as an increased number of nose pokes to incorrect locations, including blank locations. Potential alterations in response to reward-distal (initial) and reward-proximal (final) cues can be also detected (Keeler et al., 2014).

The following protocol provides instructions for the initial preparation and training of mice for operant responses in a sequential order (from left to right, as signaled by a light cue) in the touchscreen apparatus. We also show sample data obtained in wild-type control mice and introduce main parameters that can be obtained and analyzed in the task.

STRATEGIC PLANNING

Mice

The use and care of the animals must be conducted in agreement with the appropriate institutional and/or national review boards. Animals can be obtained from commercial suppliers (e.g., Jackson Laboratories, Charles River Laboratories) or bred by the laboratory. Both sexes can be tested in the touchscreen apparatus, although the presented task has thus far only been used in male mice (Frick & Berger-Sweeney, 2001; Meziane, Ouagazzal, Aubert, Wietrzych, & Krezel, 2007). The C57BL6/J strain is most commonly tested in the touchscreen apparatus and was used to obtain the sample data presented here. It is best to use young adults ($\sim$10 weeks of age), as they tend to learn faster and do not require prolonged food restriction (Creer, Romberg, Saksida, van Praag, & Bussey, 2010). However, old animals can perform successfully in the touchscreen apparatus (Kent et al., 2017), and we have tested mice up to 22 months. Mice should be at least 2 months of age.

For food restriction, it is often necessary to divide animals into smaller groups (2-3 mice per cage) or even house them individually. Single housing should be avoided if possible, as it can be stressful for mice. However, if cage-mates do not share food evenly, it may be necessary to house them singly in order to achieve and maintain the desired target weight. If single housing cannot be avoided, animals should be counterbalanced across experimental groups. With the exception of the food restriction protocol, mice should be housed under standard conditions.

For our experiments, the use and care of animals was conducted in agreement with the Canadian Council of Animal Care guidelines, and animal protocols approved by the University of Western Ontario (protocols #2016-103, 2016-104). The generation of VAChT$^{flox/flox}$ mouse lines is described in Martins-Silva et al. (2011). LoxP sequences flanking the VAChT gene do not interfere with cholinergic marker expression, and VAChT$^{flox/flox}$ mice do not differ behaviorally from wild-type littermates (Martins-Silva et al., 2011). The sample data presented were collected from 2- to 5-month-old male mice during the light cycle (N = 12).

Rewards

The touchscreen apparatus can be equipped with a solid (pellet) or liquid reward dispenser. For these experiments, we used undiluted strawberry milkshake (Neilson Dairy,

Toronto, Canada, 20 μl per single reward). Other types of sweetened liquid reward can be used, including sweetened condensed milk diluted 1:1 or more with water (Delotterie, Mathis, Cassel, Dorner-Ciossek, & Marti, 2014).

Touchscreen System

A detailed description and the availability of the apparatus can be found elsewhere (e.g., *Current Protocols* article Heath, Phillips, Bussey, & Saksida, 2016; Horner et al., 2013; Mar et al., 2013; Oomen et al., 2013; Wolf, Urbano, Ruprecht, & Leising, 2014). The apparatus used in this protocol was purchased from Campden Instruments. The Bussey-Saksida touchscreen chamber (Campden Instruments) consists of a trapezoidal arena with a touchscreen monitor (12.1-in. screen, 800 × 600 resolution) at one end and reward collection magazine (20 cm H × 18 cm L × 6-24 cm W) at the other, tapering from touchscreen to magazine. The walls of the arena are black plastic, the lid is transparent plastic, and the floor is perforated stainless steel with a stainless-steel waste tray underneath. The entire assembly is housed in a sound-attenuating chamber equipped with a fan. A click/tone generator and a video camera are mounted above the arena, adjacent to an electronic pellet dispenser. For liquid reward–equipped chambers, a peristaltic pump is positioned centrally behind the touchscreen unit. An infra-red (IR) beam is used to detect entries in the magazine, and two additional beams cross the arena to detect locomotor activity. To minimize unintended screen touches and to demarcate screen response locations, a black plastic mask is fitted in front of the touchscreen. For this protocol, we used a mask providing a row of five response locations (4 × 4 cm each) spaced 1 cm apart at 1.4 cm above the chamber floor. For a more detailed description of the chamber and mask, see Heath et al. (2016).

Cleaning Materials

The reward delivery system must be cleaned properly after completion of all daily sessions to avoid clogging. At the beginning of each session, the delivery system must be checked to ensure it is not clogged and that the animal's motivation and performance will not be disrupted.

In addition, the touchscreen chamber must be cleaned after every training session. We commonly use 70% ethanol and kitchen paper towels, but other (e.g., alcohol-free) disinfectants can be used.

IMPLEMENTATION OF THE HETEROGENEOUS SEQUENCE TASK

BASIC PROTOCOL

Food restriction and pre-training. For evaluation in the touchscreen apparatus, mice must be motivated to earn a palatable food reward (e.g., strawberry milkshake). To ensure stable motivation, mice are subjected to a mild food restriction ∼1-2 weeks before the initiation of training and throughout the testing period. Details for daily weighings and food adjustments are described below. Because mice have to be weighed and fed daily during food restriction, this period before task initiation also helps acclimate mice to the experimenter and to daily handling. After the initial food restriction, mice are habituated to the food reward and to testing in the touchscreen chamber. Proper habituation is crucial to minimize stress and anxiety that could otherwise interfere with learning and task performance.

Basic training. The basic training performed before the heterogeneous sequence task is comprised of two habituation phases followed by an initial touch phase and then a must touch phase. These phases are described further in the steps below and outlined in the flowchart in Figure 1. Starting with basic training, each mouse should receive one daily session of training/testing in the touchscreen apparatus. Mice are usually tested five to six times a week, with continued weighing and feeding performed every day at the regular/same time of day.

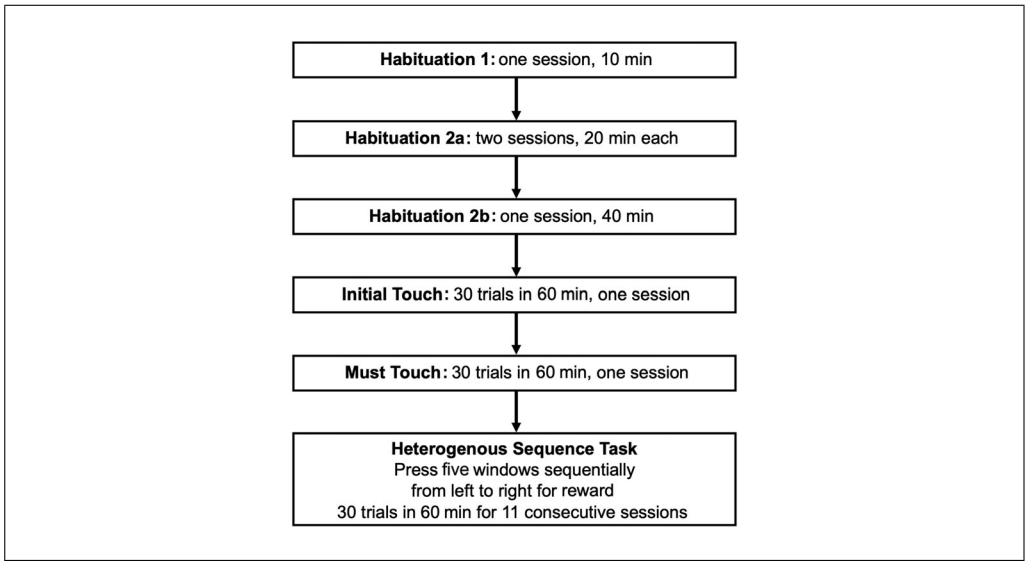

Figure 1 Flowchart summary of basic training prior the heterogeneous sequence protocol. The training consists of habituation to touchscreen chambers and training of operant responding to light stimuli presented on the touchscreen. See Basic Protocol for detailed description of individual training stages.

Heterogeneous sequence task. As presented here, the complete task is comprised of 11 consecutive sessions. Each session is ended when the animal completes 30 trials or when 60 min has elapsed, whichever is first. Sample results are discussed in Understanding Results.

Materials

Mice (typically, $n = \sim$10-15 mice; see Strategic Planning for additional details)
Food pellets (e.g., grain-based pellets, Bio-Serv)
Food reward (e.g., undiluted strawberry milkshake; see Strategic Planning)
Disinfectant, e.g., ethanol

Behavior chamber/touchscreen apparatus (see Strategic Planning), including:
- Reward magazine
- Waste tray
- Fan
- House light
- Click/tone generator
- Electronic pellet dispenser or peristaltic pump
- Infrared detector for head entry in reward magazine and movement in chamber
- Tubing
- Five-window mask
- Video camera (not required)

Windows-based PC with touchscreen software:
- ABET II (Lafayette Instrument)
- Whisker Server

Initiate food restriction and pre-training

1. Weigh mice while they are on a free-feeding diet to determine their baseline weight. For each animal, calculate a target weight range that is between 85% and 90% of the free-feeding weight.

For older and/or heavier animals (approximately above 30 g of weight), a restriction to the calculated target range may not ensure proper motivation and performance. In such cases, a target weight range between 25 and 26 g can be used.

2. To begin food restriction, remove food from all cages, leaving ~1 large pellet per mouse per cage.

3. Beginning the next day, continue daily weighing and feeding, but adjust the number and/or size of food pellets to induce a gradual weight loss until the target weight range is reached.

   Make sure weight loss is not too fast; mice should not lose >1 g per day.

   Weighing and feeding should be always done at the same time of day, optimally after completion of the daily training session.

4. Once mice reach their target weight range, maintain a stable weight throughout the experiment by weighing animals daily and adjusting food provision as needed.

   If mice appear to be competing for food, re-house them as needed.

5. Begin habituating mice to the food reward. Provide a small amount of reward in each cage every day starting ~3 days before initiation of the touchscreen experiment.

   A small Petri dish or a plastic tube cap can be used to hold the reward in the home cage.

   Be aware that the amount of food necessary to maintain the target weight may change after the beginning of the experiment, when the high-caloric food reward will be consumed.

Perform Habituation 1

6. Assign mice to individual touchscreen chambers. Maintain assignments for the duration of the experiment so a given mouse is always tested in the same chamber.

   To minimize any potential effect of different chambers on performance, assignments should be counterbalanced across experimental groups.

7. Set up the touchscreen testing apparatus with all inputs/outputs turned on to ensure animals habituate to any noise emitted. Insert a five-window mask. Open the Whisker Server and ABET II programs on the computer.

   The Whisker Server is used to control the operant chambers for behavioral experiments. It can be used to ensure all touchscreen equipment is working.

   The first habituation session is performed without reward in the food tray. In this session, mice are introduced to the touchscreen chamber.

8. Load the Habituation 1 schedule into the ABET II software ($t = 10$ min).

   The schedule is set up so that all lights are turned off and no stimulus or reward is presented.

9. Individually transfer animals to their assigned chambers, start the schedule, and allow mice to explore the environment for 10 min (Habituation 1).

10. When the session is complete, return mice to their home cages and clean the chambers with disinfectant.

    After the session is complete, a small amount of food reward can be placed in the home cage as in step 5.

Perform Habituation 2

11. On the next testing day, set up the touchscreen testing apparatus with all inputs/outputs turned on to ensure animals habituate to any noise emitted. Insert a

five-window mask. Open the Whisker Server and ABET II programs on the computer.

The second habituation session is performed with reward in the food tray. In this session, mice are taught to look for the reward in the food tray.

12. Load the Habituation 2 schedule into the ABET II software ($t = 20$ min).

    The schedule is set up so that when the mouse enters the food tray, as detected by the IR beam break, the tray light is initially turned on, a tone is played (3 kHz frequency, 1000 ms duration), and reward is delivered (6000 ms, 150 μl). When the mouse leaves the magazine, the tray light is turned off. Following a 10-s delay, the tray light is again turned on, a tone is played, and a reward is delivered (800 ms, 20 μl). If the mouse is in the reward tray at the end of the 10-s delay, an extra 1 s is added to the delay.

13. Individually transfer animals to their assigned chambers, start the schedule, and allow mice to explore the environment for 20 min (Habituation 2a).

14. When the session is complete, return mice to their home cages and clean the chambers with disinfectant.

    Starting with Habituation 2a, each stage of the protocol has a pre-defined criterion that must be fulfilled by the animal to move to the next stage. During habituation, the mouse must consume all the reward in the tray to be moved to the next session. If the criterion is not met, the session is repeated on subsequent test days until the criterion is fulfilled.

15. On the next testing day, repeat the above habituation with the same 20-min duration (Habituation 2a).

    If all of the reward was not consumed, repeat the session on subsequent test days until the criterion is fulfilled.

16. On the next testing day, repeat the above habituation but with a 40-min duration (Habituation 2b).

    If all of the reward was not consumed, repeat the session on subsequent test days until the criterion is fulfilled.

Perform Initial Touch training

17. Set up the touchscreen testing apparatus with all inputs/outputs turned on. Insert a five-window mask. Open the Whisker Server and ABET II programs on the computer.

    The Initial Touch session is performed with reward in the food tray and the house light turned off. In this session, mice are again taught to look for reward in the food magazine, but they can also receive more reward by responding to the light stimulus.

18. Load the Initial Touch schedule into the ABET II software (30 trials; $t = 60$ min). Ensure the following settings are set up:

    Image time = 30 s
    Feed pulse time = 800 ms (20 μl)
    Tone duration = 1000 ms (3 kHz frequency)
    ITI period = 20 s

    The schedule is set up so that at the beginning of each trial the stimulus is displayed randomly in one of the five windows/locations and the others are blank. After a delay (the image time), the stimulus is removed and a reward is delivered (feed pulse time). Reward delivery is accompanied by illumination of the tray light and a tone. Entering the reward tray to collect the reward turns off the tray light and starts the intertrial interval (ITI), after which another stimulus is displayed.

If the mouse touches the screen while the stimulus is displayed, the stimulus is removed, a tone is played, and 3× the reward volume is dispensed. Touching a blank window has no consequence.

19. Individually transfer animals to their assigned chambers, start the schedule, and allow mice to explore the environment for 60 min (Initial Touch).

20. When the session is complete, return mice to their home cages and clean the chambers with disinfectant.

    Mice must complete 30 trials within 60 min. The session is repeated until this criterion is achieved.

Perform Must Touch training

21. Set up the touchscreen testing apparatus with all inputs/outputs turned on. Insert a five-window mask. Open the Whisker Server and ABET II programs on the computer.

    The Must Touch session is performed with reward in the food tray and with the house light turned off. In this session, mice must respond to the stimulus in order to receive a reward. As in the previous session, the stimulus is presented in only one window at a time while the other windows are blank.

22. Load the Must Touch schedule into the ABET II software (30 trials, $t = 60$ min). Ensure that the settings are the same as in step 18.

    The schedule is set up so that the position of the stimulus in each trial is chosen pseudorandomly, such that the stimulus will not be displayed in the same position more than three times in a row. After the mouse touches the stimulus, a reward is delivered, accompanied by the tray light, tone, and initiation of the ITI. After the ITI, another stimulus is displayed. No reinforcer is delivered if the mouse touches the blank part of the screen.

23. Individually transfer animals to their assigned chambers, start the schedule, and allow mice to explore the environment for 60 min (Must Touch).

24. When the session is complete, return mice to their home cages and clean the chambers with disinfectant.

    Mice must complete 30 trials within 60 min. The session is repeated until this criterion is achieved.

Perform Heterogeneous Sequence Task

25. Commence operant response training the day after training sessions are complete. Continue food restriction and daily weighing throughout the task as described previously.

26. Set up the touchscreen testing apparatus with all inputs/outputs turned on. Insert a five-window mask. Open the Whisker Server and ABET II programs on the computer.

    The sequence task is performed with reward in the food tray and the house light turned off.

27. Load the Heterogeneous Sequence schedule into the ABET II software (30 trials, $t = 60$ min). Ensure the following settings:

    Tone duration = 1000 ms (3 kHz frequency)
    ITI period = 5 s

    The schedule is set up so that each session begins with a priming delivery of reinforcer 800 ms (20 μl). On exiting the food magazine, the first trial begins with a white square stimulus presented in first window on the left side of the screen. A correct response to each location is accompanied by a short click-like tone and

the stimulus disappearing for 500 ms. The next window is then illuminated with a white square. After the final response is made on the fifth location, the mouse must enter the reward magazine, after which the reward tone is played. This will trigger the presentation of a reinforcer 800 ms (20 µl) into the food magazine and turns on the tray light. Every new trial (i.e., stimulus appearing in the first location) is automatically initiated 5 s after reward collection (ITI = 5 s). Blank touches (i.e., responding to any blank window) are recorded but do not result in any punishment/change in task. Touches performed between individual trials (i.e., ITI touches) are also recorded.

28. Individually transfer animals to their assigned chambers, start the schedule, and allow mice to explore the environment for 60 min (Heterogeneous Sequence).

    The session is ended after a mouse completes 30 trials or after 60 min elapses (Fig. 3A).

29. When the session is complete, return mice to their home cages and clean the chambers with disinfectant.

30. Repeat task for a total of 11 consecutive sessions.

    The heterogeneous sequence task presented in this protocol consists of 11 sessions, although more can be run. The performance of wild-type control mice is known to plateau during this 11-day time frame.

SUPPORT PROTOCOL

CREATION OF THE HETEROGENEOUS SEQUENCE TASK ABET II TOUCHSCREEN SCHEDULE

The schedule for the heterogeneous sequence task was created using ABET II Touchscreen software that is used for operating Bussey-Saksida touchscreen chambers. A commercially available pretraining schedule "Must Touch" was used as a template and modified (see Fig. 2).

Materials

Windows-based PC with touchscreen software:
ABET II software (Lafayette Instrument), specifically the “Must Touch” pre-training schedule
Whisker Server

Load the software

1. Open the Whisker Server and ABET II programs on the computer.

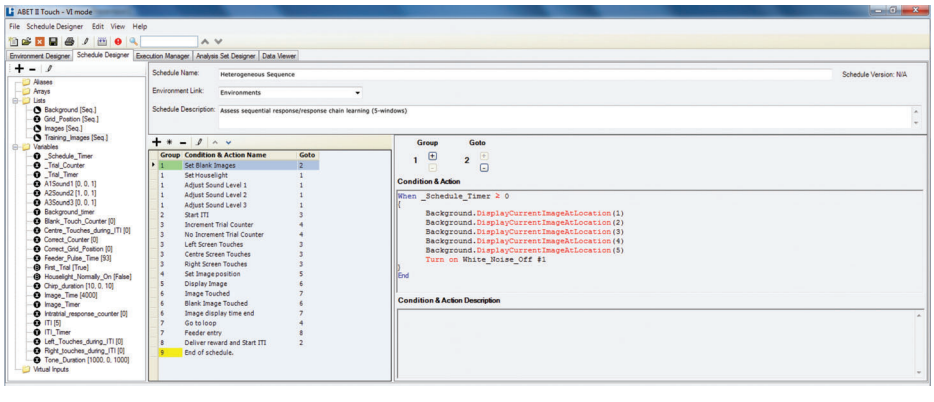

Figure 2 Heterogeneous sequence task ABET II touchscreen schedule. The touchscreen schedule was created based on the touchscreen pretraining schedule Must Touch. See Support Protocol for detailed description of modifications made to Training_Images, Variables, and Grid_Position.

2. Load the Must Touch pretraining schedule into the ABET II software.
3. Select the Schedule Designer tab.

Modify Lists-Training_Images

4. Delete the instruction "Set Image Position" from the beginning of each trial.
5. Add "Set Image Position" before the step "Display Image" so the position of the displayed cue can be changed multiple times within a single trial from window 1 to 5.

   The command of this instruction remains the same as in the original protocol.

6. In "Image Touched" of the original protocol, delete the command settings feeder duration, activating feeder, and delivering reward instructions.
7. In "Image Touched", delete the command that turns on the tray light.
8. In "Image Touched", substitute the feedback tone indicating reward delivery with a same tone with a shorter duration (a short chirp indicating a correct touch to the stimulus).
9. In "Image Touched", substitute the command to increment "Correct counter" (to count the number of correctly completed trials) with a command to increment "Intratrial response counter" (to count the responses to individual windows, gradually from 1 to 5).
10. In "Image Touched", add a command "Start background timer" to measure the time when the next image in the neighboring windows should be displayed.
11. In "Image Touched", delete the command indicating that the first trial was over in the particular session ("First_Trial = False").
12. Delete the final instruction of the original protocol "Reward Collected Start ITI" and substitute it with three additional steps: "Go to loop", "Feeder entry", and "Deliver Reward and Start".

    The instruction "Go to loop" redirects the schedule back to the instruction "Set Image Position" when the Intratrial response counter is <5 and background timer is ≥0.5 s.

    The instruction "Feeder entry" is activated when the Intratrial response counter reaches 5. After that, the cue in the last window is replaced by background and the tray light is turned on.

    The last instruction, "Deliver reward and start", is activated when the mouse enters the food magazine. After that, reward is delivered together with a feedback tone, the tray light is turned off, the intratrial response counter is reset to zero, and the counter of correct trials is incremented by one. The next trial is then started, as in the original schedule, with ITI.

Define new Variables in heterogeneous sequence task schedule

13. Insert "Background_timer", which measures the time that should elapse between the display of two neighboring cues, after the first cue is touched. Set the time to 0.5 s (see step "Go to loop").
14. Insert "Chirp_duration", which defines a short tone (10 ms) that is played whenever the correct cue is touched.
15. Insert "Intratrial_response _counter", which counts responses to the individual windows. When it reaches 5, the counter is reset and a new trial is initiated (see step "Feeder entry").

Modify Variables

16. Set the “Image_timer” value to >60 min so that it never reaches the value within the session and every cue stays in its position until it is touched or the whole session is ended.

Modify Lists-Grid_position associated with schedule

17. Change the mode of "Grid_position" to “sequential” with values 1 to 5.

This is done to ensure that the cue is not displayed randomly, as in the Must Touch protocol, but in the neighboring windows from 1 to 5.

COMMENTARY

Background Information

Touchscreen-based operant chambers are commonly used by many laboratories, as they offer some crucial advantages over other types of behavioral paradigms. Provided that multiple chambers are available, touchscreens allow for high-throughput testing of large cohorts of mice and save experimental time. More importantly, due to automatization and minimal contact between the experimenter and test animals, touchscreen-based chambers produce highly reproducible data that can be easily stored, analyzed, and directly compared over a long time period and between laboratories. To fully take advantage of this potential, a number of touchscreen-based tasks have been developed over the past years for testing various cognitive and behavioral domains in rodents. They include, but are not limited to, operant learning, motivation, cognitive flexibility, attention, spatial memory, pattern separation, and extinction (Beraldo et al., 2019; Bussey et al., 2012; Hailwood, Heath, Robbins, Saksida, & Bussey, 2018; Horner et al., 2013; Mar et al., 2013; Oomen et al., 2015). The present protocol is designed to test a relatively simple operant behavior requiring animals to emit five responses per reward to heterogeneous but stereotypical locations. This heterogeneous sequence task (as the original version was named in Keeler et al., 2014), is more difficult to learn compared to a homogeneous sequence task (commonly known in the context of motivation-focused studies as a fixed-ratio task). Properly pre-trained animals reach their maximum performance in a homogeneous sequence task during one or two sessions, whereas the plateau of performance in the heterogeneous task is reached after approximately five sessions. In a homogeneous sequence task (like heterogeneous sequence task), animals have to emit a fixed number of responses for a single reward; however, all responses are targeted to the same operandum or location. In such tasks, only one significant cue or operandum exists, which is more easily identified by the animal. In addition, a homogeneous sequence does not allow discrimination between reward-distal (initial) and reward-proximal (final) responses. For this reason, the heterogeneous sequence task described here can be particularly useful for testing a mouse’s ability to respond to cues with different proximity to the reward.

Various brain systems are implicated in the identification of significant environmental cues, including striatal dopamine (Keeler et al., 2014). Recently, we found that mice with decreased or increased dopamine release in the striatum perform worse or better, respectively, in the heterogeneous sequence task described here (Kljakic et al., unpublished data). An interesting theoretical framework that describes the potential mechanisms that underlie differential responding to cues with varying reward proximity was described by Keeler et al. (2014). The authors propose a mechanism through which the interaction between the striatal direct and indirect pathways can affect performance in the heterogeneous sequence task.

In the heterogeneous sequence task, mice are guided in their operant responses by light cues appearing in individual locations in a stereotypical order. After training, the stereotypical motor sequence might be internalized, making the cues redundant. In this context, one might expect that mice with sufficient overtraining might be able to perform the task without the guidance of light cues appearing in the correct locations. Indeed, Karly Turner and Trevor Robbins showed that rats trained using cues in the heterogeneous sequence task successfully transferred to a non-cued version of the task, eventually emitting highly stereotyped sequential responding (unpublished communication). Cued and non-cued versions of the task could be useful for studying skilled action sequences versus habits.

Table 1 Troubleshooting in the Heterogeneous Sequence Task

Problem	Possible cause	Solution
Mice unable to perform during training	Unmotivated by food reward	Ensure mice are sufficiently food restricted by reducing weight to lower end of target weight
		If mice are competing for food, isolate them in individual cages
		Present food reward in home cage between daily sessions
	Aversion to touchscreen	Perform additional habituation sessions
		Exclude mice that cannot complete training after a fixed number of sessions (5-10)
Mice that generally perform well unexpectedly struggle in one session	Hardware issues	Check that touchpad is sensitive to touch and entry into the reward tray is detected
		Check physical connections
		Restart system and run diagnostics
		Generally, hardware should be tested regularly, and there should be backup supplies such as touchscreens, connectors, and tubing
	Reward is not being delivered	Ensure there are no leaks in the reward delivery tubing and it is tightly connected to the physical touchscreen apparatus
		Flush delivery tube with hot water, then refill with reward
		If reward is still not being delivered, replace tubing
	Mice lost weight overnight	Weigh mice. If there was weight loss, top up food pellets and food reward. If weight loss continues over multiple days for mice with cage-mates, isolate them in individual cages to avoid competition for food.

Although mice at the end of the training protocol described here were still emitting a relatively large number of blank touches, more extended training could allow the use of non-cued versions of the task with mice.

Critical Parameters

Excluding animals from the experiment

Based on our experience, the present protocol is usually easily adopted by control wild-type mice subjected to mild food restriction. However, as is often the case in operant learning paradigms, certain subjects are not able to perform during training and need to be excluded from the study. A fixed maximum number of sessions for each stage of the training should be defined at the beginning of the study to exclude animals unable to complete a certain stage. Based on our experience, a maximum of five to ten sessions should be sufficient for individual stages of pre-training. However, the optimal number depends largely on characteristics of the experimental animals such as sex, age, and strain, and thus criteria need to be adjusted individually. In our experience, a drop-out rate of $\sim$10% is reasonable.

Testing mice in multiple touchscreen paradigms

Proper consideration needs to be given in cases where mice are tested in more than one touchscreen paradigm. In these cases, the order of the tasks is important, as performance in tasks may be affected by preceding paradigms (Delotterie et al., 2014). Nonetheless, examining mice in more than one touchscreen task is often beneficial and can be recommended, provided that results are interpreted with the whole training history in mind and the order of tasks is maintained across experimental groups.

Troubleshooting

For troubleshooting, see Table 1.

Understanding Results

Performance in the heterogeneous sequence task can be presented as the *time necessary to complete 30 trials* across each session conducted (Fig. 3B). This time should decrease across the training sessions (11 in this proposed protocol). *Performance speed* can be also expressed as a cumulative number of trials completed during the session time

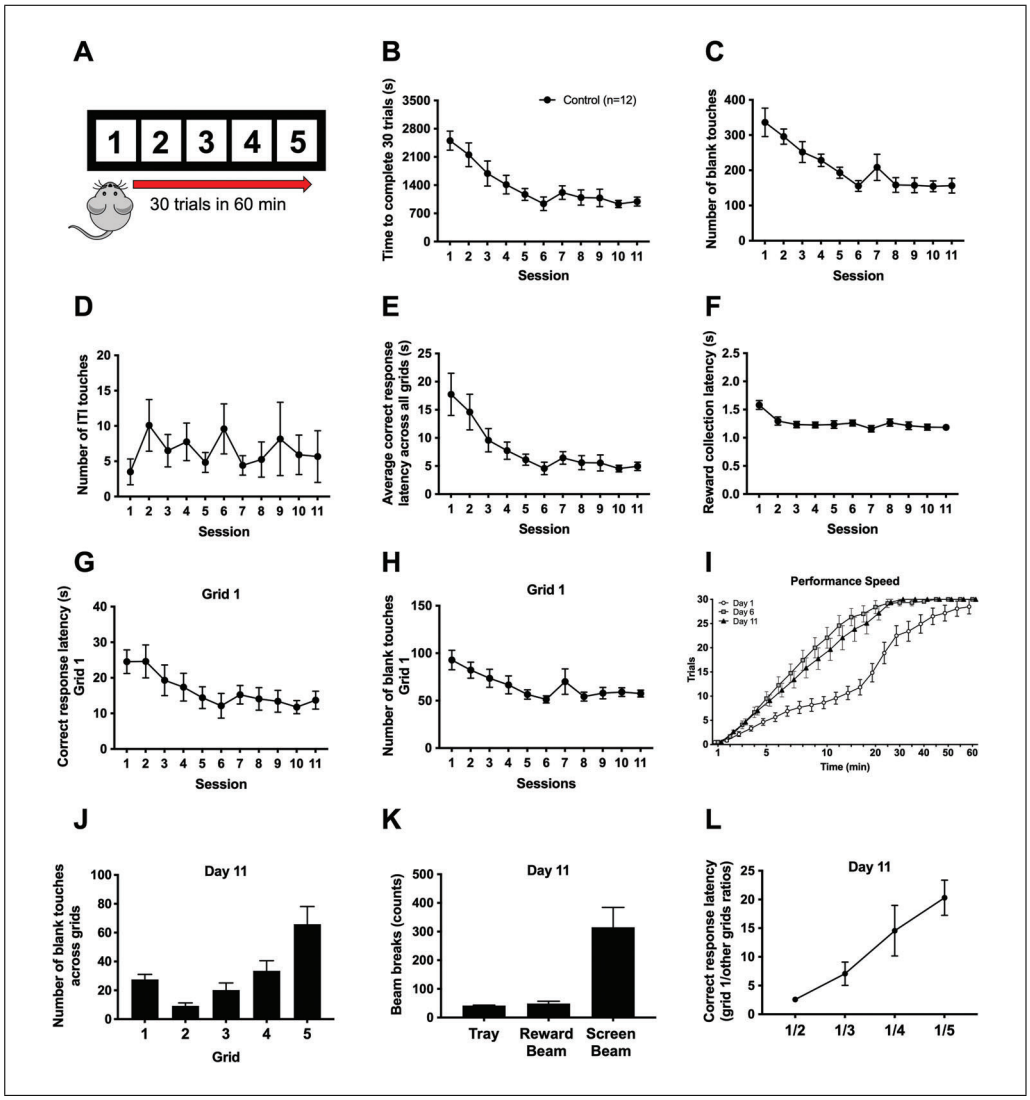

Figure 3 Representative data collected from adult male VAChT$^{flox/flox}$ mice (C57BL6/J background) performing the heterogeneous sequence task. (**A**) Schematic of heterogeneous sequence task: mice are required to make five sequential responses (nose pokes) from left to right on a white square stimulus to receive a reward. (**B-H**) Performance across 11 heterogeneous sequence training days: (**B**) Time required to complete 30 trials, (**C**) total number of blank touches, (**D**) total number of intertrial interval (ITI) touches, (**E**) average correct response latency across all five grids, (**F**) reward collection latency, (**G**) correct response latency to grid 1, and (**H**) number of blank touches on grid 1. (**I**) Performance speed on three training session days (days 1, 6, 11). (**J-L**) Parameters analyzed on day 11 of training: (**J**) Number of blank touches across all grids, (**K**) beam breaks to tray, reward, and screen, and (**L**) correct response latency as a ratio of grid 1 to other grids. Data expressed as mean ± SEM; $n = 12$ male mice, 2-5 months old.

(Fig. 3I), which gives a better overview of an animal's performance throughout the session.

Besides time, there are a number of other parameters that can be recorded and analyzed during the task. We describe the most crucial parameters in the list below.

Number of blank touches

Responses on non-illuminated windows or simply blank touches are also recorded (Fig. 3C). This number should decrease across training sessions, together with the time necessary to complete the session. The number of blank touches is the main outcome that provides information on learning and cue recognition. The time to complete sessions is more indicative of motivation and vigor, whereas the number of blank touches indicates performance accuracy and learning.

Number of ITI touches

Touches emitted during the ITI interval are inconsequential but are recorded. While this parameter often shows relatively high

variability, it is also informative. With learning, most ITI touches should be emitted to the first window where the sequence starts, and can be interpreted as anticipatory touches awaiting the start of a new trial. Thus, a lower number of ITI touches in later stages of training can indicate a learning impairment (Fig. 3D).

Correct response latency

Latency between the occurrence of the cue and the animal's response to it should decrease with learning (Fig. 3E).

Reward collection latency

Latency between the last correct response and collection of the reward in the food magazine is usually very short and should be maintained at a similar level across all days unless the mouse has a motivation deficit (Fig. 3F).

Beam breaks

Touchscreen chambers are usually equipped with infrared beams allowing the monitoring of locomotor activity during sessions (Fig. 3K). It is useful to assess the level of general activity in the task, especially in mice with impaired performance. In addition, beam breaks in the food magazine can indicate premature visits to the magazine before the whole sequence is completed.

Grid-specific parameters

As the task is performed across multiple grids, most parameters can be evaluated in a grid-specific manner. In particular, correct response latency (Fig. 3G) and number of blank touches per grid (Fig. 3H) are commonly evaluated. The correct response latency usually decreases from the initial to the final positions, in line with the reward proximity (Fig. 3L). Both of these parameters should decrease as mice learn the task.

Performance on specific training days can be also evaluated and contrasted to others. For instance, the *spread of blank touches* can be evaluated on a specific training day (Fig. 3J). It can also be established if the mice have improved performance speed as training progresses (Fig. 3I).

Time Considerations

The heterogeneous sequence protocol is rapid to implement compared to more complex touchscreen-based paradigms. The introduction of food restriction and attainment of target weight in young animals usually takes less than 1 week. Reward habituation and initial training takes a minimum of 6 days if mice are reaching the pre-defined criteria. After that, all mice require 11 days to complete the heterogeneous sequence task. After completing these 11 days, additional sessions can be introduced to treat mice with pharmacological agents. For instance, it has been established that performance in the heterogeneous sequence task is influenced by dopamine D2 ligands (Keeler et al., 2014).

Regarding daily time demands, animal weighing and feeding may take up to 30 min for an experienced researcher. The behavioral session can last for up to 60 min, but many animals require considerably less time, being as fast as 15 min.

This protocol works most efficiently if at least four touchscreen chambers are available so that four mice can be run simultaneously. It is recommended that at least ten to fifteen mice be used for analysis, which would require ∼4 hr of testing per day for 4 weeks.

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Conflict of Interest

T.J.B. and L.M.S. have established a series of targeted cognitive tests for animals, administered via touchscreen within a custom environment known as the Bussey-Saksida touchscreen chamber. Cambridge Enterprise, the technology transfer office of the University of Cambridge, supported commercialization of the Bussey-Saksida chamber, culminating in a license to Campden Instruments. Any financial compensation received from commercialization of the technology is fully invested in further touchscreen development and/or maintenance.

Data Availability Statement

Data that support the findings of this study are available in MouseBytes (*www.mousebytes.ca*). All behavioral data for the figures can be found in the following repository: *https://mousebytes.ca/comp-edit?repolinkguid=1dc0a69a-2966-4e67-89af-4d37a82f5d48*.

Each excel file provides the raw data as well as calculated parameters. The touchscreen raw data can also be downloaded directly from the Data Lab in mousebytes.ca, where other non-investigated parameters can also be extracted and examined. There is one associated experimental dataset named OK_VAChTfxControls_HeteroSequence.

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