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The Peak Interval Procedure in Rodents: A Tool for Studying the Neurobiological Basis of Interval Timing and Its Alterations in Models of Human Disease

Fuat Balcı[1, *] and David Freestone[2]

[1]Koç University, Department of Psychology, Istanbul, Turkey; [2]William Paterson University, Department of Psychology, NJ, United States

*For correspondence: fBalcı@ku.edu.tr

[Abstract] Animals keep track of time intervals in the seconds to minutes range with, on average, high accuracy but substantial trial-to-trial variability. The ability to detect the statistical signatures of such timing behavior is an indispensable feature of a good and theoretically-tractable testing procedure. A widely used interval timing procedure is the peak interval (PI) procedure, where animals learn to anticipate rewards that become available after a fixed delay. After learning, they cluster their responses around that reward-availability time. The in-depth analysis of such timed anticipatory responses leads to the understanding of an internal timing mechanism, that is, the processing dynamics and systematic biases of the brain's clock. This protocol explains in detail how the PI procedure can be implemented in rodents, from training through testing to analysis. We showcase both trial-by-trial and trial-averaged analytical methods as a window into these internal processes. This protocol has the advantages of capturing timing behavior in its full-complexity in a fashion that allows for a theoretical treatment of the data.

Keywords: Interval Timing, Peak Interval Timing, Temporal Discrimination, Fixed-Interval Schedule, Behavior, Conditioning

[Background] Organisms perceive time as if it were a sensation. It follows the same psychophysical rules as other sensations. Yet there is no sensory system that transduces physical time into a neural code. Interval timing seems to be a cognitive ability that acts a bit like a stopwatch, but the neurobiological mechanisms are largely unknown (see Balcı and Simen, 2016). How animals time intervals is one of a handful of crucial contemporary research questions studied by many research groups across the world. Our main tool for investigating time perception has consistently and for decades been the core statistical features of timed animal behavior–statistical features shared across the animal kingdom (Malapani and Fairhust, 2002; Buhusi and Meck, 2005).

Standing tall among these statistical features, scalar variability suggests that, as the time interval increases, so too does the animal's variability in their representation of it. It's Weber's Law applied to time (Gibbon, 1977) explained by the fact that the standard deviation of an animal's timed responses scales linearly with their mean (Buhusi and Meck, 2005; Buhusi *et al.*, 2009). Historically, these sorts of psychophysical and statistical regularities formed the foundation of the search for and description of an internal clock (*e.g.*, Matell and Meck, 2004; Oprisan and Buhusi, 2014; Balcı and Simen, 2016).

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Consequently, the ability to detect these statistical signatures in humans and other animals is an indispensable feature of a good testing procedure for timing. Arguably, one of the most widely utilized interval timing procedures is the Peak Interval procedure that originated in animal research (Catania, 1970; Roberts, 1981) and then later adapted for human testing (Rakitin *et al.*, 1998). In the Peak Interval (PI) procedure, a reward, usually a food pellet, is available after a fixed delay after some event, usually a stimulus. To collect the reward, the animal must make an experimenter defined response. On some trials, the reward is never delivered, regardless of when the animal responds. In this way, the procedure is rooted in Skinner's classical fixed interval (FI) schedule of reinforcement (Ferster and Skinner, 1957), except with probe trials.

When the animal's response rate is averaged over many FI trials the response curve increases smoothly (like a shallow S) over the course of the trial. On peak trials there is also a smooth decrease in the response rate after the expected reinforcement fails to occur, as if two S's lined up back to back to resemble a bell-curve.

While the standard Fixed Interval task still plays a critical role in studying the timing mechanism, its major flaw is that it only conveys half the story. The single S-curve growing toward the target time conveys the animal's growing expectation for reward. But it cannot convey exactly when the animal expects the reward. In effect, reward delivery serves as a procedural censor; it contaminates the anticipatory responding guided solely by elapsed time. Peak interval trials show the animal's expectation function that grows before the target time and shrinks after.

These peak interval (probe) trials, randomly inter-mixed with the FI trials, last much longer than the FI trials (minimum 3*FI) and reward is never delivered. Over trials, the peak of the bell-shaped response curve reflects the animal's highest expectation, an index of accuracy. The width of the response curve measures response variability, which in part reflects the animal's internal representation of the interval, elapsed time, and probably many other factors. A subject with a faulty timing mechanism but intact associative memory (*i.e.*, CS-US association) would show a flat but high response rate throughout the trial (*e.g.*, see Figure 3-top panel of Meck, 2006).

The bell-curve response gradient results from averaging the animal's responses over single trials, but does not fully represent the pattern of responding in individual trials, a difference which may be theoretically meaningful. In individual PI trials, anticipatory responding seems to reflect two different internal states. When the trial starts, subjects respond infrequently, at a low rate, called a break period. Then, usually about two-thirds of the way to the expected reward, they abruptly begin responding at a high rate --a run of responses-- presumably in anticipation of the upcoming reward. On peak interval trials, when reward is withheld, the subject abruptly transitions back to a break some time after the reward availability time. The time the run starts is called the *start time* and the time it ends is called the *stop time*. These two events capture in its most granular form the moment the animal transitions to a different internal state, guided by its internal clock(s).

The average of these two times, the *middle time,* is a proxy for temporal accuracy on a single trial. The difference between the start and stop times is the spread, which serves as a proxy for temporal precision on a single trial. The start and stop times vary from one trial to the next and these two values

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are positively correlated (Gibbon and Church, 1990). The analysis of single trial responding and correlations between the within-trial events (*i.e.*, start time, stop time, middle time, spread) has been crucial toward understanding temporal information processing and its translation into timed behavior (Gibbon and Church, 1990).

Studying the PI procedure will be helpful in understanding the brain's clock based on the meaningful psychological parameters that can be estimated from the PI data. For instance, in Scalar Timing Theory (Gibbon *et al.*, 1984), animals have a clock, a memory, and a threshold that determines when elapsed time from the clock is close enough to the stored memory of the interval. How each of the three work, and the precise relationships among them, are written as mathematical equations that can be rigorously tested and revised through experiments. The end result may be a model that closely fits the data, and explains why changes in the input affect behavior. For example, a drug that abruptly adjusts the start time without altering the stop times suggests a change to the threshold because a change in the speed of the clock or what's stored in memory would affect both the start and stop times in a predictable way. Furthermore, based on the pattern of correlations between start times, stop times, middle times, and spread one can make inferences regarding the relative contributions of different sources of noise in the operation of the internal clock. For instance, the start and stop times would be positively correlated if the variability in timing behavior originates primarily from trial-to-trial variability in clock speed and/or memory representation whereas these two measures would be negatively correlated if the variability in timing behavior originates primarily from trial-to-trial variability in response thresholds. Such models, coupled with the phenotyping of disease models, could inform us about the neural and cognitive deficits that characterize different conditions associated with disrupted time perception.

Below, we summarize how the PI procedure can be used to study both the neurobiological basis of interval timing and the psychological implications for motivation and cognition. The studies summarized below are by no means exhaustive. Their aim is to demonstrate to readers the utility of the PI procedure in different contexts.

Lesion Studies

Lesion studies have primarily focused on the striatum, prefrontal cortex, hippocampus, and midbrain dopamine neurons. From these studies, Meck (2006) showed that lesioning the dorsal striatum and substantia nigra pars compacta led to flat, not bell-shaped, response distributions which were rescued by Levodopa after SNc, but not dorsal striatal lesions. Lesioning the nucleus accumbens did not significantly change the average response gradient. Interestingly, however, subjects with nucleus accumbens lesions were not sensitive to the reward rates (1/FI). This shows up as changes in the peak response rate, which is usually proportional to the reward rate.

Hippocampal lesions shift the peak earlier (see Meck *et al.*, 1984 and 2013; Balcı *et al.*, 2009b), which is sometimes attributed to forming, retrieving, or mediating temporal memories. Other studies showed that although dorsal hippocampal lesions caused earlier peak times (Yin and Meck, 2014; Tam *et al.*, 2015), ventral hippocampal lesions caused later ones (Yin and Meck, 2014). These peak shifts are only visible on average (there is no peak in a break-run-break pattern). On individual trials, shifts in the peak

time could show up as changes in the start time, the stop time, or both. In this case, the earlier shift caused by dorsal lesions seems primarily due to earlier start times; and the later shift seems primarily due to later stop times (Yin and Meck, 2014).

Finally, several studies investigated the effect of lesioning frontal cortex on PI performance. Olton (1989) and Olton *et al.* (1988) observed a later peak. More recently, Elcoro *et al.* (2014) showed that 6-Hydroxydopamine lesions of medial prefrontal cortex led to flatter response distributions, suggesting lower precision.

Pharmacological Studies

A number of studies have shown that acute administration of dopamine agonists such as methamphetamine and cocaine resulted in dose-dependent earlier peaks (Maricq *et al.*, 1981; Meck, 1983; Abner *et al.*, 2001; Çevik, 2003; Matell *et al.*, 2006) and the acute administration of dopamine antagonists such as haloperidol resulted in later peaks (Meck, 1983 and 1996; Drew *et al.*, 2003). Because these shifts are proportional to the Peak Interval, they are sometimes interpreted as mediating the speed of an internal stopwatch, speeding it up and slowing it down, respectively (Meck, 1983 and 1996). After chronic treatment with the same agents, these shifts normalize (looks more like baseline performance). Stopping the treatment shifts the peak time in the opposite direction compared to the normalized performance (*e.g.*, a rightward shift after the termination of chronic treatments with dopamine agonists; Meck, 1983 and 1996).

A number of studies have shown that, at the single trial level, these peak shifts were actually shifts in the start times; the stop times were not affected by dopamine (*e.g.*, Taylor *et al.*, 2007; for review see Balcı, 2014), which may suggest that dopamine changes decision thresholds (possibly by altering motivational state of the subjects) rather than the clock speed. Another set of studies has shown that the effects of dopamine agonists is only observed early in training (less than 20-30 sessions), yet the same dose leads to flatter response gradients after extensive training (more than 70-180 sessions - Balcı *et al.*, 2008; Cheng *et al.*, 2007).

Fewer studies have investigated the effect of noradrenergic, serotonergic, and acetylcholinergic drugs. In one of these studies, atomoxetine,a norepinephrine reuptake inhibitor, led to more precise (narrower) response gradients (Balcı *et al.*, 2008). 5-HT1A receptor agonist 8-OH-DPAT results in earlier peaks, which was abolished by an antagonist of the same receptor (WAY-100635), even though that same antagonist did not affect peak responding on its own (Asgari *et al.*, 2006). Scopolamine, a cholinergic antagonist was found to flatten the response gradient (Abner *et al.*, 2001; Balcı *et al.*, 2008), which was rescued by physostigmine, a cholinergic agonist (Abner *et al.*, 2001). When physostigmine given by itself, it reduced the trial-to-trial variability in stop times, but not start times (Abner *et al.*, 2001; Balcı *et al.*, 2008). Chlordiazepoxide, a GABAa receptor agonist, also disrupted temporal control over responding, too (Balcı *et al.*, 2008). This set of results shows that drugs can profoundly affect the timing mechanism in all sorts of ways, perhaps promising to help uncover the neurobiological underpinnings of time perception and timed behavior.

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Other Manipulations and Models

The response gradient also changes as a function of purely psychological factors. For instance, Roberts (1981) showed that feeding the animal right before testing (prefeeding) resulted in later peaks but this effect disappeared with further training. What held throughout training was that the response rate at its peak–the peak amplitude–decreased with prefeeding. In single trial analysis, motivational manipulations usually affect the start times without modulating stop times (even with prefeeding; see Figure 2b in Plowright *et al.*, 2000). Similarly, start times shift earlier when brain stimulation is used as the reward (Ludvig *et al.*, 2007), when different amounts of reward are used (Galtress *et al.*, 2012a and 2012b), and after satiation (Balcı *et al.*, 2010b).

The Peak Interval procedure has also been used to study aging, how age affects time perception and behavior (Lejeune *et al.*, 1998; Church *et al.*, 2014; Gür *et al.*, 2019a and 2020), and as a screening tool for genetically modified animals that model human central nervous system disorders. These studies provide scientists both with information constraining our search for the timing mechanism and with new information about the disorder (*e.g.*, Balcı *et al.*, 2009a and 2010; Cordes and Gallistel, 2008; Garces *et al.*, 2018; Ward *et al.*, 2009 and 2011; Gür *et al.*, 2019b and 2019c).

The peak interval procedure also has a number of limitations. For instance, peak responding tends to become habitual with extended training, which changes how different manipulations would affect the timing behavior (Cheng *et al.*, 2007) and the typical training protocol (as presented here) has many different steps and requires training the animal over many sessions. Thus, it is important that the data of individual animals are studied on a daily basis to detect steady-state performance soon after it is established. Another possible solution is to delay habit formation by training animals on a dual peak interval procedure, where one response option (*e.g.*, left lever) is associated with one interval and another response option (*e.g.*, right lever) is associated with another interval (Balcı *et al.*, 2010a). The active response option can be presented randomly in different trials or different blocks. This also allows the researchers to assess whether the scalar property is violated in their sample or a result of an experimental manipulation. The problem of sessions-long training is exacerbated by the fact that animals need to be food- or water-deprived for the test period, which requires paying extra attention to monitoring the weight and health of the animal and taking into consideration the developmental factors if the animals start being tested young. Depending on the total duration of testing, animals can be occasionally put on one- or two-day long adlib feeding. Furthermore, it might be difficult to estimate the timing performance parameters particularly with the single-trial analysis when the response rate is very low. This constitutes an analytical constraint when the experimental manipulation results in significant reductions in response rate or when the animal is not sufficiently motivated to respond. Often, trials that have less than a predetermined number of responses are excluded from the data analysis.

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Materials and Reagents

1. Subjects

   Inbred strains of mice or rats can be used as subjects to minimize the variation within the test group. Whatever the species or strain, experimenters should confirm that the animal can identify the discriminative stimulus. For instance, because older C57Bl/6 mice have age-related auditory impairments (Idrizbegovic *et al.*, 2003); use a visual one instead If they are used in the context of aging.
2. Reinforcement

   Either liquid reinforcement such as condensed milk (*e.g.*, Nestle Carnation® condensed milk; IsosourceStandard, Nestle Health Science), a sucrose solution, or dry pellets (*e.g.*, BioServ Dustless Precision Pellets F0023) can be used as reinforcement during testing. Reinforcements should be kept in cool storage. Condensed milk should be used on the day the can is opened – spoiled reinforcement is not reinforcing.
3. Animal Feed

   In the case of food restriction, subjects will usually require sufficient supplemental standard feeding after the test session (*e.g.*, Bio-Serv, Rodent Diet). An industry standard is to keep their weights around 85% of the ad libitum weight. Growth charts can be obtained from the vendor, or from an in-house pilot study. With water restriction, the subjects will usually require sufficient supplemental water, too.

Equipment

1. Operant Boxes

   The Peak interval (PI) procedure is implemented in operant boxes (*e.g.*, ENV-307W, Med Associates) with stainless steel grid floor (*e.g.*, ENV-307W-GFW, Med Associates). Figure 1 illustrates one possible configuration of the operant box with two levers (*e.g.*, ENV-312-2W, Med Associates), four receptacles that can be lit by LEDs for local stimuli (*e.g.*, ENV-302RW, Med Associates). The head-entries to the receptacles can be detected by infrared detectors (*e.g.*, ENV-313W, Med Associates). A speaker (ENV-324W, Med Associates) and a house light (*e.g.*, ENV-315W, Med Associates) that can be used to generate global discriminative stimulus. For reinforcement, dry pellets can be delivered via pellet dispensers (*e.g.*, ENV-203-20, Med Associates), liquid reinforcers can be delivered via dippers (*e.g.*, ENV-302W-S, Med Associates).

   Note: There are other manufacturers of modular operant boxes, which can also be used to run this protocol (e.g., Harvard Apparatus, Modular Operant/Behavior Box (Panlab); Lafayette Instruments; Mouse Modular Test Chamber, Model 80015NS).

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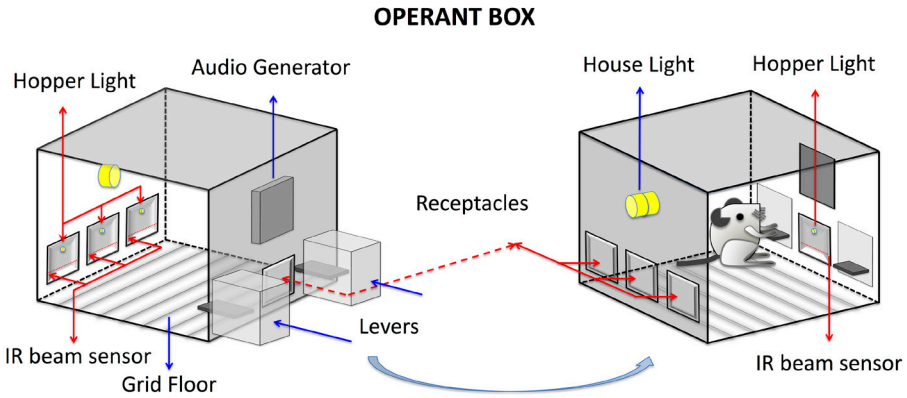

Figure 1. Illustration of a possible configuration of an operant box from two different angles

2. Sound Attenuating Cubicles

   Ideally, operant boxes should be housed in sound attenuating cubicles (*e.g.*, ENV-020M, Med Associates) to minimize the subject's distraction by outside sources during testing (*e.g.*, from other operant boxes, experimenter, *etc.*). Fans (*e.g.*, ENV-375-F, Med Associates) in each cubicle can also help. They ventilate the cubicle, and their constant whirl and humming helps muffle outside noise.
3. Housing Cages

   Animals can be kept in standard shoebox cages (*e.g.*, conventional polypropylene cages). If animals are individually housed, supplemental food and water can be given directly in the housing cage. If animals are group-housed, supplements can be given to the subjects prior to group-housing to prevent possible aggression between animals (although sometimes, animals will not fight).

Software

1. Operant boxes will be controlled either by commercially available software from the vendors of the operant boxes themselves (*e.g.*, MedPC for Med-Associates or GraphicState for Coulbourne) or custom-built generic control software (*e.g.*, Biopsychology Toolbox in Matlab Rose *et al.*, 2008).
2. Data Analysis can be easily conducted either with custom code or the freely available TSLib (for Matlab: https://github.com/CRGallistel/Contingency; for R: https://github.com/freestone-lab/TSLibrary), and can find guidance in Freestone and Balcı (2018, https://github.com/freestone-lab/timing_tasks.git).

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Procedure

A. Food or water deprivation of the subjects

1. Get the animal's ad libitum weight by weighing them over three subsequent days during which they have full access to chow and water. Use a growth chart from the animal's vendor to estimate its growth over the course of the experiment; this can be important if the animals are younger, and still growing. If one cannot be obtained, create one with a pilot group of animals. No prior handling of the animals is required as testing takes place in the operant box.

   Restriction Protocols:

   Water restriction

   Three days prior to the start of the first training session, water is restricted while maintaining the animal at 85% of its ad libitum weight. In mice, this is achieved by providing full access to an average of 4 ml water through a mash. Subjects will also be receiving liquid during testing. During water restriction, subjects have full access to food in the home cages. If the weight of an animal falls too quickly, or falls far below the 85% criterion, put the animal on ad libitum water for at least a day before starting a less restrictive water schedule for that animal.

   Food restriction

   Three days prior to the start of the first training session, chow is restricted to maintain the animal at 85% of its ad libitum weight. This is achieved by providing chow once a day about an hour after the testing session. Subjects will also receive food during testing. During food restriction, subjects have full access to water in the home cages. If subjects are young, their normal growth curves should be factored into the updating of ad lib weight. These can be obtained from the animal's vendor. If the weight of an animal falls too quickly, or falls far below the 85% criterion, put the animal on ad libitum food for at least a day before starting a less restrictive food schedule for that animal.

   It is often helpful to introduce the reinforcement before testing begins. Drop some of the reinforcement in their home cage a few days in a row before testing starts to minimize the effect of neophobia.

   Test the equipment before testing the animal

   Test each component of the operant box prior to bringing the animals in the test room. If there is any component that is malfunctioning, fix it or replace it prior to testing. Make sure that the pellet dispenser or the dipper (whichever is used) functions appropriately. It is recommended to have a number of additional units available in the lab to be able to replace the broken ones. This box test is repeated for each box every testing day. If pellet dispensers are used, make sure to collect the pellets dropped in the hopper during testing of the equipment before moving to testing

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the next subject. If there is a problem with one or more units that cannot be fixed or replaced on the same day, test the corresponding animal on the next day and record this event in the lab notebook.

The most straightforward way to test an operant chamber is to carefully design and write a test protocol using the software that controls the boxes (*e.g.*, MedPC). For example, write a protocol that turns on each output sequentially when some input is triggered. This way, one can distinguish faulty input from output.

B. Training Phase 1

2. Place the animal in the operant box and close the doors. Before closing the door, make sure the animal's tail is not near the latches to prevent injury to the animal. This step is repeated on each test day. At the beginning of testing, animals might be reluctant to get into the operant chamber when the cage is tilted toward the opening of the operant box. Carefully pick the animal up by its tail and place it in the operant chamber.

   Below, we provide two methods for training the animal on a Peak Interval procedure. The first is simpler and usually results in very clean data. The second is more involved, but more safely guards against those few animals who need more, or more careful, training.

   The goal of the simpler method is to start as close to the Peak Interval task as possible, minimizing both the number of training phases and the differences between phases.

   Training starts with Delay Conditioning: A long exponentially distributed intertrial interval begins the session. Longer intertrial intervals, relative to the trial duration, will lead to faster learning (Gibbon and Balsam, 1981; Ward *et al.*, 2013). The trial starts with the onset of a stimulus, usually a light or a tone. The light or tone lasts for the FI used during the eventual peak interval task, then it turns off and a reward is immediately delivered. If the eventual FI is very long (say, several minutes), it may be a good idea to start with a shorter delay and adjust toward the long FI over a few days. Wherever possible, it is better to start with the final FI.

   Then, transition to the Peak Interval task (Step B8 below). Some may want to start with a few days of FI training without peak trials, but it is not strictly necessary. The benefit of starting with a Delay conditioning procedure is twofold: first, it teaches the animal the true FI interval right away, rather than asking the animal to learn it through trial-and-error. Second it allows the researcher to measure anticipatory entries into the food cup as a measure of acquisition.

Fixed Time 60 s concurrent schedule

The goal of the less simple method is to take smaller and more incremental steps toward the final peak task. In doing so, it ensures that each animal has completed each step before moving to the next one. This is helpful in a few cases in which the animal is slower to learn, or in cases where the researcher is uncertain about how slow acquisition will be, for example, if working with an unfamiliar strain of mice.

Magazine training

3. Deliver reward in the corresponding hopper every 60 s (called an FT60 procedure). If the reward is delivered via a dipper, then the dipper stays up for 60 s and the cup is refilled and presented again to the animal. If the liquid reward is presented through peristaltic pump or if it is in pellet form, the reward can accumulate over trials until it is consumed by the animal. This magazine training will last for at least one 20-minute-long session. Note that 0.7 ml of sucrose water indicated in Gür *et al.*, 2019b; 2019c (that were used as the reference studies for this protocol) was the approximate total amount of water received during a Magazine Training session (it was mistakenly expressed as the amount delivered every 40 s).

   If reward is delivered via dipper, it should be presented long enough for the animal to notice and move to the dipper from the Lever (in subsequent the Lever Press Training, the animal will need to do this). If an animal does not spend time in the magazine where dipper is raised, the reinforcement presentation time can be extended. If there are infra-red beam detectors at the feeding hopper, the dipper can be kept available for a fixed duration after the detection of a nose poke.

   After the termination of the session, check for any pellets or liquid reinforcement accumulated in the feeding hopper and remove them (this is not an issue for reward deliveries via dipper). Then check how many times the animal has entered the feeding hopper.
4. At the end of the session, use a cumulative record to chart the animal's responses over the session, steeper slopes indicate higher response rates. It is often very clear if (and when) the animal acquired a response. After experience, the researcher can usually tell more quickly and easily by the total number of responses in the session (but a cumulative record is still important to catch inconsistencies in their behavior throughout the session). If needed, repeat the magazine training for that animal on the subsequent day.

Lever Press Training

5. The lever is inserted in the operant box and it is retracted either after 60 s or after it is pressed (detected by mechanical switches or break of infrared beam). When one of these events take place, the lever is retracted and the reward is delivered. If the reward is delivered via a dipper, then make sure to leave enough time for the animal to reach it (*e.g.*, 3 s) or rely on the head entry to the feeding hopper as described in Step B3. Stationary levers can also be used, in which case it will be available at all times. The lever press training continues typically for 60 min.

   A crucial point is that lever press training should continue until the animal is reliably pressing the lever. This may be done by examining a raster plot or cumulative record; it may be done by a statistical test, say, on the difference in the number of responses per trial against the number of responses in the intertrial interval; or it may be done with a simple criteria: continue until the animal presses the lever for at least a number of times (*e.g.*, 40 or 60), or obtains a certain amount of food, in an hour-long session for two subsequent sessions.

   Some subjects may take longer to train than others. There are a few simple techniques for

helping them along. First, remove the FT component, and introduce a Fixed Ratio schedule in which every lever press is reinforced (FR1). If this doesn't help, try shaping the lever press response by hand while the FR1 schedule is in effect. If this fails, it may be time to exclude the animal from the study; it may be an outlier. In this case, record the details in a laboratory notebook, and remember to report it in the paper. In our experience, subjects rarely need more than 2-3 sessions of FR training, and rarely need more than 2 sessions of shaping.

Nose poke training

6. The nose-poke hopper is lit and it is turned off either after 60 s or after the subject makes a nose-poke (detected by the break of infrared beam). When one of these events take place, the light is turned off and the reward is delivered. If the reward is delivered via a dipper, then make sure to leave enough time for the animal to reach it (*e.g.*, 3 s) or rely on the head entry to the feeding hopper as described in Step B3. The nosepoke training continues typically for 60 min. The nose-poke training continues until the animal nosepokes a number of times (*e.g.*, 40 or 60) in a session for two subsequent sessions. Either Step B5 or Step B6 will be realized based on the choice of the experimenter regarding the form of response.

Fixed Interval (FI) Training

7. A trial is initiated with the presentation of the discriminative stimulus. The stimulus stays for a minimum of the FI and it is turned off contingent upon the first response following FI. The first response following the FI is reinforced (some also require that the response occur earlier than 3xFI). The experimenter might choose to present the stimulus longer than 3xFI, but this typically not necessary. After each trial, an exponentially distributed inter-trial interval (ITI) is introduced. This is important to reduce how predictable the next trial is. This training phase, including only simple Fixed Interval trials, is usually conducted daily for at least 5 sessions before introducing peak trials. Use raster plots to track their acquisition. The data should be studied on a daily basis with simple visual or statistical summaries that showcase the most raw form of the data possible. If a scalloped responding is not observed in any of the subjects, FI training can be extended. Figure 2A illustrates the typical pattern of responding observed in FI trials.

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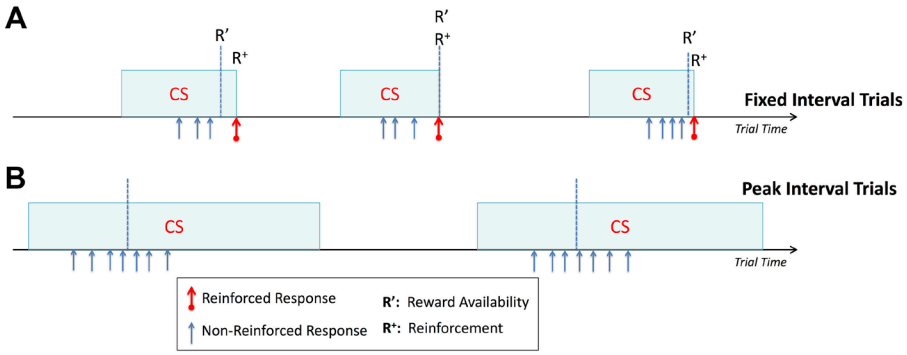

Figure 2. Illustration of fixed interval and peak interval trials and the typical form of responding that is observed in these two different trial types

Peak Interval (PI) Testing

8. Peak Interval (PI) trials are randomly mixed with FI trials. In PI trials, the stimulus lasts at least three times longer than the FI and the reinforcement is omitted, regardless of the animal's behavior. The proportion of FI to PI trials can change between 1:1 to 1:3. A lower proportion of peak trials will facilitate the acquisition of peak interval responding but because we typically focus on PI trials in analysis, this will also result in less data per session (Kaiser, 2008 and 2009). The duration of the PI trials can also be varied between trials or sessions (but at least 3xFI). Figure 2B illustrates the typical pattern of responding observed in PI trials.
9. After each session, gently remove the animal from the operant box and place it promptly either in the holding case or the home cage available nearby. Wipe the grid with a 70% ethanol solution to minimize olfactory cues between different sessions. Provide supplemental food or water about an hour after the test session.

Figure 3 summarizes the key steps of the PI procedure in the form of a decision-tree to be used during the implementation of the protocol.

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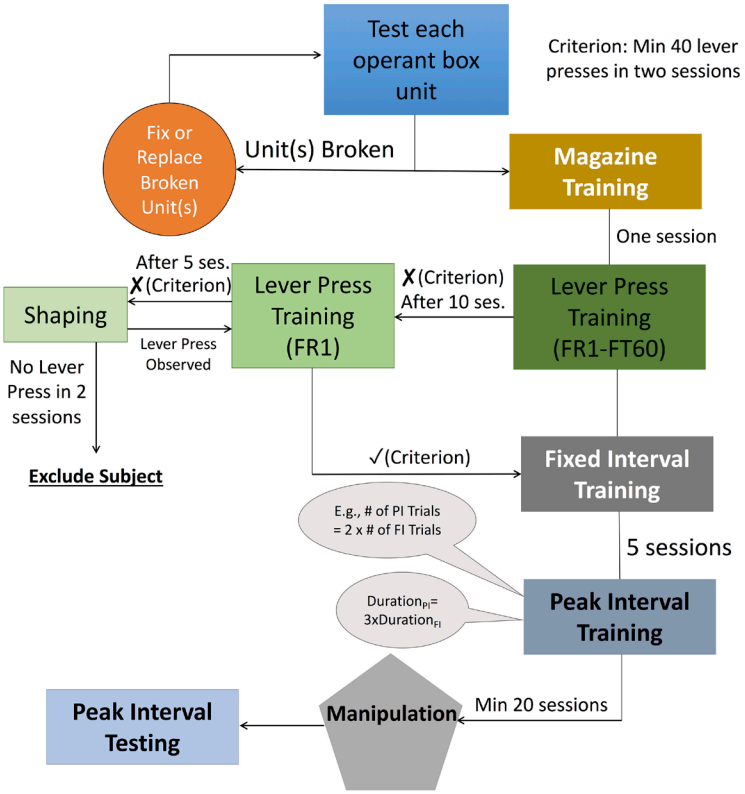

Figure 3. An example illustration of the peak interval testing steps and contingencies

Note: The criteria for switching between different states can be decided on by the experimenter. This figure will appear as an animation when viewed here through a web browser.

Data analysis

Analytical Approaches:

Analysis of the peak response curve

1. Peak response curves can be characterized by fitting unimodal distribution functions (such as Gaussian, Gamma, Inverse Gaussian, Weibull) to the averaged response curve. The choice of the function to be used to characterize the response curve can be based on measures of goodness of fit (*e.g.*, omega squared; log likelihood), or based on theoretical considerations (Guilhardi *et al.*, 2007). The mode of the function fit to the data can be used as an estimate of the peak time, whereas its coefficient of variation (CV) can be used as an estimate of temporal precision.

   The same estimates can be gathered using nonparametric techniques, that is, without fitting a function to the data. For example, one could choose to report the location of the amplitude of the response gradient as the peak time and the difference between the locations of a particular value prior and after the peak as the spread. Because the data is often a little noisy, one typically uses algorithms that smooth it out in order to find the peak or spread (*e.g.*, Roberts, 1981; Balcı *et al.*, 2009c). Some have averaged multiple points on the response gradient (*e.g.*, 60%, 70%,

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80% of the amplitude; Balcı *et al.*, 2008; Figure 1). Make sure that the smoothing algorithm does not introduce bias in the estimates. Figure 4B shows an example response gradient from one mouse (averages are shown in blue and smoothened data are shown in red).

Analysis of the individual-trial data

2. The primary aim of the individual trial analysis is to estimate the trial time at which animals shift from break to a run (start time) and the run back to a break (stop time). A number of approaches have been suggested in the literature. In this paper, we present one of the most widely used methods originally proposed by (Church *et al.*, 1994).

   This method segments the single trial data into three periods: The period before the run–the first break–should have lower-than-average response rates; the run should have higher-than average response rates; and the second break should again show lower-than-average response rates. Any other pattern of behavior does not follow the traditional break-run-break pattern, although a small proportion of various other patterns do occur over the course of an experiment. Let r be the overall response rate in that trial, r_1 be the response rate of the (potential) first segment, and t_1 be the duration of the first segment. The quantity $t_1(r - r_1)$ measures how low the response rate was during the first break relative to the average, scaled by how long the break lasted. Graphically, it's the area of a rectangle of width t_1 and height $(r - r_1)$. Similar computations will be applied to the run period $t_2(r_2 - r)$ and the second break period $t_3(r - r_3)$. Thus, maximizing t1(r – r1) + t2(r2 – r) + t3(r – r3) will generally provide a good estimate of the start and stop times. Figure 4A shows the raster plot of data from one mouse, where blue lines correspond to nose pokes, green circles show the start times and red circles show the stop (in terms of 1 s bins).

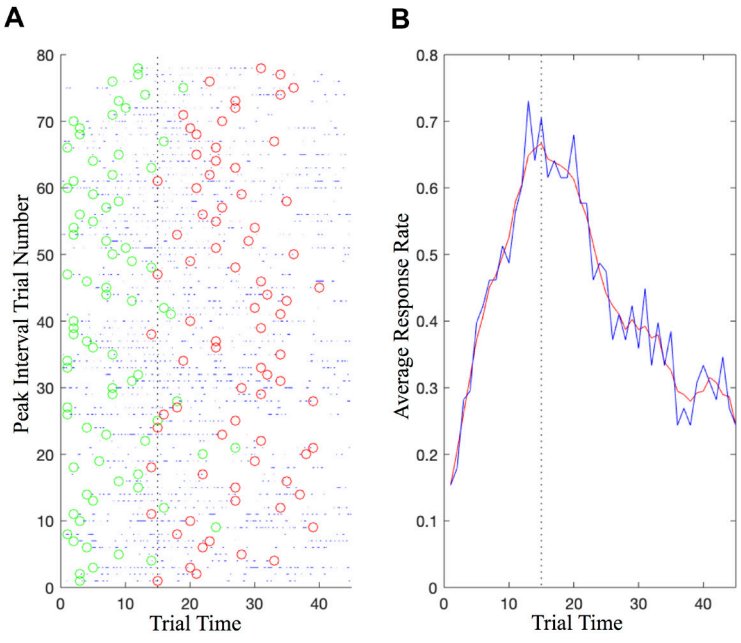

Figure 4. Example mouse data. A. Example raster plots (nose pokes) in peak interval trials

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(steady state) from one mouse at steady state (data pooled across 10 sessions). B. Example response gradient (blue) from the same mouse with the smoothened response gradient (red) superposed on the average response gradient. Dotted vertical lines show the reward availability time (FI = 15s). Data come from a pilot study conducted at Dalhousie University (unpublished) with a 2 month of Nrxn1$^{+/-}$ mouse on a B57BL/6J background. The actual experiment is ongoing. All procedures were approved by Dalhousie University Committee on Laboratory Animals (protocol #18-096).

Another approach uses Maximum likelihood methods to find the start and stop times. Here, the researcher makes parametric assumptions about the way the animals respond in each segment, for example, that the interresponse times are always exponentially distributed, but shorter during a run than a break. The start and stop times correspond to the response times that maximize the likelihood of the data assuming three segments split at those start and stop points (rather than split somewhere else or not at all) (*e.g.*, Balcı *et al.*, 2009c). The main advantage of this approach over non-parametric ones is that it uses likelihood ratios to detect changes. This way, one can remove the trials without a clearly defined break-run-break pattern. The researcher is free to choose what constitutes a clear break-run-break pattern by choosing a looser or more strict likelihood ratio. This criterion should be justified and reported in the paper. Lastly, Bayesian methods can be used in place of maximum likelihood estimates. This gives the full posterior distribution for the start and stop times, which generally provides more information than point estimates alone.

In order to avoid those trials in which animals did not show temporal control in their responding, and provided that they constitute only a fraction of the trials, researchers might choose to further exclude those trials in which the start time is longer than the FI or the stop time is shorter than the FI. It is important that this exclusion choice is made prior to data analysis. We find it useful to exclude trials where start-stop spread is smaller than 5% of the FI. The corresponding estimates gathered from single trial data and response curves have been shown to be correlated (*e.g.*, Balcı *et al.*, 2008), which should also be checked in studies that employ both methods of analysis.

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The protocol was derived from a number of previous studies (Balcı *et al.*, 2008 and 2009c; Gür *et al.*, 2019b and 2019c).

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Competing interests

The authors declare that they have no conflict of interest.

Ethics

All procedures were approved by the Institutional Animal Care and Use Committee at PsychoGenics, Inc and Dalhousie University Committee on Laboratory Animals (protocol #15-097 & protocol #18-096).

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