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Serotonin 1B receptor effects on response inhibition are independent of inhibitory learning

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Abstract

Impulsivity is frequently defined in terms of deficits in instrumental response inhibition, when the inability to withhold an action produces a negative outcome. There are many behavioral and cognitive constructs which theoretically could contribute to disordered impulsivity, including Pavlovian responding, which few studies have considered in this context. In the present set of studies, we examine Pavlovian inhibitory learning and excitatory responding in a mouse model for dysregulated impulsivity, specifically, mice lacking the serotonin 1B receptor (5-HT_{1B}R). Consistent with previous results, we show that these mice display increased impulsivity as measured by premature responding in the operant 5-choice serial reaction time test. In a Pavlovian conditioned inhibition paradigm, they also show a decreased ability to withhold responding, but importantly have an intact ability to learn inhibitory associations. In a Pavlovian appetitive conditioning experiment, 5-HT_{1B}R knockout mice show normal responding under a positive contingency schedule, however, they display increased responding to cues presented on an independent schedule from reinforcement in a zero contingency schedule. Interestingly this difference does not occur when the cues are explicitly unpaired in a negative contingency schedule, nor during a 25% reinforcement schedule. Overall, while our results show that the deficits in operant response inhibition in mice lacking 5-HT_{1B}R are likely not due to Pavlovian inhibitory or excitatory learning, it is relevant to consider associative learning in the context of dysregulated impulsive behavior.

Keywords

5-HT_{1B} receptor; impulsive action; instrumental conditioning; classical conditioning; conditioned inhibition; Pavlovian appetitive conditioning

1. Introduction

Impulsivity is a complex construct which is a major component of many psychiatric disorders, including attention deficit hyperactive disorder (ADHD), schizophrenia, substance

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use disorders, and gambling disorders (Dalley & Robbins, 2017; Mestre-Bach et al., 2020; Ouzir, 2013; Robbins et al., 2012). The diversity in the presentation of impulsivity across these disorders likely arises from its multiple, independent subcomponents. These different aspects of impulsivity have dissociable behavioral and biological underpinnings in humans and preclinical models (Dalley & Robbins, 2017; MacKillop et al., 2016; Nautiyal et al., 2017; Zeeb et al., 2013, 2016). One component of impulsivity is impulsive choice, which includes decreased tolerance for delays and risky decision making, as famously measured in the Marshmallow Test (Mischel et al., 1972). Another distinct category of impulsive behavior is impulsive action, which is characterized by deficits in response inhibition, including difficulty stopping, omitting, or delaying responding. Each of these components of impulsivity are also *themselves* complex phenotypes with a number of contributing factors, including components of learning and memory. For example, elevated impulsive action could be the result of deficits in learning inhibitory associations rather than the inability to inhibit an action. A better understanding of how differences in associative learning could support alterations in impulsive behavior may be helpful in delineating neural circuits which underlie pathological levels of impulsivity.

Limited research has considered how deficits in Pavlovian responding may contribute to standard assays of impulse control (Sosa & dos Santos 2018). Of particular interest for impulsive action is inhibitory learning, given that exhibiting inhibition first requires an understanding of the inhibitory association. Inhibitory learning is commonly assessed by Pavlovian conditioned inhibition, which develops when a cue predicts the absence of reinforcement that would otherwise be expected (Pavlov, 1927; Rescorla, 1969b). In clinical populations, lower conditioned inhibition is associated with schizotypy (Migo et al., 2006), and violent offenders with personality disorders (often characterized by high levels of impulsive behavior) have deficits in conditioned inhibition such that an inhibitory stimulus had little effect on decreasing excitatory responding (He et al., 2011). Additionally, normal adolescent development is associated with increased impulsivity and reward sensitivity (Casey & Jones, 2010; Somerville et al., 2011), and interestingly, adolescent rats take longer to discriminate between trial types in negative occasion setting, another form of inhibitory learning (Meyer & Bucci, 2014b, 2017b). However, a direct examination of conditioned inhibition in an animal model for increased impulsive action may clarify relevant underlying behavioral mechanisms of the disordered impulsivity.

Another aspect of classical conditioning which could be affected in subjects predisposed to impulsivity is excitatory responding, when cues predict reinforcement independent of action. Thus, there are conceivably several behavioral mechanisms which could affect appetitive responding in both operant tests of impulsivity and Pavlovian appetitive conditioning behavior. For example, increasing the subjective value of reward could enhance responding during the presentation of a predictive cue. Associative learning theories suggest that reinforcer magnitude influences the rate of learning and level of responding in classical conditioning (Rescorla & Wagner, 1972), and increased reward sensitivity is prevalent in populations with increased impulsivity (Dissabandara et al., 2014; Jonker et al., 2014; Kamarajan et al., 2015). Thus, changes in reward processing could support changes in both operant and classical conditioning in individuals with pathological levels of impulsivity.

A wealth of preclinical and clinical studies have identified varied neural mechanisms underlying impulsivity, and have more recently dissociated their contributions to different components of impulsivity. In the present study, we focus on the role of serotonin signaling, which has emerged as a candidate for the modulation of the impulsive action component, particularly. For example, global reductions of serotonin levels generally increase impulsive action (Winstanley et al., 2004; Worbe et al., 2014), and several serotonin receptors, including serotonin 1B, 2A and 2C regulate impulsive action (Fink et al., 2015; Higgins et al., 2017; Nautiyal et al., 2017). In particular, strong translational evidence points to the role of the serotonin 1B receptor (5-HT_{1B}R) in a number of phenotypes associated with increased impulsivity. For example, single nucleotide polymorphisms in the gene encoding this receptor are associated with cocaine, alcohol, and heroin abuse, and impulsive-aggressive behaviors (Cao et al. 2013; Contini et al. 2012; Zouk et al. 2007; Proudnikov et al. 2006). Additionally, mice lacking the 5-HT_{1B}R show increased impulsive action, increased cocaine self-administration, and deficits in response inhibition in instrumental tests of impulsive action (Nautiyal et al., 2015, 2017; Rocha et al., 1998).

The goal of the present experiments was to determine whether impulsive action modulated by serotonin may be subserved by deficits in classical conditioning. We examined Pavlovian inhibitory learning and excitatory responding for appetitive cues in mice with a global knockout of the 5-HT_{1B}R, which produces deficits in assays aimed at measuring impulsive action. First, we used the operant 5-choice serial reaction time test (5CSRTT) to show that mice lacking the 5-HT_{1B}R have increased impulsive action as measured by premature responding. Next, we examined changes in responding during classical conditioning for inhibitory associations in a Pavlovian conditioned inhibition test and excitatory associations in Pavlovian appetitive conditioning experiments, with various schedules of reinforcement. We find that, in addition to elevated impulsive action, mice lacking the 5-HT_{1B}R show deficits in inhibitory responding and changes in excitatory responding under certain conditions in tests of classical conditioning. Overall, this work demonstrates the importance of examining how differences in learning Pavlovian associations may be important for the interpretation of measured deficits in impulsivity.

2. Materials and Methods

2.1 Mice

Animals were bred in the vivarium at Dartmouth College and were weaned at postnatal day (PN) 21 into cages of 2–4 same sex littermates. All mice were maintained on a 12:12 light-dark cycle and on *ad libitum* chow and water until experimental operant behavioral testing began at 10–14 weeks. Groups of mice lacking expression of 5-HT_{1B}R and littermate genetic controls were generated by crossing the floxed tetO1B mouse model to a βActin-tTS mouse line (tetO1B+/+ females crossed to tetO1B+/+::βActin-tTS+ males), as previously reported (Nautiyal et al., 2015). All procedures were approved by the Dartmouth College Institutional Animal Care and Use Committee.

2.2 5-Choice Serial Reaction Time Test (5CSRTT)

2.2.1 Mouse Touchscreen Operant Chambers—Behavioral training and testing for the 5CSRTT was conducted in four identical mouse Bussey-Saksida touchscreen operant chambers (Lafayette Instruments Co., Lafayette, IN). Each apparatus consisted of a sound attenuating chamber with a fan for ventilation/ background noise reduction enclosing a trapezoidal operant area with black plastic walls, a perforated stainless-steel floor, and a clear plexiglass roof. A speaker and LED houselight were attached to the ceiling of the sound attenuation chamber, directly above the operant arena (the houselight was off unless otherwise specified). A touchscreen (30.7 cm, 800 × 600 resolution) located at the front of the arena was covered by a black plastic mask with 5 square openings (4 × 4 cm each, spaced 1 cm apart, 1.5 cm above floor; '5 choice' mask; Lafayette Instruments Co., Lafayette, IN) to define response areas and reduce accidental background touches. A feeder with an LED light was located at the back end of the chamber, with undiluted evaporated milk (Nestle Carnation) reward delivered by a liquid pump. Infrared beams were positioned at the front and back of the chamber, as well as in the feeder. Stock behavioral programs (5-Choice Serial Reaction Time Task for Mouse Touch Screen Systems and ABET II) were executed by the ABET II software (Lafayette Instruments Co., Lafayette, IN) and Whisker Server (Cardinal & Aitken, 2010).

2.2.2 Initial Touchscreen Chamber Training—5CSRTT training and testing were run 5 days a week, with procedures modified from Fletcher et al. 2013. Mice lacking 5-HT_{1B}R expression (males=4, females=5) and genetic controls (males=3, females=4) were maintained at approximately 90% of their free-feeding weight, with *ad lib* water provided throughout the experiment. For initial touch training (5-choice Mouse Initial Touch Training), a 4 × 4 cm white stimulus appeared for 30s randomly at one of the 5 response windows on the touchscreen. At stimulus offset, the LED in the feeder turned on and reward was delivered (280 ms pump time, 7 μl). The light turned off after reward retrieval and the next stimulus was presented. If the mouse touched the correct stimulus window during the 30s presentation, 3x reward (840 ms pump time, 21 μl) was delivered in the lit feeder. After all mice reached a criterion of 30 trials in 30 minutes (2 days), they moved on to must touch training (5-choice Mouse Must Touch Training). In these sessions, the stimulus was presented randomly at one of the 5 response windows, and remained present until the mouse responded in the correct window. Reward (840 ms pump time, 21 μl) was delivered in the lit feeder, and the LED turned off after retrieval. After an ITI of 5s, the next stimulus was presented. After all mice reach a criterion of 20 trials in 30 minutes (2 days), they moved on to 5CSRTT training.

2.2.3 Training to Baseline for 5CSRTT—For training in the 5CSRTT (5-choice Mouse Touch basic), each session began with a priming reward delivery (840 ms pump time, 21 μl) delivered in a lit feeder. Following reward retrieval, the LED turned off and a 5s ITI began. Then, the white light stimulus was randomly presented at one of the 5 touchscreen windows. The stimulus durations during 5CSRTT training were 32s (4 days), 16s (2 days), 8s (2 days), 4s (2 days), 2s (2 days), 1.8s (2 days), 1.6s (2 days), 1.4s (2 days), 1.2s (3 days), and 1s (3 days; baseline stimulus). The stimulus duration was reduced over training when all mice achieved at least >65% accuracy (correct trials/(correct + incorrect

trials)) and <40% omissions (missed trials/presented trials). A nosepoke response to the correct stimulus window within the time of presentation plus a 5s limited hold after the stimulus presentation ended resulted in immediate reward delivery in the feeder (as well as the removal of the stimulus if it was still present). Reward retrieval triggered the start of the ITI. Nose poke responses to any window during the ITI were considered premature responses, the houselight turned on and there was a 5s timeout. After the timeout was over, a response in the feeder initiated the next trial's ITI. If a mouse responded in the incorrect window, or if no responses were made during the stimulus presentation and limited hold, a 5s timeout was initiated (see Fig 1A for trial structure). The session ended after 100 trials or 30 min. Data for 5CSRTT training were averaged for each stimulus duration, with days following a break in training (i.e. following a weekend) excluded.

2.2.4 5CSRTT Tests: Long ITI and Short Variable Stimulus Test—

The same cohort of mice described in 5CSRTT then were used in two manipulations of the 5CSRTT paradigm. Mice were trained with a 1s stimulus for at least 3 days before each test session. For the Long ITI manipulation, the procedure was the same as the baseline 5CSRTT training except the ITI was extended to 9s. For the Short Variable Stimulus manipulation (5-choice Mouse Var2) the procedure was the same as the baseline 5CSRTT training except the stimulus duration for each trial was shortened to variable 0.8s, 0.6s, 0.4s, or 0.2s durations. Data for each test was analyzed as a difference from the previous day's baseline performance and averaged over 2 separate test sessions.

2.3 Behavioral Apparatus and Initial Training for Classical Conditioning Tests

Classical conditioning studies (Conditioned Inhibition, Pavlovian Appetitive Conditioning, and 100% versus 25% Reinforcement experiments) were conducted in eight identical operant chambers individually enclosed in ventilated, sound attenuating isolation boxes (Med Associates, St. Albans, VT). Each operant chamber consisted of stainless-steel modular walls, stainless-steel bar floors, and a noseport receptacle for the delivery of liquid reward by a dipper (undiluted evaporated milk; 0.02ml cup volume). Head entry into the reward port was detected by an infrared beam break. The chamber also contained two stainless steel levers placed 2.2 cm above the chamber floor on either side of the reward port, though these were not used in the present study. A houselight and speaker were located on the upper portion of the wall opposite the reward port. A computer equipped with MED-PC IV (Med Associates Inc., St Albans, VT) computer software delivered stimuli and collected behavioral data. Training and testing were run 5–7 days a week, and mice were maintained at approximately 90% of their free-feeding weight, with ad lib water was provided throughout the experiment. Before classical conditioning testing, all mice were trained to retrieve an evaporated milk reward through head entry into the reward port.

2.4 Conditioned Inhibition

Mice lacking 5-HT_{1B}R expression and genetic controls were split into experimental (5-HT_{1B}R KO: males=3, females=9; genetic control: males=5, females=8) and procedural control (5-HT_{1B}R KO: males=3, females=8; genetic control: males=5, females=8) conditions for a conditioned inhibition experiment, with procedures modified from Bonardi

et al. 2010. Each session for this experiment was about 70 min long, and the houselight was off unless otherwise specified.

2.4.1 Preexposure—All mice were preexposed to the inhibitor stimulus (X; houselight), the experimental excitor inhibitor compound stimulus (AX; 75 dB 10 Hz click and houselight), and the control excitor inhibitor compound stimulus (BX; 75 dB white noise and houselight) in the absence of reward over 2 sessions. Each session had 10 trials of each trial type presented for 20s, randomly ordered with an average 120s variable ITI (range 69.8–206s).

2.4.2 Excitor Training—To increase excitor trial responding, all mice were trained with the experimental excitor stimulus (A; 75 dB 10 Hz click) and the control excitor stimulus (B; 75 dB white noise) with 5s reward delivery at offset over 4 sessions. Each session had 15 trials of each trial type presented for 20s, randomly ordered with an average 120s variable ITI (range 69.8–206s). Note that stimuli were not counterbalanced between mice, but both excitor cues were of the same sensory modality.

2.4.3 Conditioned Inhibition Training—Next, mice were trained for 15 sessions on conditioned inhibition. All mice were presented with rewarded trials for both excitor cues (A+, B+). Mice in the experimental condition also had inhibitor trials with an excitor-inhibitor compound stimulus (AX-). A Pavlovian differential conditioning procedure was used as a conservative procedural control condition where the excitor trials were interspersed with inhibitor trials with no reward (X-) (see Fig 2A, Conditioned Inhibition Training). Each session had 10 trials of each the 3 trial types presented for 20s, randomly ordered with an average 120s variable ITI (range 69.8–206s). Data was recorded as the total duration of responding in reward port during the cue presentation minus duration of responding during the immediately preceding 20s of ITI (elevation score; there were no significant differences between groups during this pre-trial period). Results for the B+ trials are not shown, as they are used as a control only for the summation test and were not statistically different from A+ trial results in the procedural controls.

2.4.4 Summation Test—Following 15 sessions of conditioned inhibition training, behavior was assessed in summation tests over 2 sessions. These tests were completed in the absence of reward delivery. Each session had 15 trials each of 20s presentations of the control excitor (B) and the control excitor inhibitor compound (BX), with an average 120s variable ITI (range 69.8–206s; see Fig 2A, Summation). Data from the summation test was averaged over the 2 sessions and recorded as a suppression ratio: duration of response during BX/(B+BX) responding. A suppression ratio of 0.5 would indicate equal responding to B and BX, whereas 0 would indicate no responding to BX.

2.4.5 Retardation of Acquisition Test—Finally, mice were tested for retardation of acquisition over 4 sessions. Each session had 30 trials which consisted of a 20s presentation of the inhibitor cue immediately followed by a 5s reward presentation (X+; houselight) (see Fig 2A, Retardation). The ITI was a variable average 120s (range 69.8–206s). Data was recorded as the total duration of responding in reward port during the cue presentation minus

duration of responding during the immediately preceding 20s of ITI (elevation score; there were no significant differences between groups during this pre-trial period).

2.5 Pavlovian Appetitive Conditioning

Separate groups of mice were tested in positive contingency (5-HT_{1B}R KO: males=2, females=6; genetic control: males=4, females=5), zero contingency (5-HT_{1B}R KO: males=6, females=9; genetic control: males=11, females=5), and negative contingency (5-HT_{1B}R KO: males=4, females=5; genetic control: males=5, females=5) conditions for a Pavlovian appetitive conditioning experiment over 9 sessions, with procedures modified from Ward et al. 2012. The houselight was on for the duration of each session. For mice in the positive contingency condition, each trial consisted of an 8s conditioned stimulus (CS; 75 db white noise) followed by a 5s reward delivery, with an average 80s variable ITI (range 4.4–201s). For the negative contingency condition, mice had the 8s CS and the 5s reward delivered on separate schedules each with an average 80s variable ITI (range 4.4–201s), such that the CS-CS and US-US intervals were completely independent of one another. For mice in the negative contingency condition, each trial consisted of a randomly selected 8s CS or 5s reward presented after an average 40s variable ITI (range 2.2–100.5s). For every condition, there were 40 CS presentations and 40 reward presentations, with each session lasting around 62 minutes. Data was recorded as the duration of responding in the reward port over the duration of the cue minus duration of responding over the immediately 8s of ITI (elevation score; there were no significant differences between groups during this pre-trial period).

2.6 100% versus 25% Reinforcement

Naive mice were tested either in 100% reinforcement (5-HT_{1B}R KO: males=3, females=5; genetic control: males=4, females=4) or 25% reinforcement (5-HT_{1B}R KO: males=4, females=4; genetic control: males=4, females=4) conditions over 9 sessions. The houselight was on for the duration of each session. For the mice in the 100% reinforcement condition, each of 20 trials consisted of an 8s CS (75 db white noise) followed by a 5s reward delivery, with an average 180s variable ITI (inclusive of cue; range 177.8–182.8s). For the 25% reinforcement condition, mice had the 8s CS presented with an average 45s variable ITI (inclusive of cue; range 42.8–46.8s). 80 total CS presentations occurred, with one out of every 4 CS presentations randomly reinforced at offset by a 5s reward delivery. For both conditions, there were 20 rewards in total delivered in each session, with each session lasting around 64 minutes. Data was recorded as duration of responding in the reward port minus duration of responding over the immediately 8s of ITI (elevation score; there were no significant differences between groups during this pre-trial period).

2.7 Statistical Analysis

Data was analyzed using the car and ez packages in the R statistical software (Fox et al., 2016; Lawrence, 2016; R Core Team, 2019). For the 5CSRTT, premature responses, omission rate, and accuracy rate were analyzed with a two-way mixed ANOVA for training (10 stimulus durations × 2 genotypes) and with two-tailed, unpaired ttests comparing genotypes for the tests. For conditioned inhibition training, response duration was analyzed with a four-way mixed ANOVA (15 sessions × 2 experimental conditions × 2 trial types

× 2 genotypes). Because we expected genotype differences to present in responding during inhibitor trials (X- for control, AX- for experimental group), a three-way mixed ANOVA was also performed as a planned comparison of just the inhibitor trials during training (15 sessions × 2 conditioned inhibition conditions × 2 genotypes). The summation test suppression ratios were analyzed with a two-way independent measures ANOVA (2 experimental conditions × 2 genotypes) while retardation response duration was analyzed with a three-way mixed ANOVA (4 sessions × 2 experimental conditions × 2 genotypes). Two-way mixed ANOVAs were also used for response duration elevation score for each of the three Pavlovian Appetitive Conditioning experiment contingency conditions (9 sessions × 2 genotypes). Response duration across cue presentation was also analyzed in two-way mixed ANOVAs with data collapsed across session for each of the three contingency conditions (8 seconds × 2 genotypes). A three-way mixed ANOVA was used to analyze response duration elevation score in the 25% versus 100% Reinforcement experiment (9 sessions × 2 experimental conditions × 2 genotypes). Response duration across cue presentation was also analyzed for this experiment in a three-way mixed with data collapsed across session (8 seconds × 2 experimental conditions × 2 genotypes). All data was first analyzed with sex included as a factor, but there were no significant fully powered effects found so data was collapsed across sex for all reported statistics.

3. Results

Mice lacking the 5-HT_{1B}R are more impulsive than controls. Specifically, in the 5CSRTT measuring impulsive action, they showed increased premature responding during the training sessions (Fig 1B; $F_{1,14}$=5.79, p=0.031 for main effect of genotype). However, this effect decreased over training, ($F_{9,126}$=31.39, p<0.001 for main effect of stimulus length; $F_{9,126}$=3.03, p=0.003 for interaction), as all mice improved task performance by reducing premature responses. Additionally, omission rate for all mice increased as the stimulus length decreased and the task became more difficult, however, mice lacking the 5-HT_{1B}R omitted less than controls toward the end of training (Fig 1C; $F_{9,126}$=36.35, p<0.001; $F_{9,126}$=2.72, p=0.006 for interaction; $F_{1,14}$=1.77, p=0.204 for main effect of genotype). Finally, accuracy rate increased overall across training, (Fig 1D; $F_{9,126}$=18.29, p<0.001), and importantly there were no observed genotype differences ($F_{1,14}$=0.39, p=0.545 for main effect; $F_{9,126}$=1.58, p=0.128 for interaction), which is commonly interpreted to rule out attention deficits (T. W. Robbins, 2002; Turner et al., 2016). These results suggest that mice lacking 5-HT_{1B}R show increased impulsivity which can be ameliorated to control levels with extended training.

We next performed two tests to determine if the differences in premature responding would reemerge in different manipulations of the 5CSRTT. First, we extended the ITI from 5s to 9s to make the waiting period longer, and therefore more difficult for mice to withhold responding. Under these conditions, 5-HT_{1B}R knockout mice had higher premature responding compared to control mice (Fig 1E; $t_{13.9}$=−2.63, p=0.020), indicating that they were less able to withhold responding under the pressure of a longer wait period. They also had fewer omission trials (Fig 1F; $t_{14.0}$=2.58, p=0.022), as seen during the training sessions, but had similar accuracy (Fig 1G; $t_{7.3}$=0.53, p=0.609). Next, we varied the stimulus duration to make it shorter and unpredictable, increasing the attention requirement by requiring faster

responding. Importantly, there were no genotype differences in accuracy (Fig 1J; $t_{8.1}$=0.20, p=0.849). Consistent with the behavior in the extended ITI test, mice lacking the 5-HT_{1B}R had increased premature responses compared to controls (Fig 1H; $t_{9.8}$=−2.96, p=0.015), with decreased omission rate (Fig 1I; $t_{9.9}$=2.45, p=0.035). These data suggest that mice susceptible to impulsive action show increased premature responding when pressured to respond quickly in response to an increased demand on attentional resources.

To assess whether the increases in impulsive action seen in these mice could be influenced by inability to learn inhibitory associations and/or general increased activity in response to excitatory cues, we used appetitive classical conditioning experiments. First, we used a Pavlovian conditioned inhibition paradigm to determine if mice lacking 5-HT_{1B}Rs show differences in inhibitory learning or in responding to inhibitory cues. We found that they showed deficits in response inhibition, rather than inhibitory learning during training for conditioned inhibition. A planned comparison of inhibitor trials (X- for procedural control, AX- for experimental group; Fig 2B) revealed significant main effects of genotype ($F_{1,45}$=4.32, p=0.043) and condition ($F_{1,45}$=24.43, p<0.001), and a trend toward a genotype by condition interaction ($F_{1,45}$=3.21, p=0.080). Since the conditioned inhibition procedural control can sometimes generate inhibition due to the negative contingency between the X-cue and reward (Rescorla, 1969b, 1969a), the main effect of genotype suggests that mice lacking the 5-HT_{1B}R have deficits in response inhibition, in both the procedural control and experimental conditions. Overall during training, all mice increased responding in the goal location during excitor (A+) trials and decreased responding during nonrewarded (X-, AX-) trials (Fig 2B,C, data shown averaged over days, note different scales; $F_{1,45}$=159.97, p<0.001 for main effect of trial type; $F_{14,630}$=11.39, p<0.001 for main effect of day; $F_{14,630}$=12.21, p<0.001 for trial type × day interaction). This indicates that all mice were able to discriminate between trial types, with mice in the experimental condition able to learn that the conditioned inhibitor indicated the reward was not coming. There was also a significant interaction between experimental condition and trial type such that mice in the experimental group had increased responding for the excitor-inhibitor (AX-) compound compared to the procedural control group's response to the inhibitor (X-) cue alone, suggesting that they had some remaining excitation to the excitor (A) cue despite the pairing ($F_{1,45}$=6.88, p=0.012). All other effects were nonsignificant (ps>0.05).

Increased responding to inhibitory compounds during training (i.e. Fig 2B) could reflect either a deficit in learning of inhibitory associations or in the expression of that learning. Given that mice lacking 5-HT_{1B}R had reduced response inhibition to the inhibitor trials, we performed two tests to directly assess their learning of the inhibitory association. In a summation test, we examined whether the inhibitor (X) could transfer to another excitor cue (B), that was not previously presented in conjunction with the inhibitor during training. Interestingly, despite the genotype differences in inhibitor responding during training, we found no significant effects of genotype on suppression ratio during summation (Fig 2D; $F_{1,45}$=0.39, p=0.533 for main effect; $F_{1,45}$=0.48, p=0.491 for interaction). Mice in the experimental condition had lower responding to the excitor-inhibitor (BX) compound, though this effect did not reach statistical significance ($F_{1,45}$=4.01, p=0.051), potentially due to a reduced suppression ratio in the procedural controls due to an acquired latent inhibition to X- during training. Finally, in a retardation of acquisition test, we tested for differences in

responding when the previously inhibitory cue was then rewarded, making it positively associated with reward (X+). There were no differences in learning rate between the conditions (Fig 2E; $F_{3,135}$=1.30, p=0.276 for session × condition interaction), however, mice in the conditioned inhibition experimental condition had overall lower responding indicating that the previously inhibitory cue acquired less excitatory meaning, suggesting successful conditioned inhibition learning ($F_{1,45}$=11.64, p=0.001). All mice increased responding to the previously inhibitory cue (X+) over retardation sessions ($F_{3,135}$=19.76, p<0.001), and, as in the summation test, there were no significant main effects or interactions with genotype (all ps>0.05). The collective data from this conditioned inhibition experiment indicate that despite decreased response inhibition during training, 5-HT_{1B}R knockout mice display evidence of learned inhibitory associative relationships as demonstrated by normal performance in the summation and retardation of acquisition tests.

Given that the deficits in response inhibition are unlikely due to deficits in inhibitory learning, we next examined the role of the 5-HT_{1B}R in modulating responding to appetitive cues. We hypothesized that increased cue reactivity could contribute to the increased impulsive action in the 5CSRTT as well as the deficits in withholding responding shown in the conditioned inhibition test. Even though we did not see genotype effects in the excitor trials in conditioned inhibition, we reasoned that this could have been due to a ceiling effect or an inability to capture the subtleties of initial approach behavior. Therefore, we conducted an experiment to explore differences in Pavlovian appetitive conditioning by measuring responding to cue presentation. First, in a positive contingency condition, we examined responding at the reward receptacle over the duration of a cue that always predicted reward at offset (Fig 3A inset). We found that all mice tended to increase responding to the paired cue over training days (Fig 3A; $F_{8,120}$=15.72, p<0.001), with no genotype difference ($F_{1,15}$=0.39, p=0.542 for main effect; $F_{8,120}$=0.50, p=0.857 for interaction). When we examined responding in a second by second analysis across the duration of the cue, as expected mice increased responding as reward approaches toward offset (Fig 3B; $F_{7,105}$=121.10, p<0.001 for main effect of second in cue), again, with no group differences ($F_{1,15}$=0.39, p=0.542 for main effect of genotype; $F_{7,105}$=0.09, p=0.999 for interaction). Next, in a zero contingency condition, with the cue and the reward on independent interval schedules (Fig 3C inset), mice decreased responding to cue over training (Fig 3C; $F_{8,232}$=7.88, p<0.001). Mice lacking the 5-HT_{1B}R, however, showed increased responding compared to controls, which was maintained across days of training ($F_{1,29}$=4.66, p=0.039 for main effect; $F_{8,232}$=1.05, p=0.396 for interaction). Analysis across the duration of the cue revealed that the increased responding occurs at cue onset (Fig 3D; $F_{7,203}$=48.01, p<0.001 for main effect of second in cue; $F_{1,29}$=4.66, p=0.039 for main effect of genotype; $F_{7,203}$=3.81, p<0.001 for interaction). While this supports the idea that mice lacking 5-HT_{1B}R are more reactive to cues alone, it is also possible that the increased responding in 5-HT_{1B}R knockout mice in the zero contingency condition was due to the low probability of the cue and the reward overlapping or happening in sequence. To address this possibility, we next presented the cue and reward as explicitly unpaired in a negative contingency design, such that there was always an ITI between any cue or reward presentation (Fig 3E inset). Again, cue responding decreased over days (Fig 3E; $F_{8,136}$=14.31, p<0.001), but there were no differences between the two genotype groups

($F_{1,17}$=0.01, p=0.912 for main effect; $F_{8,136}$=0.92, p=0.505 for interaction). Analyzed across seconds in the cue, we found a main effect of second and interaction such that there was still a small effect of genotype at cue onset (Fig 3F; $F_{7,119}$=12.09, p<0.001 for main effect of second in cue; $F_{7,119}$=2.13, p=0.045 for interaction), but no overall main effect of genotype ($F_{1,17}$=0.01, p=0.912). Given the diminished effect of 5-HT_{1B}R on responding to cues when they were always separated from reward, it is possible that the increased responding under a zero contingency schedule is instead due to differential responding for weakly predictive pairings, where there is a low probability of the cue predicting the outcome.

Therefore, we lastly tested whether the genotype difference in cue reactivity in the Pavlovian appetitive conditioning experiment was due to differences in responding to a weakly predictive cue by systematically controlling the probability of the cue predicting reward. We reduced the predictive strength of the cue by making the probability of the cue predicting the reward 25%, and compared this to a control 100% reinforced condition (Fig 4A insets). Overall responding during the cue increased over training in both conditions, but was lower in the 25% compared to the 100% reinforced condition as expected (Fig 4A; $F_{8,224}$=22.99, p<0.001 for main effect of session; $F_{1,28}$=4.94, p=0.035 for main effect of condition), with no significant main effects of genotype or other interaction (all ps>0.05). When analyzed over the seconds of the cue, we similarly found that mice in both reinforcement conditions increase responding toward the end of the cue (Fig 4B; $F_{7,196}$=145.83, p<0.001 for main effect of second in cue), but the rate of this elevation differed such that mice in the 100% condition reach maximum responding earlier in the cue than those in the 25% condition ($F_{1,28}$=4.94, p=0.035 for main effect of condition; $F_{7,196}$=2.93, p=0.006 for second × condition interaction). Again, there were no main effects or interactions with genotype (all ps>0.05). This suggests that the difference between genotypes in the independent interval Pavlovian appetitive conditioning condition was not due to altered responding for fully or partially predictive cues.

4. Discussion

Overall, our data suggest a role for the 5-HT_{1B}R in operant responding in tests of impulsivity as well as responding in classical conditioning paradigms. Specifically, mice lacking the 5-HT_{1B}R demonstrate elevated impulsive action in the 5CSRTT, as measured by premature responding, which is consistent with our previous studies of impulsive action in this model (Nautiyal et al., 2017). Additionally, these mice do not show deficits in performance accuracy in the 5CSRTT, which is often interpreted as normal attention (T. W. Robbins, 2002; Turner et al., 2016). Inattention and impulsivity are key characteristics of ADHD (Nigg, 2016), and it is possible that in some cases, impulsive action may arise through a decreased ability to attend properly to cues and respond at the correct time. Our data suggests that this is not the case in these mice, however, it is still possible that attentional changes such as sensitivity for cue detection could contribute to these results. Next, we used a Pavlovian conditioned inhibition test to show that all mice were able to learn inhibitory associations, and during training discriminated between excitatory versus inhibitory trials. However, mice lacking the 5-HT_{1B}R show increased responding to inhibitor trials during training compared to controls, suggesting a deficit in response inhibition consistent with the impulsive action phenotype. Interestingly, this difference in

responding was not seen in the summation test when the control excitor-inhibitor (BX-) compound was presented in extinction conditions with no rewards presented during the session. This is consistent with our prior findings that 5-HT_{1B}R knockout mice have increased instrumental responding in tests of motivation, but show normal extinction of responding in the absence of reward, which is also dependent on intact inhibitory learning (Bouton et al., 2021; Desrochers et al., 2021). Therefore, we suggest that the increased responding during conditioned inhibition training is reflective of differences in responding when there is the potential for rewarded trials.

Beyond conditioned inhibition, negative occasion setting could also be an important inhibitory classical conditioning consideration in the context of impulsivity. The trial structure of this procedure may more closely mimic the 5CSRTT given that the inhibitory cue precedes the normally excitatory cue rather than being simultaneously presented, as in conditioned inhibition. The negative occasion setting procedure has been used in preclinical work, including in adolescent rats (a developmental period characterized by increased impulsivity; Meyer & Bucci, 2014, 2017a, 2017b), adult rats with decreased prefrontal cortex activity and increased nucleus accumbens activity to mimic the imbalance present during adolescence (Meyer & Bucci, 2016), as well as spontaneously hypertensive rat model for ADHD (Bucci et al., 2008). Conditioned inhibition, on the other hand, is more similar to the Go/No-go test of impulsive action in which mice withhold responding during no-go cues presented simultaneously with a lever operand (in the absence of the no-go cue, presses the lever gives reward). Interestingly we have also previously reported deficits in the Go/No-go task in mice lacking the 5-HT_{1B}R (Nautiyal et al., 2017). It could be useful to study multiple different kinds of inhibition in the same preclinical model for impulsivity, as negative occasion setting and conditioned inhibition can be biologically dissociated (MacLeod & Bucci, 2010; Meyer & Bucci, 2014a). Whether performance in either, or both, of these procedures is impacted in a model could suggest which brain regions and circuits could be driving impulsivity as well.

To consider the role of the 5-HT_{1B}R in responding to classically conditioned excitatory cues, we measured Pavlovian appetitive conditioning behavior under various contingencies, as well as responding in 100% versus 25% reinforcement schedules. We found that mice lacking the 5-HT_{1B}R show no differences in responding to cues in a positive contingency reinforcement schedule, but did have increased responding to cues that were presented in the context of reward, but were not explicitly predictive of reward (a zero contingency condition). Interestingly, the increased responding to cues did not occur when the cues and rewards were separated by an ITI in a negative contingency condition or when the mice were on a 25% reinforcement schedule. There were some procedural differences between the contingency experiments and the 100% versus 25% reinforcement to maintain session length; there were fewer US presentations and a longer US-US interval in the 100% versus 25% reinforcement experiment. It is possible that a difference in partial reinforcement would only emerge with shorter interval timings, so future experiments could explore the effects of ITI length on partial reinforcement in this model. If there is no difference in partial reinforcement as our data suggest, then the increased responding in the 5-HT_{1B}R may be something else unique to the zero contingency condition, including the variable orientations of the CS and US presentations, including the presence of trials similar to backwards or

trace conditioning. Future studies could explore the effect of 5-HT_{1B}R on trace conditioning, where there is a delay between cue offset and the onset of reinforcement. We would expect mice lacking the 5-HT_{1B}R to have increased responding to the cue over longer delay periods compared to controls. Interestingly, in support of this hypothesis, in adolescence, rats have enhanced trace conditioning compared to preadolescent and adult controls (Hunt et al., 2016).

One potential unifying explanation for the increased responding to cues in the zero contingency condition and the increased operant responding in tests of impulsive action is that the absence of the 5-HT_{1B} receptor could alter perception of timing. If the ITI of the 5CSRTT is perceived as being shorter than it actually is, animals may respond prematurely. Similarly, in the conditioning paradigms, if the time between cue and reinforcement was subjectively reduced, the associative strength of that cue may be increased, resulting in the increased responding to cues when there is a weak temporal relationship with reward. This would also be consistent with previous findings that mice lacking the 5-HT_{1B}R maladaptively respond early in an instrumental differential reinforcement of low-rate responding paradigm, resulting in a left shifted response distribution, i.e. earlier time of peak responding (Nautiyal et al., 2017). Additionally, there are previously reports suggesting a role for serotonin signaling in modulating temporal perception and discrimination (Asgari et al., 2006; Halberstadt et al., 2016). Interestingly, this explanation would conform with our previous results showing the effects of 5-HT_{1B}R in a delay discounting test, in which we report that mice lacking the 5-HT_{1B}R actually have no differences in rate of delay discounting, but in fact have a higher preference for a larger reward regardless of delay (Nautiyal et al., 2017). This could feasibly be due to a subjective shortening of time perception such that delays for seems shorter and are therefore more tolerated (Paasche et al., 2019; Wittmann & Paulus, 2008).

An alternative explanation for the effect of 5-HT_{1B}R on behavioral responding is that the phenotype is the result of generalized hyperactivity. However, we find that our data do not support this interpretation. First, in the 5CSRTT training, mice lacking the 5-HT_{1B}R learned to inhibit premature responding over time. If impulsive action were the result of general increased activity, we would expect to see this behavior persist over all sessions. Additionally, in the classical conditioning paradigms, all responding was measured as an increase from ITI responding to control for potential differences in baseline responding, so it is unlikely hyperactivity contributed to these results. Finally, we have previously reported no effect of 5-HT_{1B}R knockout on open field activity (Nautiyal et al., 2017). More plausibly, changes in reward processing, as we have previously reported (Desrochers et al., 2021), could alter responding in both operant and classical conditioning experiments. If mice lacking the 5-HT_{1B}R have increased subjective valuation of reward or increased motivation for reward, this could enhance the salience of excitatory cues (Rescorla & Wagner, 1972), potentially enhancing the relative associative strength over the temporal separation of the cue and reward in the independent interval condition. In this interpretation, it is possible that the deficits in response inhibition seen in the 5CSRTT and conditioned inhibition experiments could occur without changes in inhibitory processing and could alternatively be the result of increased reward drive (Desrochers et al., 2021).

5. Conclusion

Overall, these studies show that serotonin signaling through the 5-HT_{1B}R influences cue reactivity in both excitatory and inhibitory contexts, despite intact inhibitory learning. Additionally, the conditioned inhibition and Pavlovian appetitive conditioning experiments demonstrate that increased impulsivity may be seen in differences in responding in classical conditioning, in the absence of action-based consequences. However, the extent to which the operant and Pavlovian effects seen in mice lacking the 5-HT_{1B}R have similar underlying behavioral and neural mechanisms remains unclear. It is possible that 5-HT_{1B}R plays a role in distinct systems supporting these different behaviors, so subsequent experiments could examine the potential convergence of neural circuits using tissue-specific manipulations of serotonin signaling. More broadly, we suggest that careful designed and analyzed behavioral testing could contribute to a better understanding of the underlying cognitive and neural mechanisms of impulsivity, as well as characterization of clinical presentation and preclinical models. Specifically, combining tests of classical conditioning, especially Pavlovian conditioned inhibition, with traditional operant-based tests of impulsivity may be important to gain insight into the learning processes which contribute to deficits in response inhibition.

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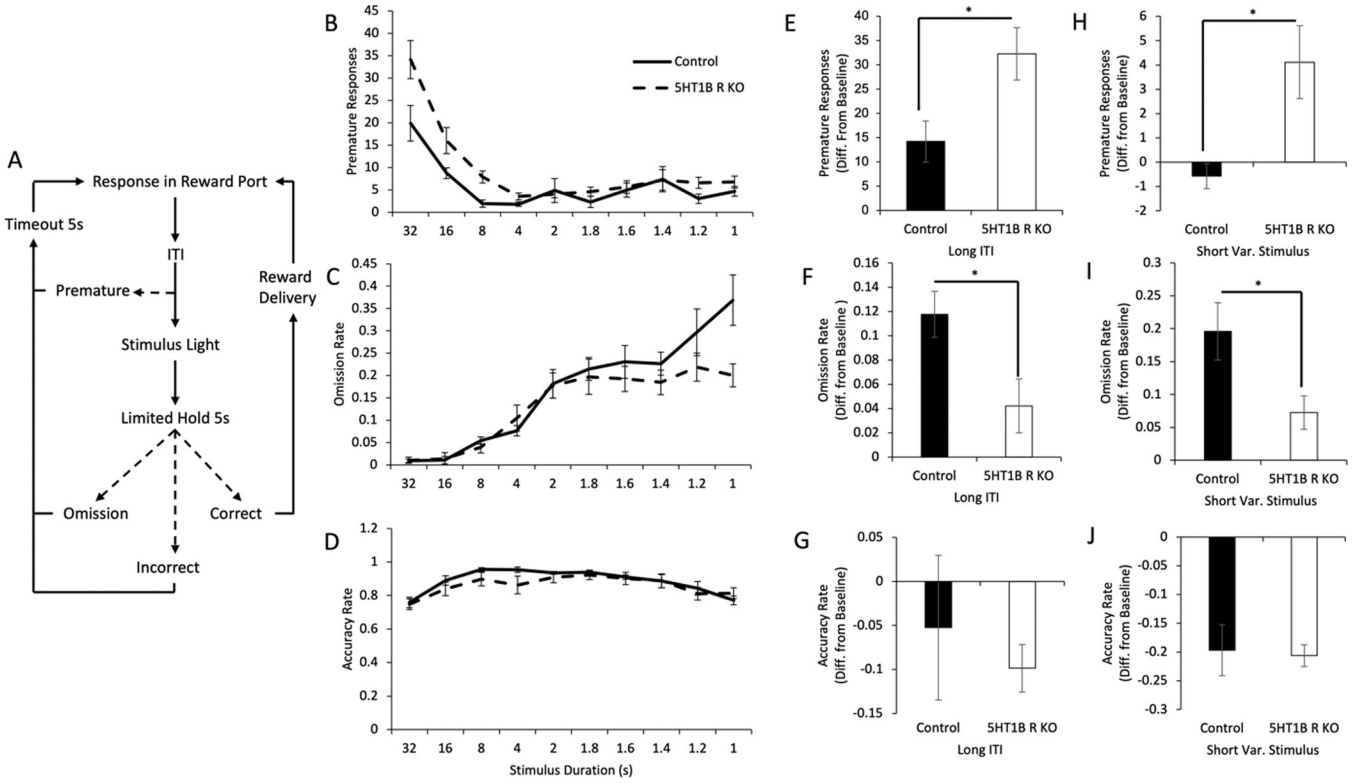

Figure 1. An absence of 5-HT 1B R causes impulsive responding in the 5-choice serial reaction time test.

A) A diagram of the 5CSRTT trial structure. During training, the ITI period was 5s and the stimulus began at 32s and decreased based on group performance until a baseline of 1s was achieved. The Long ITI test increased in the ITI to 9s, and the Short Variable Stimulus test decreased the stimulus duration (0.2s, 0.4s, 0.6s, 0.8s, randomly ordered), with all other parameters the same as the baseline procedure. The total premature responses for B) training and difference from baseline premature responses for E) Long ITI and H) Short Variable Stimulus tests are shown in the top row. Proportion of total trials in which the mouse did not respond for C) training and difference from baseline omission rate for F) Long ITI and I) Short Variable Stimulus tests in the center row. Proportion of correct non-omission trials in which the mouse for D) training and difference from baseline accuracy rate for G) Long ITI and J) Short Variable Stimulus tests in the bottom row. * $p<0.05$. All data are groups means +/− SE.

A

	Conditioned Inhibition Training	Summation	Retardation
Procedural Control	A+ (Click+) B+ (Noise+) X- (Light-)	B (Noise) BX (Noise/Light)	X+ (Light+)
Experimental	A+ (Click+) B+ (Noise+) AX- (Click/Light-)	B (Noise) BX (Noise/Light)	X+ (Light+)

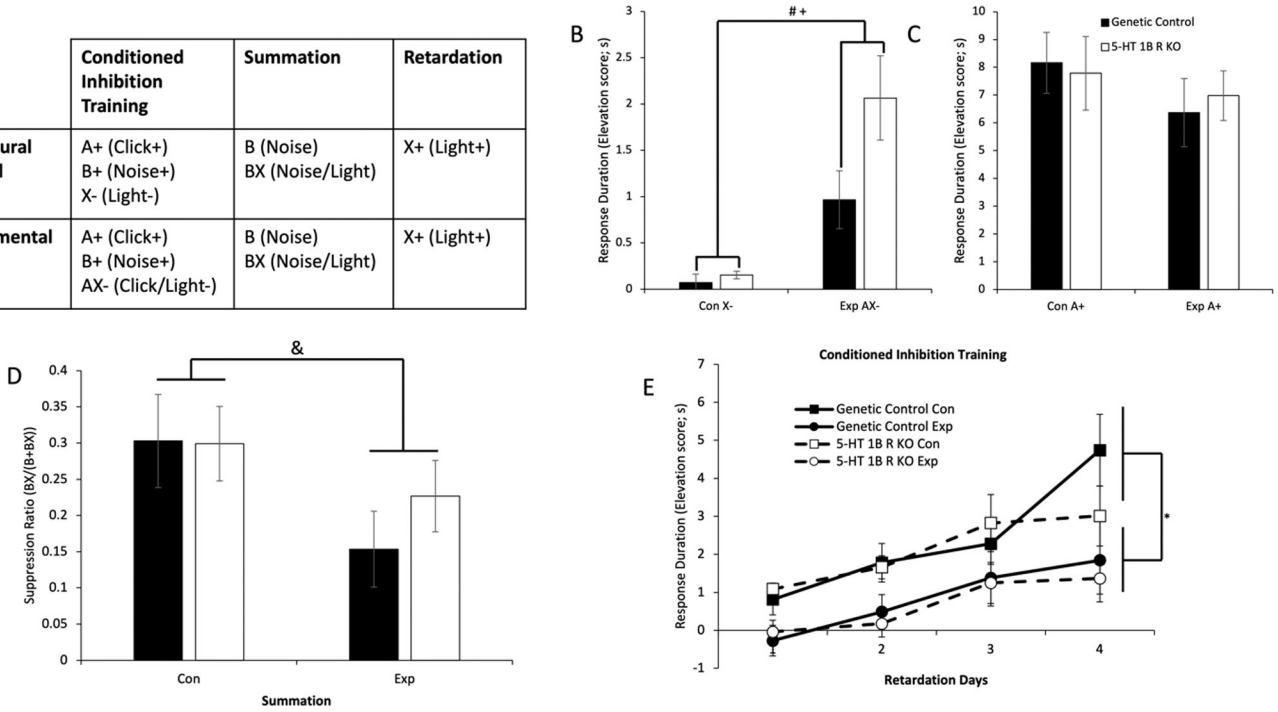

Figure 2. Mice lacking 5-HT 1B R have deficits in response inhibition during training but intact inhibitory learning in tests of conditioned inhibition.

A) Trial types presented to the conditioned inhibition procedural control and experimental groups during training (note B+ trial data not shown), summation, and retardation. Elevation score (average response duration during cue-average preceding ITI responding) averaged over days for B) inhibitor and C) excitor A trials during conditioned inhibition training. D) Suppression ratio (duration of responding during BX/(B+BX)) for the summation test. E) Response duration elevation score over days for X+ trials for the retardation of acquisition test. & p of main effect of condition =0.051; * p of main effect of condition <0.05; # p of main effect of genotype <0.05; + p of interaction =0.080. All data are groups means +/− SE.

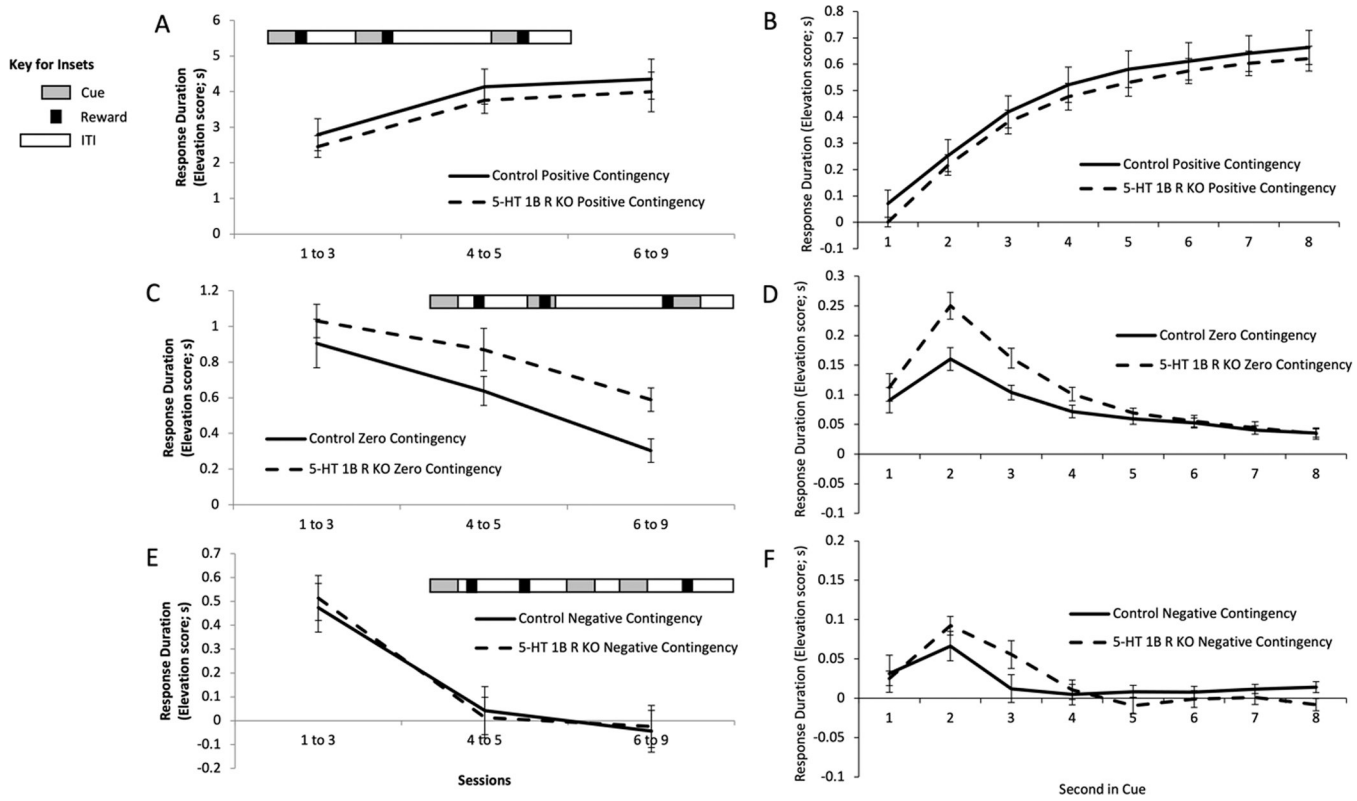

Figure 3. In appetitive Pavlovian conditioning, mice lacking 5-HT 1B R in a zero contingency condition have elevated responding to cue onset.

Total cue response duration elevation score over sessions for A) positive, C) zero, and E) negative contingency conditions. Response duration elevation score across seconds in the cue for B) positive, D) zero, and F) negative contingency conditions (averaged over training days), with insets showing the general trial structure for the conditions. All data are groups means +/− SE.

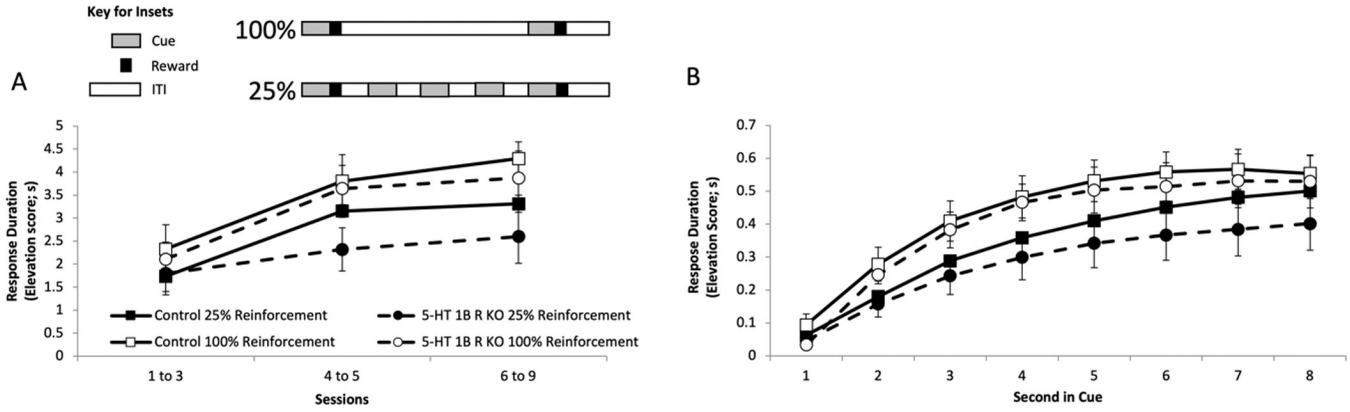

Figure 4. Mice lacking 5-HT 1B R do not show differences in approach behavior for 100% or 25% reinforced cues.

A) Total cue response duration elevation score over sessions for 100% reinforcement and 25% reinforcement conditions. B) Response duration elevation score across seconds in the cue for 100% reinforcement and 25% reinforcement conditions (averaged over training days), with insets showing the general trial structure for the conditions. All data are groups means +/− SE.