

## Research report

## Distinguishing between predictive and incentive value of uncertain gambling-like cues in a Pavlovian autoshaping task

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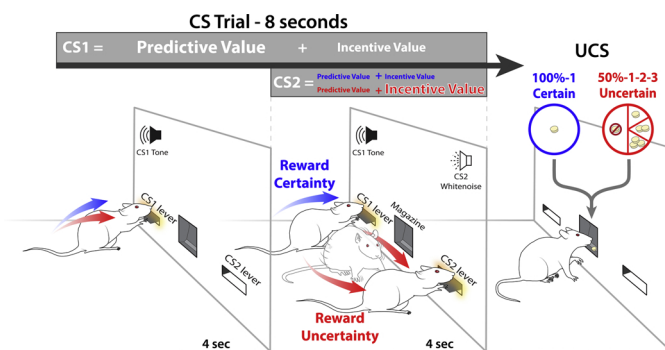
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## GRAPHICAL ABSTRACT



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## ABSTRACT

The flashing lights and celebratory sounds that dominate slot-machine gambling are believed to promote engagement and motivation to keep playing. However, these cues are often presented in the absence of reward, and previous research suggests that this reward uncertainty, which degrades their predictive value, also increases their incentive value. Here, we used autoshaping to tease apart the impact of reward uncertainty on the predictive and incentive value of a conditioned stimulus (CS) using serial cues. Each CS trial began with the presentation of a predictive CS1, followed by a CS2, holding primarily incentive value, because of its proximity to sucrose reward delivery, under Certain (100%-1) or Uncertain (50%-1-2-3) reward conditions. Subsequently, we tested the impact of amphetamine and nicotine on cue attraction, and the ability of these cues to either serve as a conditioned reinforcer, or promote motivation for sucrose during a progressive ratio task. Finally, we measured anxiety behavior, and examined its interaction with each cue and uncertainty. Our results suggest that reward uncertainty increases attraction to the incentive CS2 and its ability to trigger motivation and reward-seeking. However, although the CS2 is largely ignored under Certain conditions, both CS1 and CS2 become conditioned reinforcers for both groups. Finally, amphetamine reduced the attraction of the CS1 for both groups but had no effect on the attraction of the CS2. These results suggest that reward uncertainty recruits and increases the incentive value of cues with limited predictive value and highlights the distinction between cue attraction, reward-seeking and conditioned reinforcer properties.

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## 1. Introduction

Gambling Disorder is characterized by repeated problematic gambling behavior that causes significant problems or distress [1]. Studies have reported that 78.4% of Americans have gambled at least once in their lifetime, with 2% characterized as suffering from gambling disorder [2]. In many games of chance, particularly slot machines, external cues such as flashing lights and celebratory sounds are repeatedly presented during “winning” outcomes and are believed to play a crucial role in gambling involvement [3–6]. Repeated exposure and pairing of these visual and auditory “win” cues causes them to be attributed with high levels of incentive salience, making them ‘wanted’ and capable of triggering intense bouts of craving for gambling [7,8]. Cues that reliably predict reward are attractive and become imbued with incentive and predictive properties [9]. However, in slot machine gambling, many of these cues are repeatedly presented even in the absence of a winning outcome [5,10]. While their presentation in the absence of reward is believed to play an important role in engaging and motivating players [3], it also results in a degradation of these cues’ predictive value. This is the case under conditions of reward uncertainty, such as in slot machine gambling, where cues are associated with high levels of uncertainty in both the probability and magnitude of reward. Nevertheless, despite diminished predictive value, cues paired with reward uncertainty seem to acquire high levels of incentive value. Attribution of incentive value to a cue or conditional stimulus (CS) can be measured as sign-tracking (sniffing, nibbling and biting a cue), in contrast to goal-tracking (sniffing and nibbling the location of reward delivery during CS presentation), using a Pavlovian autoshaping paradigm [11,12]. Previous studies have shown that reward uncertainty can render cues more attractive [13], and can recruit and ascribe incentive value to distal cues that are otherwise ignored [14]. Reward uncertainty can also increase the proportion of individuals that direct their attention towards cues (sign-trackers) [15], resulting in a combination of increased sign-tracking and reduced goal-tracking [13,16,17].

Under conditions of reward uncertainty, cues therefore appear to gain greater incentive value despite diminished predictive value. However, how these opposing changes affect cue attraction can be hard to disentangle. It has been suggested that whether an animal assigns predictive or incentive value to a cue can be examined based on the form of the conditioned response elicited when the cue is presented, and whether the cue becomes a conditioned reinforcer, as measured through a conditioned reinforcement task [18]. Alternatively, the degree of incentive and predictive value imparted to a cue can be manipulated through experimental design. For example, previous studies have shown that the predictive and incentive value of cues can be examined by presenting two cues in a sequence prior to reward [19–21]. In this scenario, the first cue (CS1) carries the majority of the predictive value since it signals the onset of a trial that culminates in the presentation of the unconditioned stimulus (UCS) [22]. A second cue (CS2) is then presented after the CS1 but before the UCS. This second cue holds little to no additional predictive value, but instead is thought to carry mostly incentive value because of its temporal proximity to the reward [20,23–26]. Here, the CS2 is believed to carry more incentive value than predictive value, although it is unclear whether it carries similar amounts of incentive value as the CS1. On the one hand, it could be argued that the CS1 carries diminished incentive value in a serial design, as it more immediately predicts the presentation of the CS2, which is a stimulus without any intrinsic incentive value, unlike the sucrose reward [23–25]. Yet recent findings have suggested that although a reward-proximal CS2 might initially carry more incentive value, the more distal CS1 might over time come to acquire the most incentive value [27]. Despite this, electrophysiological recordings made by Tindell and colleagues have shown that increases in mesolimbic dopamine activity, either through acute amphetamine administration or prior amphetamine sensitization, shifts the neuronal activity of the ventral pallidum away from predictive coding of the CS1 and towards

incentive coding of the CS2 [20]. This suggests that manipulations that increase the attribution of incentive salience may do so primarily for the CS2.

Here, we used a similar design employing sequential cues to examine the impact of reward uncertainty on the attraction to cues bearing different degrees of predictive and incentive value. Rats were exposed to a Pavlovian Conditioned Approach (PCA) or autoshaping task, consisting of a series of CS trials, each lasting a total of 8 s. Each CS trial began with the presentation of a CS1 (lever + auditory cue) that predicted the onset of the CS trial, followed by the presentation of a more reward-proximal CS2 (different lever + different auditory cue). Each CS trial concluded after 8 s by a UCS outcome (sucrose pellet) under either Certain or Uncertain reward conditions. Certain reward conditions consisted of the delivery of a single sucrose pellet on 100% of CS trials, while Uncertain reward conditions consisted of the delivery of 1, 2 or 3 sucrose pellets on 50% of trials and no sucrose pellets on the remaining 50%. In Experiment 1, each CS trial consisted of the Sequential presentation of 4 s of CS1 followed by 4 s of CS2. In Experiment 2, animals were initially presented with 4 s of CS1 but were then presented with a Choice, consisting of the dual presentation of CS1 and CS2 during the last 4 s of the CS trial. Finally in Experiment 3, the first 5 days of conditioning consisted of the Sequential presentation of CS1 followed by CS2, as in Experiment 1, whereas the last 5 days of conditioning consisted of the Choice design, as seen in Experiment 2. For each experiment, cue attraction for each rat was measured as the amount of lever presses on either CS1 or CS2, or magazine entries into the food cup during each 8-second CS trial. The ability of either CS1 or CS2 to act as a conditioned reinforcer was then measured using a conditioned reinforcement task. For animals in Experiments 1 and 2, the impact of amphetamine or nicotine on cue attraction was then tested under autoshaping conditions, and their reward-seeking and motivation for reward was assessed under a fixed ratio (FR1) and a progressive ratio (PR). Finally, all animals were tested for anxiety using an elevated plus maze both before and after all behavioral conditions, and the impact of high and low anxiety on cue attraction was examined.

Since reward uncertainty degrades the predictive value of a cue, yet appears to ascribe more incentive value to reward cues, we expected to see strong differences in the attraction towards the CS1 and CS2, in the form of sign-tracking, between animals under Certain and Uncertain reward conditions. Specifically, we hypothesized that animals under reward uncertainty (1) would show greater attraction to the CS2, (2) would ascribe strong conditioned reinforcing properties to both CS1 and CS2, (3) would demonstrate greater motivation for reward, and (4) would show differences in cue attraction based on their levels of anxiety.

## 2. Materials & methods

### 2.1. Animals and housing conditions

Female Sprague Dawley rats (N = 48, weight: 220–300 g) purchased from Envigo and bred in house were housed in groups of two or three in a reverse 12-h light/dark cycle at 21 °C constant temperature. Prior to food restriction, rats had ad libitum access to chow (LabDiet, Teklad) and tap water. Prior to the start of behavioral experiments, animals were handled and habituated for 2–3 days, and were food-restricted to 85–90% of initial body weight. All procedures were approved by the Institutional Animal Care and Use Committee at Wesleyan University.

### 2.2. Anxiety: elevated plus maze

#### 2.2.1. Apparatus

The Elevated Plus Maze (EPM) is a known behavioral measure used to index levels of anxiety in rodents [28]. As previously described [16,17] the apparatus contained two perpendicular platforms that

intersect to form a plus-shaped symbol. The plus-shaped maze was elevated 97 cm off the floor and consisted of four arms measuring 40 cm in length and 15 cm in width. The four arms consisted of two “closed” arms, partially enclosed by dark, non-transparent walls on three sides, and two “open” arms that were exposed, without walls. Each arm was located directly across from its matching arm with a 15 cm x 15 cm square open intersection in the middle, joining each arm of the maze. An overhead infrared video camera (Advidia™) was used for visualization and recording of behavior during each 5 min session.

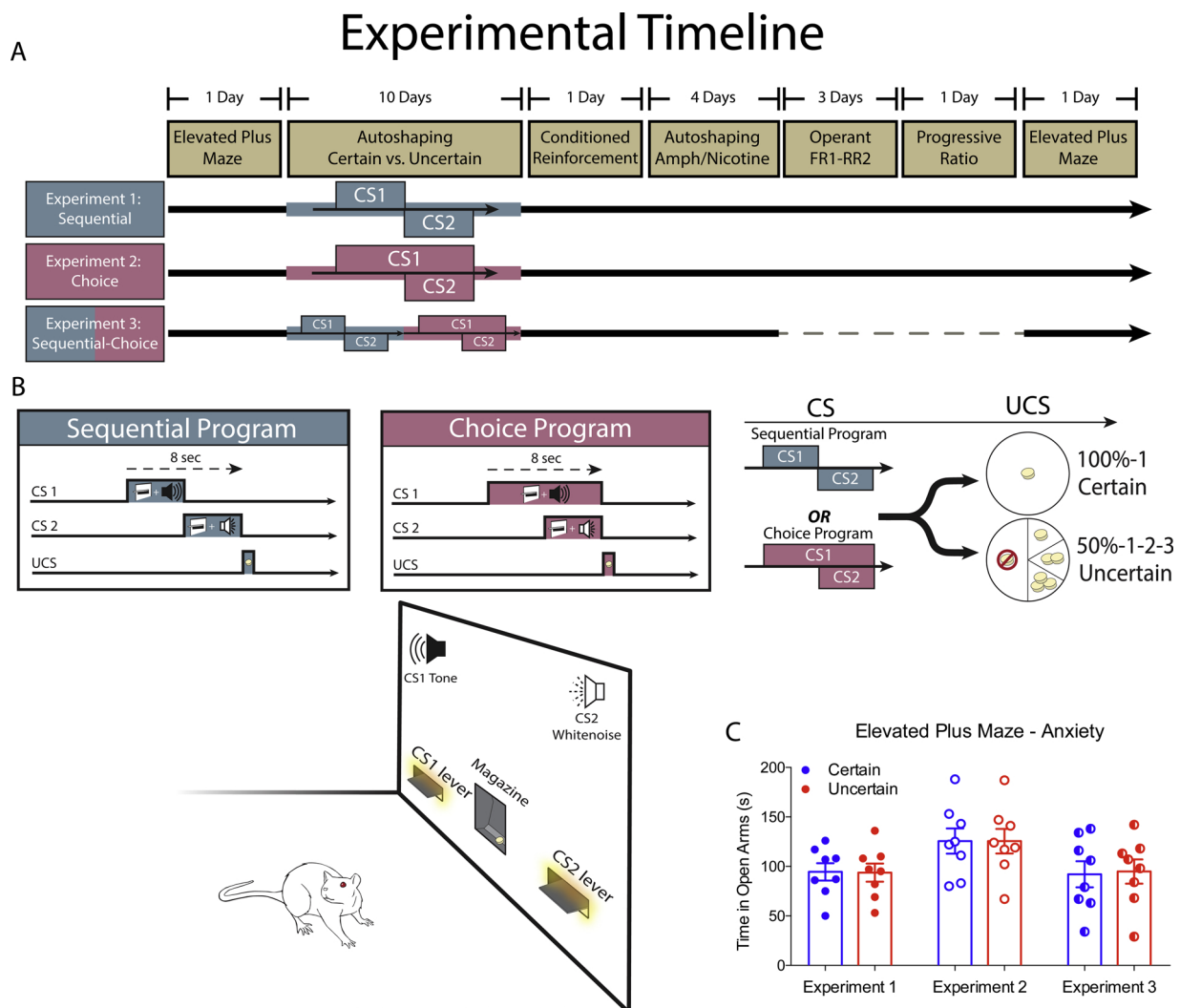
### 2.2.2. Procedure

Rats were placed in the center of the maze, with the head and tail facing the open arms. Exploratory behavior was assessed for a period of 5 min under red light conditions. After each trial, rats were returned to their home cage, and the apparatus was cleaned with 90% Versa-Clean™ Multi-Purpose Cleaner. Videos were manually scored by an investigator blind to the experimental conditions. Time spent and entries into the two closed and two open arms were recorded for each animal's

first five minutes on the maze. An arm entry was recorded when all four paws of the animal were located in one arm. The entry ended when all four paws of the animal were no longer in the arm. The amount of time spent on the two open arms was summed and used as a measure of anxiety, where open arm time inversely correlates with anxiety [16,28,29].

### 2.3. Groups and conditions

Rats were initially divided across three experiments (N = 16 for each experiment), where each experiment differed by the sequence with which cues were presented during autoshaping (Fig. 1A). Within each experiment, rats were further divided into two groups (N = 8 for each group) according to baseline anxiety levels (EPM), so that anxiety was matched between groups within a given experiment. The two groups differed by reward condition (Certain: 100%-1 or Uncertain: 50%-1-2-3) according to the probability and magnitude of reward delivery per CS trial during autoshaping (Fig. 1B).



**Fig. 1. Experimental timeline of each experiment and initial measures of anxiety.** A) An overview of the experimental timeline of Experiments 1, 2, and 3, showing the order and duration of each individual task. B) Animals were exposed to either a Sequential or Choice design during autoshaping. The Sequential Design presents the predictive CS1 for the first 4 s followed by the incentive CS2 during the last 4 s of each 8 s CS trial. In the Choice Design, the CS1 is presented for the full 8 s and the CS2 is introduced only during the last 4 s. During autoshaping animals were exposed to either Certain (100%-1) or Uncertain (50%-1-2-3) reward conditions. A diagram shows the arrangement of the food dish (magazine) and relative position of CS1 and CS2 (side counterbalanced). C) Anxiety was initially measured using the Elevated Plus Maze, where greater time spent on the open arms reflected lower anxiety. Open arm time was used within each experiment to assign animals to either Certain or Uncertain conditions so that anxiety was matched between groups.

## 2.4. Pavlovian and operant conditioning

### 2.4.1. Apparatus

All testing was conducted in Med Associates Inc.<sup>™</sup> modular test chambers (25.8 × 32.2 × 33.2 cm) with metal bar floors, two modular front and back walls and two plexiglass walls, as previously described [30]. Each chamber was equipped with two retractable levers located on the front wall of the chamber, either side of a recessed magazine dish, which delivered 45 mg sucrose pellets (TestDiet, St. Louis, MO, USA), and was equipped with an infrared beam and sensor to record head entries. Auditory speakers at the top of the chamber delivered a 2.9 kHz tone or white noise (Fig. 1B). For the conditioned reinforcement session, the back wall was outfitted with three nose poke holes (two active on the left and right side, and one inactive in the center). During this time, the food cup on the front wall was covered with a custom metal plate. MedPC<sup>®</sup> software automatically recorded lever presses, nose pokes, and magazine entries across all sessions. Chambers were placed in sound-attenuating cabinets to reduce ambient light and noise. Red LED lights were mounted on the wall inside the cabinet and were turned on during all sessions.

### 2.4.2. Autoshaping

Two days prior to autoshaping, all animals were exposed to sucrose pellets in their homecage in order to reduce neophobia. The following day, rats underwent one day of magazine training which consisted of a 30 min training session where rats were habituated to the environment of the testing chamber and received 30 sucrose pellets from the magazine dish on a 45 s variable intertrial-interval (VI-45; 15–75 sec). Rats in all experiments then underwent 10 consecutive days of autoshaping, with each session consisting of 36 conditioned stimulus (CS) trial presentations (VI-45), and lasting approximately 30–35 minutes. Each CS trial lasted 8 s and predicted the delivery of sucrose pellets as an unconditioned stimulus (UCS). Pellets were dispensed according to two reward conditions: Certain (100%-1) and Uncertain (50%-1-2-3). In the 100%-1 reward condition, each CS trial resulted in the delivery of 1 sucrose pellet to the magazine dish. In the 50%-1-2-3 reward condition, half of the CS trials (18 trials; order randomized) resulted in the delivery of 0 sucrose pellets, while the other half of the CS trials (18 trials) resulted in the delivery of 1, 2, or 3 sucrose pellets, with equal probability. The 50%-1-2-3 reward condition created uncertainty in both the probability and magnitude of reward delivery. However, despite the different reward conditions, all rats received 36 pellets and 36 CS presentations by the end of each autoshaping session and were therefore equally exposed to both the CS and UCS rewards. Throughout each session, lever responses and head entries into the magazine were recorded but had no programmed consequence.

In order to tease apart the impact of reward uncertainty on the predictive and incentive value of a cue, each Pavlovian CS trial consisted of the presentation of two separate cues (lever + sound) prior to the UCS. Each 8 s CS trial began with the presentation of an initial CS1 (left or right illuminated lever + tone or white noise, counter-balanced) which predicted the onset of the CS trial and bore the majority of UCS predictive value [22]. Halfway into the CS trial, after 4 s of presentation of the CS1, a second cue (right or left illuminated lever + white noise or tone), referred to as CS2, was presented for an additional 4 s. The CS2 carried little to no additional predictive value, since any predictive value it might have carried was already overshadowed by the presentation of the CS1 to signal the initiation of the CS trial. Instead, the CS2 carried primarily incentive value due to its greater proximity to the UCS reward delivery. This is based on previous studies that have shown that the incentive impact of Pavlovian cues gradually rises and focuses as CSs become more temporally proximal to the reward [20,26,31]. Finally, the goal dish was present at all times during the CS trial and was used as a contextual cue most proximal to the reward.

At the end of the 8 s CS trial, any and all levers currently extended

were retracted and all auditory cues were silenced. This was immediately followed by the delivery of a UCS consisting of 0, 1, 2 or 3 pellets depending on whether an animal was under Certain or Uncertain reward conditions. The manner and timing with which the CS1 and CS2 were presented was varied across three experimental designs.

**2.4.2.1. Experiment 1: sequential design.** The Sequential design aimed to examine if animals under Certain reward conditions would primarily show attraction to a predictive CS1 and mostly ignore a less informative CS2, and whether animals under Uncertain reward conditions would demonstrate similar levels of attraction to both CSs (Fig. 1B). Here, each CS trial consisted of the initial presentation of the CS1 lever + auditory cue for 4 s. After those initial 4 s, the CS1 ended and was immediately followed by the presentation of the CS2 (opposite) lever + auditory cue for 4 s. Four seconds later, the CS2 ended, and the UCS was delivered according to reward condition.

**2.4.2.2. Experiment 2: choice design.** The Choice design aimed to determine whether reward uncertainty would result in sufficient cue attraction being assigned to a secondary CS2 to draw attention away from the predictive CS1 cue. As such, each CS trial began with the presentation of the CS1 that lasted 8 s. After 4 s, and while the CS1 was still present, the CS2 cue was presented for 4 s. Four seconds later, both the CS1 and CS2 ended and the UCS was presented. The CS1 therefore initially predicted the onset of the CS trial and was present alone for 4 s. Thus for the last 4 s of the 8 s CS trial, both the CS1 and CS2 were presented concomitantly.

**2.4.2.3. Experiment 3: sequential then choice design.** In order to compare the relative amount of incentive value attributed to the CS2 versus the CS1 under Certain and Uncertain conditions, animals were initially exposed to the Sequential design for the first 5 days of training, and then switched to the Choice design for the last 5 days of autoshaping. Animals were therefore initially exposed to CS trials that consisted of 4 s of CS1 followed by 4 s of CS2. Starting on Day 6, CS trials still began with the presentation of the CS1, however the CS1 remained present for a full 8 s, with the CS2 being presented during the last 4 s of the CS trial. Initial exposure to the Sequential design was intended to promote attribution of incentive value to the CS2. Subsequent exposure to the Choice design created a scenario that simultaneously juxtaposed the CS1 and CS2 during the last 4 s of each CS trial and allowed animals to direct their attention back to the CS1 if it carried more incentive value.

**2.4.2.4. Sign-tracking and goal-tracking.** Although the delivery of reward was independent of behavior, all rats typically developed a conditioned response after initial training by interacting (e.g. sniffing, nibbling, biting, pressing) with the CS lever and/or magazine dish, resulting in two distinct conditioned responses (CR): sign-tracking and goal-tracking. These behaviors may be quantified as a measure of the incentive salience attributed to that cue and reveal individual differences in cue attraction [9]. An animal's response bias towards either cue was determined using the following equation (LP-ME)/(LP + ME) derived from the Pavlovian Conditioned Approach (PCA) index [32], with scores ranging from 1 to -1. Animals with a strong preference for the lever had a response bias between 1 and 0.5 and were classified as sign-trackers, whereas goal-trackers had a response bias between -0.5 and -1. An individual was classified as an intermediate if it directed its responses to both the lever and the food cup and had a response bias between 0.5 and -0.5. An animal's phenotype was based on responses during the CS presentations of the last day (Day 10) of Pavlovian autoshaping. When calculating the response bias for a particular lever, the equation was modified to focus on both lever presses (LP) and magazine entries (ME) during that particular time window. For example, response bias for CS1 in Experiment 1 would be calculated as (CS1LP-CS1ME)/(CS1LP + CS1ME), where CS1ME equaled the number of magazine entries performed exclusively for



the duration of the CS1 presentation, in this case, the first 4 s of the CS trial. However this approach to calculating response bias could not reliably be applied when more than three options are available to the rat, as is the case during the last 4 s of the CS trial in Experiment 2. Instead the response bias is best calculated using an equation that simultaneously includes all three factors (CS1, CS2 and ME), as is outlined below.

**2.4.2.5. Comparing attraction for cues bearing predictive, incentive or reward delivery value.** Our aim was to compare the attraction and interaction animals performed with three major targets during each CS trial, notably the CS1, CS2 and magazine dish. In order to do so we adapted a prior novel 'profile analysis' used to assess the coding properties of Ventral Pallidum neurons to three separate temporal events [20]. This 'profile analysis' generates a unitary vector that takes into account data from all three separate factors, and allows for a more accurate portrayal of an individual's response bias. For example, in cases where both CS1 and CS2 were simultaneously present (e.g. Choice Design), an animal might principally focus on the CS1 lever, thereby performing no CS2 lever responses or magazine entries during the last 4 s when the CS2 is present. The result would be that a numeric value for CS2 response bias could not be calculated because the denominator, CS2LP + CS2ME, equaled 0. Although response bias can determine overall sign-tracking and goal-tracking behavior, it can only do so reliably when only two options are present (e.g. lever and food cup). In contrast, this novel behavioral profile analysis vector accounts for CS1, CS2 and magazine responses, and allows for 0 values in each and any factor.

In each experiment, we therefore used the level of responding as lever presses on either CS1 or CS2, or head entries into the goal dish to compute the relative attraction of each component at different points during the 8 s of cue presentation (e.g. first 4 vs. last 4 s of each CS trial). We denote each animal's attraction pattern to the CS1, CS2, and magazine as  $x$ ,  $y$ , and  $z$ , respectively. With these coordinates, and based on equations from Tindell and colleagues [20], we created a two dimensional vector ( $\alpha$ ,  $\beta$ ) representing the relative attraction to these cues, where  $\alpha = (2y - x - z)/2$  and  $\beta = \sqrt{3}(x - z)/2$ . The magnitude of this vector  $r = \sqrt{(\alpha^2 + \beta^2)} = \sqrt{[(x - y)^2 + (y - z)^2 + (z - x)^2]}/2$  is modulated by the relative attraction to each of the three stimuli. Its direction is  $\theta = \tan^{-1}(\frac{\beta}{\alpha})$ , and represents an animal's preference for either of the three stimuli (CS1, CS2, magazine). Thus for  $\theta = 0^\circ$ , this would imply that  $y > x = z$  suggesting that attraction and responding was greatest for CS2. Similarly,  $\theta = 120^\circ$  would imply primary attraction to the CS1, whereas  $\theta = 240^\circ$  would suggest a principal attraction towards the magazine. Therefore while a  $\theta = 0^\circ$  or  $120^\circ$  would suggest an animal presented as a sign-tracker,  $\theta = 240^\circ$  would designate a primarily goal-tracker phenotype. Consequently, an animal with  $\theta = 60^\circ$  would be expressing a sign-tracker phenotype with a split attraction between the CS1 and CS2, as might be the case if each cue is presented independently for a similar amount of time, as in Experiment 1 where CS1 and CS2 are introduced sequentially for 4 s each. Group Profile Vectors for Certain and Uncertain groups were calculated using the mean of CS1, CS2 and magazine responses as the  $x$ ,  $y$  and  $z$  coordinates for that particular group on a given day or period of time. Whereas primary attraction for a cue's predictive value anticipates Group Profile Vectors predominantly directed towards CS1 ( $180^\circ - 60^\circ$ ), with  $CS1 > CS2 > \text{magazine}$ , dominant attraction towards a reward-proximal cue would predict Group Profile Vectors predominantly directed towards the CS2 ( $60^\circ - 300^\circ$ ) where  $CS2 > CS1 > \text{magazine}$ . Of particular interest here, is the ability of reward uncertainty to shift Group Profile Vectors away from CS1 attraction and towards greater CS2 attraction.

#### 2.4.3. Conditioned reinforcement

Following 10 days of autoshaping, rats completed a one-day conditioned reinforcement task (30 min) to assess the relative incentive

value of both the CS1 and CS2 and to measure their ability to reinforce a novel operant (nose-poking) response. Rats were given the opportunity to work on a Fixed Ratio 1 (FR1) schedule for the presentation of either the CS1 or CS2 lever + auditory cue. The session began with the illumination of three nose poke ports on the back wall (Fig. 6 A). Entry into either the left or right nose poke ports resulted in the presentation of an illuminated lever and its associated auditory cue for 4 s. Whether the left or right nose port on the back wall produced the presentation of the left or right lever on the front wall was counterbalanced across subjects. The center nose port served as a control and had no programmed consequence. After 30 min, the session ended, and the ports became inactive. Med-PC software automatically recorded the number of nose pokes per port and lever presses.

#### 2.4.4. Autoshaping: amphetamine and nicotine priming

Following conditioned reinforcement, the impact of amphetamine and nicotine on attraction to the CS1 and CS2 in autoshaping was ascertained across Certain and Uncertain conditions. Rats in Experiments 1 and 2 underwent four additional days of autoshaping, in which all four sessions were preceded by a single injection, 15 min prior to the start of the autoshaping session. On the first two days, rats received injections of saline (1 ml/kg, SC) to habituate animals to injections and establish baseline behavior. On the third and fourth day, rats received either an amphetamine (0.5 mg/kg, SC) or nicotine (0.3 mg/kg, SC) injection (order counterbalanced).

#### 2.4.5. Instrumental training and progressive ratio

Motivation for the sucrose reward and reward-seeking was assessed using operant responding and a progressive ratio paradigm. The operant training procedure consisted of one day of Fixed Ratio 1 (FR1) training, two days of Random Ratio 2 (RR2), and one day of progressive ratio (PR), each session lasting 30 min. As in autoshaping, the front wall contained two metal levers on either side of the magazine dish corresponding to the CS1 and CS2 levers. Each session began with the simultaneous presentation of both CS1 and CS2 levers, and reward contingencies on each given day applied equally to both levers. The FR1 reward contingency required rats to execute one lever press for the delivery of one sucrose pellet. Following the FR1 task, rats completed a two-day RR2 task, which required rats to complete between 1 and 3 lever presses to obtain a single sucrose pellet. After RR2, rats completed a one-day PR task with both levers presented simultaneously. Progressive ratio assessed rats' willingness to expend effort in order to obtain a sucrose reward. The number of presses required to obtain a single sucrose pellet increased on an exponential progressive ratio schedule (1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95 ...) determined by the equation (progressive ratio =  $[5e^{(\text{reward number} \times 0.2)}]$  - 5) and rounded to the nearest integer [33–35]. Med-PC recorded the number of rewards earned and lever presses an animal completed on either lever during the task. The highest number of lever presses completed to obtain a single sucrose pellet, was used as a measure of breakpoint associated with either the CS1 or CS2 lever.

#### 2.5. Test for anxiety post experiment

Levels of anxiety were once again measured following exposure to autoshaping, conditioned reinforcement, and instrumental training. To create a sense of novelty and reduce habituation to the contextual cues associated with the open arms of the elevated plus maze, the apparatus was relocated to the opposite side of the testing room, and the arms of the maze were rotated  $45^\circ$  about the origin [17]. Post-conditioning testing followed the same procedure as initial pre-conditioning sessions and consisted of a one-day session on the elevated plus maze lasting 5 min.

## 2.6. Statistical analyses

Data from all tasks were analyzed using one-way/repeated measures ANOVAs or paired/unpaired t-tests (IBM SPSS 25 and Graphpad PRISM 7), where appropriate. Further analysis between groups was performed using post-hoc analyses (Tukey's HSD). In order to accurately represent cue attraction across each experiment, relative to the duration of each cue, the number of lever presses and magazine entries was standardized by calculating responses per second of cue presentation divided by the number of cue presentations (LP/Sec/CS or ME/Sec/CS). Comparison of Group Profile Vectors was done using multivariate ANOVAs. K-means clustering based on anxiety data during the initial elevated plus maze test was used to separate animals into high and low anxiety groups within Experiments 1 & 2. All analyses were two-tailed and performed at a level of significance of  $p < 0.05$ .

## 3. Results

### 3.1. Anxiety

In order to establish baseline levels of anxiety, animals were run through a 5 min session in an elevated plus maze (EPM). The amount of time spent on the two open arms was summed and used as a measure of anxiety, with greater time spent on the open arms indicating lower levels of anxiety [28]. For each separate experiment, the amount of time spent on the open arms was used to assign the rats to two groups ( $N = 8$  each) with similar means and variances. Each group was then randomly assigned to either Certain (100%-1) or Uncertain (50%-1-2-3) reward conditions. Prior to each experiment, Certain and Uncertain groups displayed no difference in anxiety across the three experimental designs (Expt 1:  $F_{(1,14)} = 0.059$ ,  $p = 0.954$ ; Expt 2:  $F_{(1,14)} = 0.007$ ,  $p = 0.994$ ; Expt 3:  $F_{(1,14)} = 0.153$ ,  $p = 0.881$ ; Fig. 1C). There was no overall difference between reward conditions across all experiments combined (Reward Condition:  $F_{(1,42)} = 0.004$ ,  $p = 0.947$ ). However, there was a difference between experiments in initial anxiety scores (Experiment:  $F_{(1,42)} = 5.051$ ,  $p = 0.011$ ) such that animals in Experiment 2 displayed lower levels of anxiety than animals in Experiment 1 (Tukey's HSD:  $p = 0.025$ ) and Experiment 3 ( $p = 0.022$ ; Fig. 1C).

### 3.2. Autoshaping

#### 3.2.1. Sign-trackers and goal-trackers and overall conditioned approach

During the autoshaping task, animals were trained through repeated CS-UCS pairings to associate the delivery of sucrose pellets with a CS (lever + auditory cue) trial consisting of a CS1 followed by a CS2 under either a Certain or Uncertain reward condition (Certain: 100%-1; Uncertain: 50%-1-2-3). Animals were also classified as sign-trackers (STs), goal-trackers (GTs), or intermediates (INTs), by calculating their response bias (LP-ME)/(LP + ME) based on their lever presses (LP) and magazine entries (ME) during CS presentations on day 10 of autoshaping.

Throughout ten days of autoshaping, the number of magazine entries and lever presses during each CS trial was recorded for each session. Across all three experiments, animals typically decreased magazine entries, while increasing lever presses. In the Sequential Design (Experiment 1), there was a significant increase in total lever presses and decrease in total magazine entries across the 10 days of autoshaping in both the Certain (Response Type:  $F_{(1,7)} = 24.060$ ,  $p = 0.002$ ; Day:  $F_{(9,63)} = 3.989$ ,  $p = 0.000$ ; Day x Response Type:  $F_{(9,63)} = 9.882$ ,  $p = 0.000$ ; Fig. 2A) and Uncertain group (Response Type:  $F_{(1,7)} = 78.461$ ,  $p = 0.000$ ; Day:  $F_{(9,63)} = 5.362$ ,  $p = 0.000$ ; Day x Response Type:  $F_{(9,63)} = 19.907$ ,  $p = 0.000$ ; Fig. 2B). Examination of the animals' response bias (LP-ME/LP + ME) indicates that, across the ten autoshaping days, both Certain and Uncertain groups similarly developed a strong sign-tracking phenotype (Day:  $F_{(9,126)} = 37.202$ ,  $p = 0.000$ ; Group:  $F_{(1,14)} = 1.916$ ,  $p = 0.188$ ; Day x Group:  $F_{(9,126)} =$

1.269,  $p = 0.260$ ; Fig. 2C).

Lever-pressing acquisition in Experiment 2 (Choice Design) closely resembled that of Experiment 1 as animals in both the Certain and Uncertain groups significantly increased total lever presses while decreasing total magazine entries (Certain: Day:  $F_{(9,63)} = 4.468$ ,  $p = 0.000$ ; Response Type:  $F_{(1,7)} = 54.360$ ,  $p = 0.000$ ; Day x Response Type:  $F_{(9,63)} = 12.407$ ,  $p = 0.000$ ; Uncertain: Day:  $F_{(9,63)} = 10.578$ ,  $p = 0.000$ ; Response Type:  $F_{(1,7)} = 9.439$ ,  $p = 0.018$ ; Day x Response Type:  $F_{(9,63)} = 9.305$ ,  $p = 0.000$ ; Fig. 2D-E), and exhibited strong sign-tracking tendencies (Response Bias: Day:  $F_{(9,126)} = 24.405$ ,  $p = 0.000$ ; Group:  $F_{(1,14)} = 1.804$ ,  $p = 0.201$ ; Day x Group:  $F_{(9,126)} = 1.474$ ,  $p = 0.242$ ; Fig. 2F).

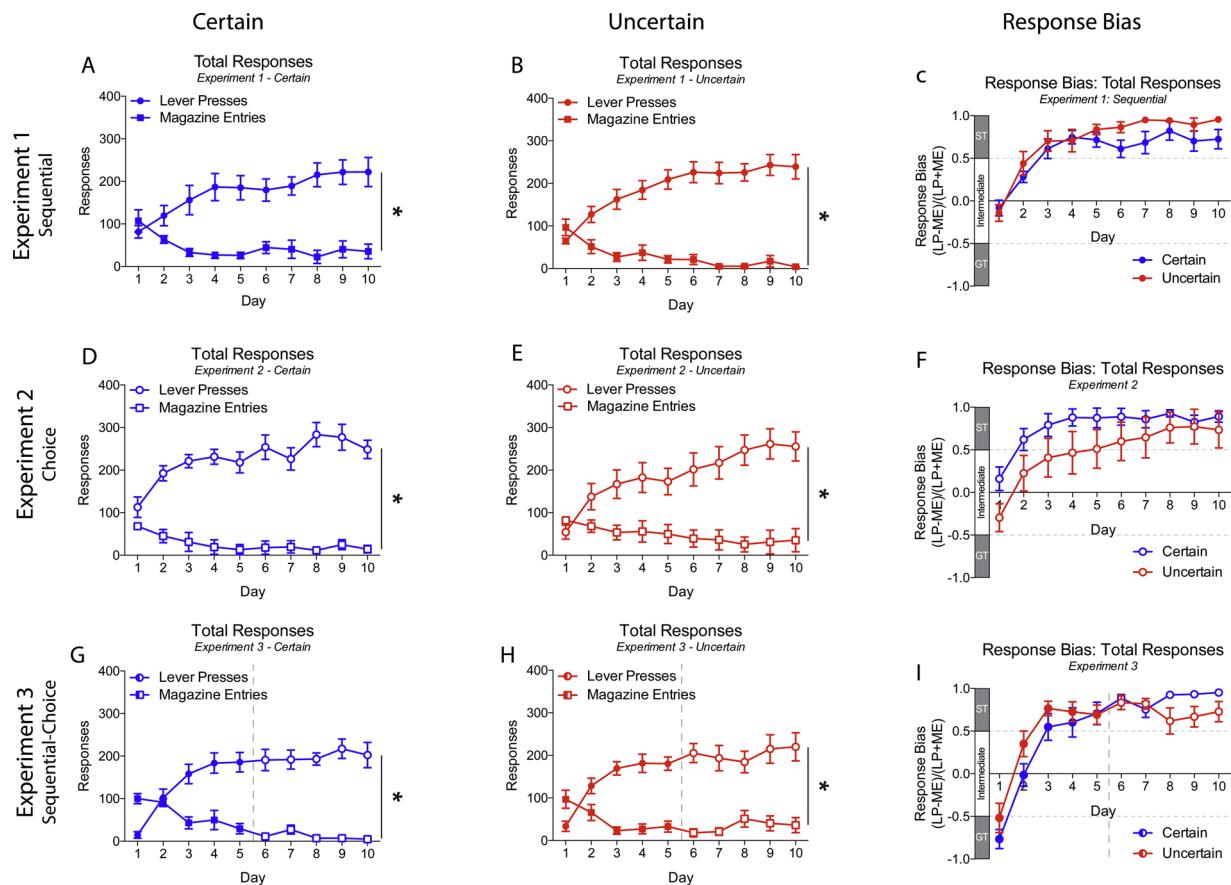
Finally, similar results were found for Experiment 3 (Sequential-Choice Design), with animals significantly increasing total lever presses and decreasing total magazine entries (Certain: Day:  $F_{(9,63)} = 5.966$ ,  $p = 0.000$ ; Response Type:  $F_{(1,7)} = 35.348$ ,  $p = 0.001$ ; Day x Response Type:  $F_{(9,63)} = 25.839$ ,  $p = 0.000$ ; Uncertain: Day:  $F_{(9,63)} = 4.359$ ,  $p = 0.000$ ; Response Type:  $F_{(1,7)} = 24.100$ ,  $p = 0.002$ ; Day x Response Type:  $F_{(9,63)} = 18.502$ ,  $p = 0.000$ ; Fig. 2G-H). Whereas both groups developed a strong sign-tracking phenotype across days (Response Bias: Day:  $F_{(9,126)} = 65.222$ ,  $p = 0.000$ ; Group:  $F_{(1,14)} = 0.018$ ,  $p = 0.896$ ; Fig. 2I), animals under the Uncertain condition appeared to initially develop sign-tracking faster, but then showed a mild decrease after day 5 when the manner with which the CS1 and CS2 were presented changed (Day x Group:  $F_{(9,126)} = 3.939$ ,  $p = 0.003$ ). Across all three experiments, the large majority of the animals had developed a strong sign-tracking phenotype by day 10 (87.5%; 42 out of 48), with only very few animals still expressing an intermediate (10.4%; 5 out of 48) or goal-tracking (2.1%; 1 out of 48) phenotype.

#### 3.2.2. Attraction to CS1 and CS2 under Certain or Uncertain reward conditions

Lever presses during the CS1 and CS2 cue presentation were recorded separately to measure cue-specific behavior. Responses per session were transformed into lever presses per second per CS presentation (LP/sec/CS), to allow for a more standardized comparison of lever interaction regardless of the duration of each cue presentation. Specifically, this enabled us to directly compare lever presses during the 8-second CS1 presentation to lever presses during the 4-second CS2 in Experiment 2. It also allowed for a direct comparison of responses to the CS1 in Experiment 3 when its duration changed from 4 to 8 s.

**3.2.2.1. Experiment 1: Cue attraction is greatest for a Predictive CS1 cue under Certain but not Uncertain conditions.** In Experiment 1, the CS1 and CS2 cue were presented sequentially for 4 s each in order to assess the relative degree of attraction attributed to each cue under Certain and Uncertain reward conditions. The rate of CS1 and CS2 LP/sec/CS across the ten days of autoshaping was compared within reward condition. While the Certain group increased responding for both the CS1 and CS2 across days and did so at similar rates (Day:  $F_{(9,63)} = 12.468$ ,  $p = 0.000$ ; Day x Lever:  $F_{(9,63)} = 0.270$ ,  $p = 0.981$ ), they showed a strong preference for the CS1, with reduced interaction with the CS2 (Lever:  $F_{(1,7)} = 11.652$ ,  $p = 0.011$ ; Fig. 3A). In contrast, animals in the Uncertain condition showed increased attraction for both the CS1 and CS2 across days but did not show a significant preference for either cue across the ten days of autoshaping (Lever:  $F_{(1,7)} = 0.037$ ,  $p = 0.853$ ; Day:  $F_{(9,63)} = 20.508$ ,  $p = 0.000$ ; Day by Lever:  $F_{(9,63)} = 1.707$ ,  $p = 0.106$ ; Fig. 3B). This suggests that under Uncertain reward conditions a similar degree of attraction is attributed to both the CS1 and CS2, compensating for any loss in attraction assigned to the CS2 due to its lower predictive value.

Analysis of the response bias for both groups highlighted differences in cue-triggered responding for CS1 and CS2. In particular, both Certain and Uncertain groups began with similar but greater attraction for the CS1 than the CS2 on Day 1 (Group:  $F_{(1,14)} = 0.615$ ,  $p = 0.446$ ; CS Type:  $F_{(1,14)} = 50.867$ ,  $p = 0.000$ ; Fig. 3C-D), initially displaying on average



**Fig. 2. Total Lever Presses, Magazine Entries and Response Bias during autoshaping for all Experiments.** A–C) Experiment 1 (Sequential Design): Both Certain and Uncertain groups performed significantly more lever presses, relative to magazine entries, resulting in a response bias indicating that most rats became sign-trackers, regardless of reward condition. D–F) Experiment 2 (Choice Design): Both groups predominantly displayed lever-pressing over magazine entries during CS trials, again showing a strong response bias towards sign-tracking behavior. G–I) Experiment 3 (Sequential-Choice Design): As above, animals in both groups developed a strong preference for lever-pressing over entering the magazine, despite changing the design mid-training. Rats in both reward conditions demonstrated strong sign-tracking behaviors during both the Sequential and Choice Design.

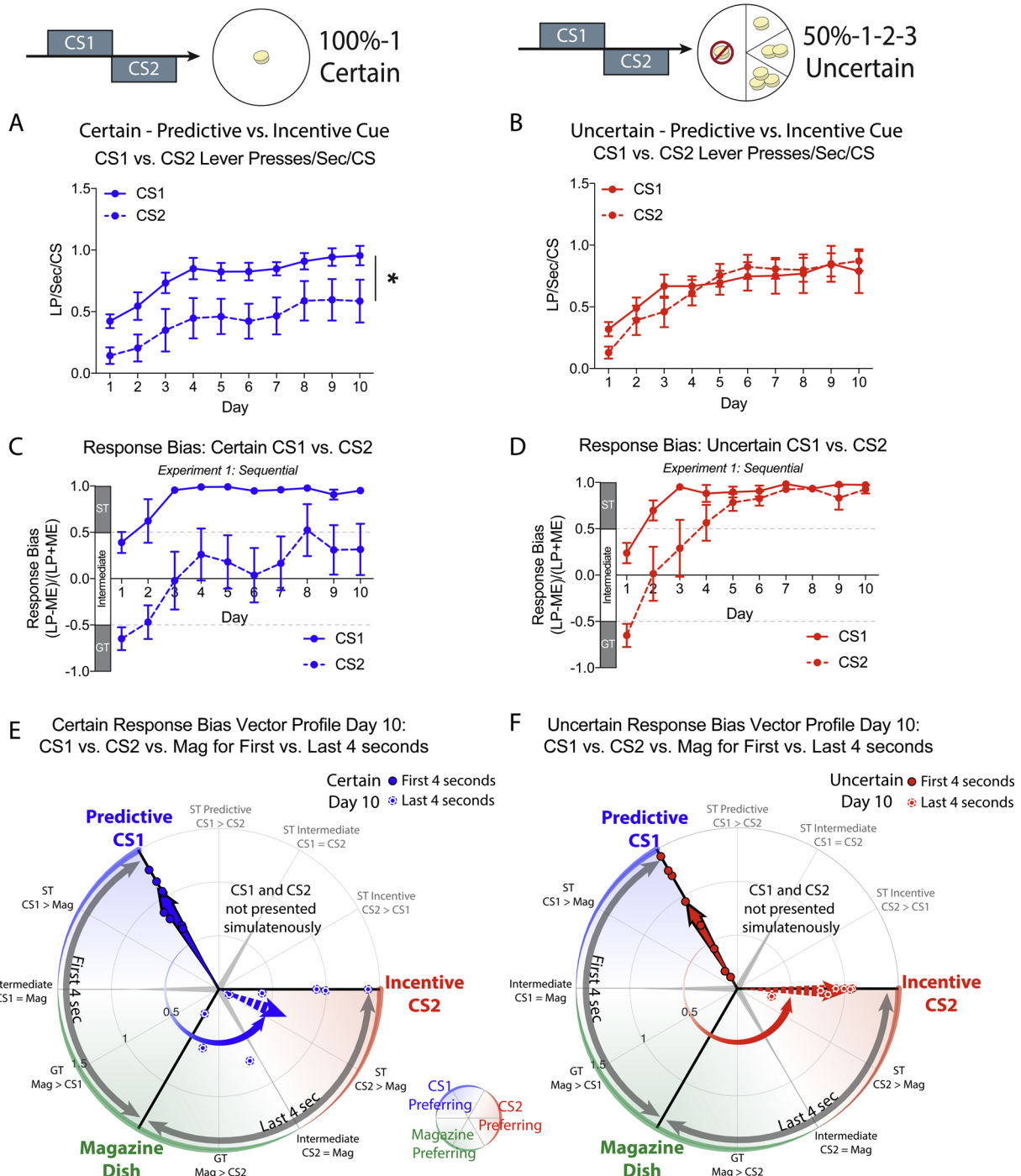
an intermediate response to the CS1, but a goal-tracking response for the CS2. This greater tendency to sign-track towards the CS1 rather than the CS2 was apparent for both groups during the first 5 days of autoshaping (Group:  $F_{(1,14)} = 0.7$ ,  $p = 0.416$ ; CS Type:  $F_{(1,14)} = 27.786$ ,  $p = 0.000$ ). However, each group displayed a different pattern of behavior during the last five days of autoshaping (Group:  $F_{(1,14)} = 4.924$ ,  $p = 0.044$ ; CS Type:  $F_{(1,14)} = 8.379$ ,  $p = 0.012$ ; Group  $\times$  CS Type:  $F_{(1,14)} = 5.657$ ,  $p = 0.032$ ). In particular, while the Certain group was still more attracted to the CS1 than CS2, displaying sign-tracking for the CS1 and an intermediate phenotype for the CS2 (CS Type:  $F_{(1,7)} = 7.239$ ,  $p = 0.031$ ; Fig. 3C), the Uncertain group displayed a similar sign-tracking phenotype to both CS1 and CS2 (CS Type:  $F_{(1,7)} = 1.676$ ,  $p = 0.236$ ; Fig. 3D).

The difference between the response to the CS1 and CS2 for animals under Certain or Uncertain conditions can be exemplified by their Vector Profile on Day 10 (Fig. 3E–F). In particular, whereas both Certain and Uncertain animals show a strong preference for the CS1 during the first 4 s of the CS trial, there is a distinct difference when the CS2 is presented during the last 4 s. Notably all Uncertain animals show a strong attraction to the CS2 (Fig. 3F), whereas at least 3 out of 8 (37.5%) animals exposed to Certain reward conditions primarily focus their behavior towards the magazine (Fig. 3E).

**3.2.2.2. Experiment 2: reward Uncertainty ascribes value to the CS2, which is ignored under Certain conditions.** To assess whether the attraction for the CS2 cue was powerful enough to draw animals away from the predictive CS1 cue, the CS1 cue was introduced first and remained

present for 8 s, with the CS2 occurring alongside the CS1 during the last 4 s of the CS trial. For comparison, lever responses were transformed into LP/Sec/CS since the CS1 (8 s) and CS2 (4 s) were presented for different amounts of time during each CS trial. Animals in both Certain and Uncertain groups significantly increased responding on the CS1 across days (Day:  $F_{(9,126)} = 15.450$ ,  $p = 0.000$ ; Fig. 4A), and did so at the same rate (Day  $\times$  Group:  $F_{(9,126)} = 0.535$ ,  $p = 0.847$ ). However, animals under Certain conditions developed a stronger preference for the CS1 across 8 s than their Uncertain counterparts (Group:  $F_{(1,14)} = 5.220$ ,  $p = 0.038$ ). This can be explained by the presentation of the CS2 during the last 4 s of the CS1, which was only ascribed with incentive value by animals under Uncertain reward conditions (Group:  $F_{(1,14)} = 5.382$ ,  $p = 0.036$ ; Day  $\times$  Group:  $F_{(9,126)} = 3.653$ ,  $p = 0.000$ ; Fig. 4B), drawing these animals away from the CS1. The result was a significant increase in responding on the CS2 for animals exposed to Uncertain reward conditions (Day:  $F_{(9,63)} = 3.499$ ,  $p = 0.001$ ), whereas animals under Certain conditions virtually ignored the CS2 across all 10 days (Day:  $F_{(9,63)} = 0.468$ ,  $p = 0.891$ ), only performing approximately one tenth of the behavior performed by the Uncertain group. Closer examination of responding on the CS1 during the first and last half of the CS trial shows that during the first 4 s, when only the CS1 is present, there is no group difference in the amount of attraction garnered by the CS1 (Group:  $F_{(1,14)} = 0.895$ ,  $p = 0.360$ ; Fig. 4C). In contrast, during the last 4 s of the CS trial, when animals were faced with a choice between the CS1 and the CS2, Uncertain animals displayed a significant decrease in attraction to the CS1 (Group:  $F_{(1,14)} = 9.148$ ,  $p = 0.009$ ; Fig. 4D).

## Experiment 1: Sequential



**Fig. 3. Analysis of CS1 vs CS2 responses for Experiment 1: Sequential Design.** A) Rats in the Certain reward condition performed more lever-presses per second per CS trial for the CS1 over the CS2, B) while animals in the Uncertain reward condition interacted with both CS1 and CS2 levers equally. C) Under Certain conditions, animals developed a sign-tracking phenotype for the CS1 but were intermediates when the CS2 was present. D) In contrast, rats in the Uncertain reward condition developed a sign-tracking response to both CS1 and CS2. E-F) Vector Profile of the response bias on Day 10 for either the CS1, CS2 or magazine dish. Arrows represent average group vector for the first (full) vs the last (dashed) 4 s of the CS trial. Concentric circles represent the average response per second per CS for either CS1, CS2, or magazine dish from 0 to 1.5 Responses/Sec/CS. The upper right quadrant is blank as CS1 and CS2 were never simultaneously presented in the Sequential design and thus no vector profile could exist in that zone. Each Vector space contains three main poles representing greatest amount of responding directed towards the Predictive CS1, Incentive CS2 or Magazine Dish. During the first 4 s (outer grey arrow labeled 'First 4 s') both Certain (blue) and Uncertain (red) animals directed all their behavior towards the predictive CS1. During the last 4 s (outer grey arrow labeled 'Last 4 s'), all Uncertain animals shifted (circular arrow) their focus towards the CS2, whereas a proportion of Certain animals (3 out of 8) directed their responses preferentially towards the magazine dish.



The way in which the presentation of the CS2 differentially affects the two groups can be observed by examining the response bias vector profile during the first 4 s (when only the CS1 and magazine are present) and the last 4 s (when the CS2 is introduced in the presence of the CS1 and the magazine) of the CS trial on Day 10 of autoshaping. This analysis reveals that the presentation of the CS2 during the last 4 s of the CS trial differentially impacts how the two groups assign incentive value and responding across the CS1, CS2 and magazine (Group x Response Type x First/Last 4 s:  $F_{(2,28)} = 5.2$ ,  $p = 0.012$ ). In particular, presentation of the CS2 during the last 4 s dramatically shifts the response profile of the Uncertain group towards the CS2 (Response Type x First/Last 4 s:  $F_{(2,14)} = 7.367$ ,  $p = 0.007$ ; Fig. 4F), creating an average response bias vector that favors the incentive value of the CS2 over the predictive value of the CS1 (dashed red arrow). Whereas animals exposed to Certain reward conditions, show no noticeable effect on the cue-triggered responding (Response Type x First/Last 4 s:  $F_{(2,14)} = 0.016$ ,  $p = 0.984$ ; Fig. 4E), and maintain an average response bias vector that strongly favors attraction towards the predictive CS1.

**3.2.2.3. Experiment 3: only under reward uncertainty does the incentive value attributed to the CS2 persist despite being challenged by the CS1.** Experiment 3 (Sequential-Choice) examined the impact of reward uncertainty on the acquisition of incentive value to a primarily incentive CS2, and its ability to retain incentive value when the presence of the predictive CS1 was extended to compete with the CS2. To do so, animals were initially exposed to 5 days of autoshaping under Sequential conditions as in Experiment 1. Here, the CS1 was available during the first 4 s and then replaced by the CS2 during the last 4 s to promote attribution of incentive value to the CS2 in the absence of the CS1. Starting on day 6, animals were suddenly exposed to the Choice condition, as in Experiment 2, whereby the CS1 remained extended for 8 s, and the CS2 was jointly present with the CS1 only during the last 4 s. This meant that while animals may have attributed incentive value to the CS2 during the first 5 days of autoshaping, when the CS2 was presented alone during the last 4 s of each CS trial, starting on day 6, the degree of incentive value attributed to the CS1 came to challenge that of the CS2.

Across the first five days of autoshaping, while animals were sequentially exposed to the CS1 then to the CS2, both Certain and Uncertain groups increased their responding on both CS1 and CS2 (Group:  $F_{(1,14)} = 0.210$ ,  $p = 0.654$ ; Day:  $F_{(4,56)} = 59.689$ ,  $p = 0.000$ ; CS Type:  $F_{(1,14)} = 2.022$ ,  $p = 0.177$ ; Fig. 5A-B). However it seemed that animals increased their responding per second for the CS2 at a higher rate across days than for the CS1 (CS Type x Day:  $F_{(4,56)} = 3.74$ ,  $p = 0.009$ ), and this trended towards significance only under Uncertain reward conditions (CS Type x Day: Uncertain:  $F_{(4,28)} = 2.703$ ,  $p = 0.051$ ; Certain:  $F_{(4,28)} = 1.426$ ,  $p = 0.251$ ). This suggests that conditions of reward uncertainty may have begun promoting greater incentive salience attribution to the CS2 than to the CS1.

Responding on the CS1 and CS2 was examined during the transition from Day 5 to Day 6 to assess the impact of presenting the CS1 for 8 rather than 4 s, thus allowing it to compete for incentive value with the CS2. Most notably, allowing the CS1 to compete for incentive value had an immediate impact on responding for animals under Certain reward conditions (Group x CS Type x Day:  $F_{(1,14)} = 5.062$ ,  $p = 0.041$ ). In particular, Certain animals displayed a precipitous drop on Day 6, to 1/10th of their responding rate for the CS2 on Day 5 (CS Type x Day:  $F_{(1,7)} = 20.696$ ,  $p = 0.003$ ; CS2 LP/Sec/CS: Day 5 M = 0.70; Day 6 M = 0.06). This occurred despite showing no change in their responding rate for the CS1, which effectively more than doubled their total responses on the CS1 as it was now available for 8 rather than 4 s (CS1 LPs: Day 5 M = 85; Day 6 M = 181.125; Fig. 5A). This meant that animals exposed to Certain reward conditions virtually ignored the CS2 now that the CS1 was also present. In contrast, animals exposed to Uncertain reward conditions appeared unaffected by the presence of the CS1 during the last 4 s of the CS trial (CS Type x Day:  $F_{(1,7)} = 1.273$ ,

$p = 0.296$ ; Fig. 5B), and instead mostly continued to attribute incentive value to the CS2.

The stark difference seen between the two groups on Day 6 as a result of suddenly giving animals a choice to approach either the CS1 or CS2 during the last 4 s continued across the four subsequent days until Day 10. In particular, animals exposed to Certain reward conditions directed almost exclusively their attention towards the CS1 (CS Type:  $F_{(1,7)} = 97.316$ ,  $p = 0.000$ ; CS Type x Day:  $F_{(4,28)} = 0.689$ ,  $p = 0.606$ ; Fig. 5A). Whereas animals under Uncertain reward conditions ascribed similar levels of attention to both the CS1 and CS2 (CS Type:  $F_{(1,7)} = 0.594$ ,  $p = 0.466$ ; CS Type x Day:  $F_{(4,28)} = 1.818$ ,  $p = 0.153$ ; Fig. 5B). Notably, this occurred despite the CS1 being continually present during each CS trial, suggesting that the CS2 garnered sufficient incentive value to draw animals away from the CS1 even when it was not retracted after 4 s.

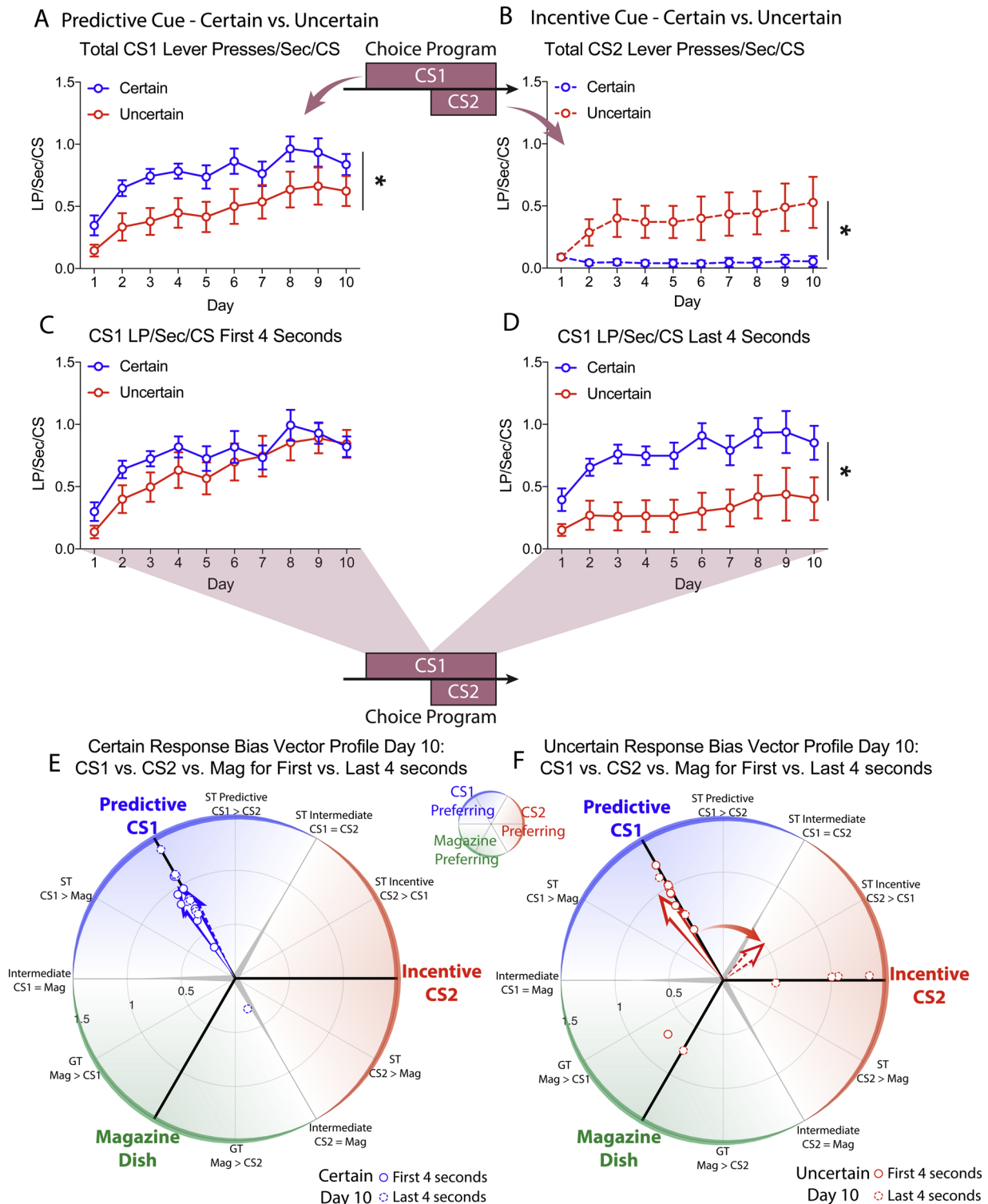
Further analysis of the profile vector for each group between Days 5 and 6 reveals that extending the CS1 for the full 8 s of the CS trial on Day 6 had a large effect on responding for animals under Certain, but not Uncertain reward conditions (Group x Day:  $F_{(1,14)} = 5.608$ ,  $p = 0.033$ ; Group x Day x Response Type:  $F_{(2,28)} = 4.443$ ,  $p = 0.021$ ; Fig. 5C-D). In particular, whereas the change in conditions affected the response pattern of animals under Certain reward conditions (Day x Response Type:  $F_{(2,14)} = 17.67$ ,  $p = 0.000$ ; Fig. 5C), by shifting responding towards the CS1, it had no effect on the pattern of responding under reward Uncertainty (Day x Response Type:  $F_{(2,14)} = 1.516$ ,  $p = 0.254$ ; Fig. 5D). This effect was even further illustrated when comparing only the last 4 s of each CS trial, where animals could initially only respond on the CS2 or the magazine on Day 5, but were given additional access to CS1 starting on Day 6 (Group x Day x Response Type:  $F_{(2,28)} = 7.408$ ,  $p = 0.003$ ; Fig. 5E-F). This analysis highlights the sudden transition under Certain reward conditions from having a primary attraction to the CS2 on Day 5 to having an almost exclusive attraction to the CS1 once it becomes available (Fig. 5E). In contrast, under reward uncertainty, a majority of animals (5 out of 8) retained a preference towards the CS2 on Day 6 (Fig. 5F).

### 3.3. Conditioned reinforcement

Following the tenth day of autoshaping, rats underwent a single conditioned reinforcement test to assess the ability of the CS1 or CS2 to act as a conditioned reinforcer. In particular, this test examined whether cues could acquire reinforcing properties even under conditions of reward uncertainty where they possess limited predictive value. The back wall of the chamber was equipped with three nosepoke ports, one which triggered a brief 4 s presentation of the CS1 (lever + auditory cue), while another triggered a presentation of the CS2 (Fig. 6A), neither of which resulted in the delivery of a sucrose reward. Finally, the center nosepoke port acted as a control and had no programmed consequence.

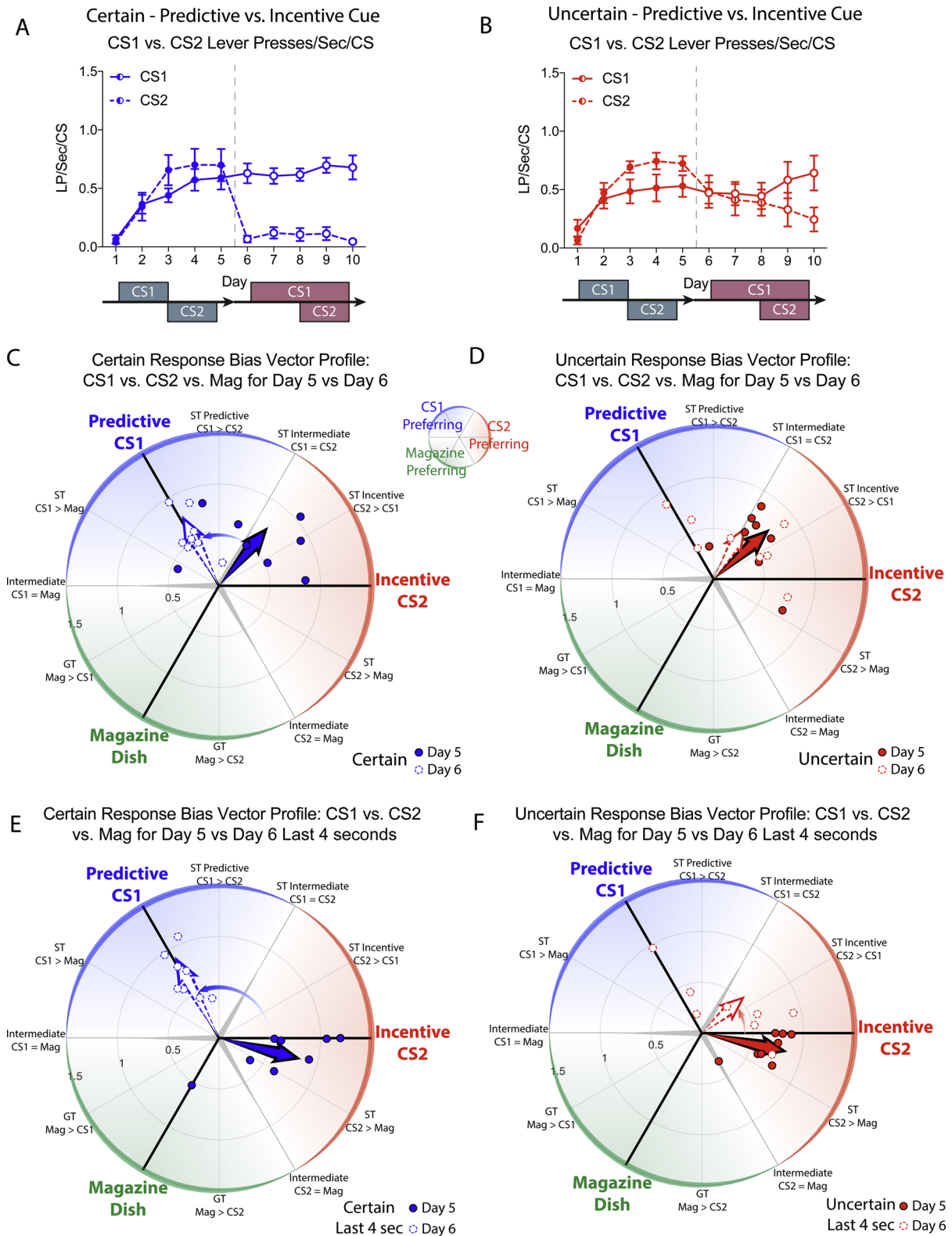
In Experiment 1 (Sequential), rats in both Certain and Uncertain conditions showed more interest for the nosepokes associated with the presentation of the CS1 or the CS2 over the control nosepoke port (Nosepoke:  $F_{(2,28)} = 32.330$ ,  $p = 0.000$ ; Group:  $F_{(1,14)} = 0.013$ ,  $p = 0.909$ ; Nosepoke x Group:  $F_{(2,28)} = 0.748$ ,  $p = 0.483$ ; Fig. 6B), suggesting that these cues had become conditioned reinforcers, despite lower predictive value under Uncertain conditions. In particular, animals under Certain reward conditions responded more for CS1 and for CS2 than on the control nosepoke (CS1-Control:  $t_{(7)} = 4.012$ ,  $p = 0.005$ ; CS2-Control:  $t_{(7)} = 4.604$ ,  $p = 0.002$ ). However, Certain animals also showed a preference for the CS1-paired noseport over the CS2-paired one (CS1-CS2:  $t_{(7)} = 2.615$ ,  $p = 0.035$ ), suggesting that the CS1 had acquired stronger reinforcing properties. In contrast, under Uncertain reward conditions, although animals favored the active nosepoke ports over the control (CS1-Control:  $t_{(7)} = 7.159$ ,  $p = 0.000$ ; CS2-Control:  $t_{(7)} = 6.869$ ,  $p = 0.000$ ), they did not show a strong preference for either, suggesting that the CS1 and CS2 had acquired similar reinforcing properties (CS1-CS2:  $t_{(7)} = 1.981$ ,  $p = 0.088$ ; Fig. 6B).

## Experiment 2: Choice



**Fig. 4. Analysis of CS1 and CS2 responses for Experiment 2: Choice Design.** A) Animals in the Certain reward condition demonstrated a significantly greater attraction to the predictive cue (CS1). B) In contrast, rats in the Uncertain reward condition performed significantly greater CS2 lever presses, while animals in the Certain reward condition almost entirely ignored the CS2. C) Breakdown of responding to the CS1 into the first and last four seconds shows that both groups displayed a similar rate of responding to the CS1 when it was the only cue present, D) but that responding on the CS1 significantly decreased in the Uncertain group during the last 4 s, as these animals presumably shifted their attention to the CS2. E) Analysis of vector profiles shows that under Certain reward conditions, there was no change in response between the first and last 4 s of the CS trial, with attention focused almost exclusively on the CS1. F) In contrast, under Uncertain reward conditions, the presentation of the CS2 shifted behavior away from the CS1 and resulted in behavior primarily directed towards the CS2.

# Experiment 3: Sequential-Choice



(caption on next page)

**Fig. 5. Analysis of CS1 and CS2 responses for Experiment 3: Sequential-Choice Design.** A) Animals in the Certain reward condition initially demonstrated similar attraction to both CS1-CS2 during the first 5 days (Sequential), but immediately shifted their behavior away from the CS2 and back to the CS1 when the Choice design was introduced on Day 6 of autoshaping. B) In contrast, rats in the Uncertain reward condition maintained equivalent attraction to the CS1 and CS2 across all 10 days of autoshaping, irrespective of the design and duration of the CS1 presentation. C) Analysis of vector profiles across the entire CS trial between Day 5 and Day 6, shows that under Certain reward conditions there is a significant shift in response towards the CS1 when the Choice program is introduced on Day 6, with all animals becoming CS1-preferring. D) In contrast, under Uncertain reward conditions, this change in conditions had virtually no effect on the average response bias. E) A more detailed analysis of the last 4 s of each CS trial shows that under Certain reward conditions, animals go from largely directing their attention towards the CS2 on Day 5, to exclusively responding to the CS1 on Day 6. F) In contrast, under Uncertain reward conditions, most animals (5 out of 8) maintained their preference for the CS2 during the last 4 s of the CS trial, despite the presence of the CS1 starting on Day 6.

In Experiment 2 (Choice), animals were trained during autoshaping with a concomitant presentation of the CS1 and CS2 during the last 4 s of each CS trial. Under these conditions, Certain animals entirely ignored the CS2, whereas reward uncertainty appeared to promote attraction to the CS2. Despite these striking differences in attraction to the CS2 during autoshaping, it appears that both Certain and Uncertain groups developed a strong preference for responding in the active nosepoke ports over the control port (Nosepoke:  $F_{(2,28)} = 16.071$ ,  $p = 0.000$ ; Group:  $F_{(1,14)} = 0.352$ ,  $p = 0.562$ ; Nosepoke x Group:  $F_{(2,28)} = 0.590$ ,  $p = 0.561$ ; Fig. 6C). In particular, under reward uncertainty, animals responded more on the CS1- and CS2-associated port than the control (CS1-Control:  $t_{(7)} = 4.065$ ,  $p = 0.005$ ; CS2-Control:  $t_{(7)} = 3.809$ ,  $p = 0.007$ ), but showed no preference for either (CS1-CS2:  $t_{(7)} = 1.033$ ,  $p = 0.336$ ; Fig. 6C), suggesting that both developed similar reinforcing properties. Surprisingly, under Certain reward conditions, animals responded more for the CS2 than on the control port (CS2-Control:  $t_{(7)} = 3.261$ ,  $p = 0.014$ ), suggesting that it had acquired conditioned reinforcer properties despite being virtually ignored during autoshaping. In addition, responding for the CS2 was similar to that of the CS1 (CS1-CS2:  $t_{(7)} = 0.699$ ,  $p = 0.507$ ), although the preference for the CS1-paired port over the control only trended towards significance (CS1-Control:  $t_{(7)} = 2.219$ ,  $p = 0.062$ ).

Finally, in Experiment 3, where animals were initially exposed to the CS1 and CS2 sequentially and were then given a choice between the CS1 and CS2 during the last four seconds, both the CS1 and CS2 appeared to garner conditioned reinforcing properties relative to the inactive nosepoke (Nosepoke:  $F_{(2,28)} = 35.307$ ,  $p = 0.000$ ; Group:  $F_{(2,28)} = 3.375$ ,  $p = 0.088$ ; Nosepoke x Group:  $F_{(1,14)} = 1.652$ ,  $p = 0.215$ ; Fig. 6D). Although there was a trend for animals in the Uncertain group to perform more nosepokes for the CS1 and CS2 than their Certain counterparts, this trend failed to reach significance (Group:  $F_{(1,14)} = 3.698$ ,  $p = 0.075$ ). Again, as in Experiment 2, despite large differences in each group's response to the CS2, especially during the last 5 days of autoshaping, animals displayed conditioned reinforcement for the CS1 and CS2 compared to the control nosepoke in both Certain (CS1-Control:  $t_{(7)} = 6.373$ ,  $p = 0.000$ ; CS2-Control:  $t_{(7)} = 5.012$ ,  $p = 0.002$ ; CS1-CS2:  $t_{(7)} = 1.41$ ,  $p = 0.201$ ) and Uncertain groups (CS1-Control:  $t_{(7)} = 5.362$ ,  $p = 0.001$ ; CS2-Control:  $t_{(7)} = 6.882$ ,  $p = 0.000$ ; CS1-CS2:  $t_{(7)} = 0.074$ ,  $p = 0.943$ ).

Across all three experiments, each animal consistently responded on either the CS1 or CS2 lever when it was presented, suggesting that they were nosepoking specifically to gain access to the cues. There were however no differences in the number of CS1 and CS2 cue presentations obtained across all three experiments for either group (Group:  $F$ 's  $< 2.634$ ,  $p$ 's  $> 0.126$ ), irrespective of the cue type (CSType x Group:  $F$ 's  $< 1.073$ ,  $p$ 's  $> 0.317$ ). Instead there was a trend, only in Experiment 1, for animals to obtain more CS1 cue presentations, independent of their assigned reward condition (CSType:  $F_{(1,14)} = 4.234$ ,  $p = 0.059$ ; Experiment 2 & 3:  $F$ 's  $< 0.010$ ,  $p$ 's  $> 0.926$ ).

### 3.4. The effect of amphetamine and nicotine on CS1 and CS2 attraction

Following conditioned reinforcement, animals in Experiments 1 and 2 received two days of autoshaping to re-establish conditioned approach behavior, under either the Sequential or Choice conditions, respectively. On days 3 and 4, animals received an injection of either

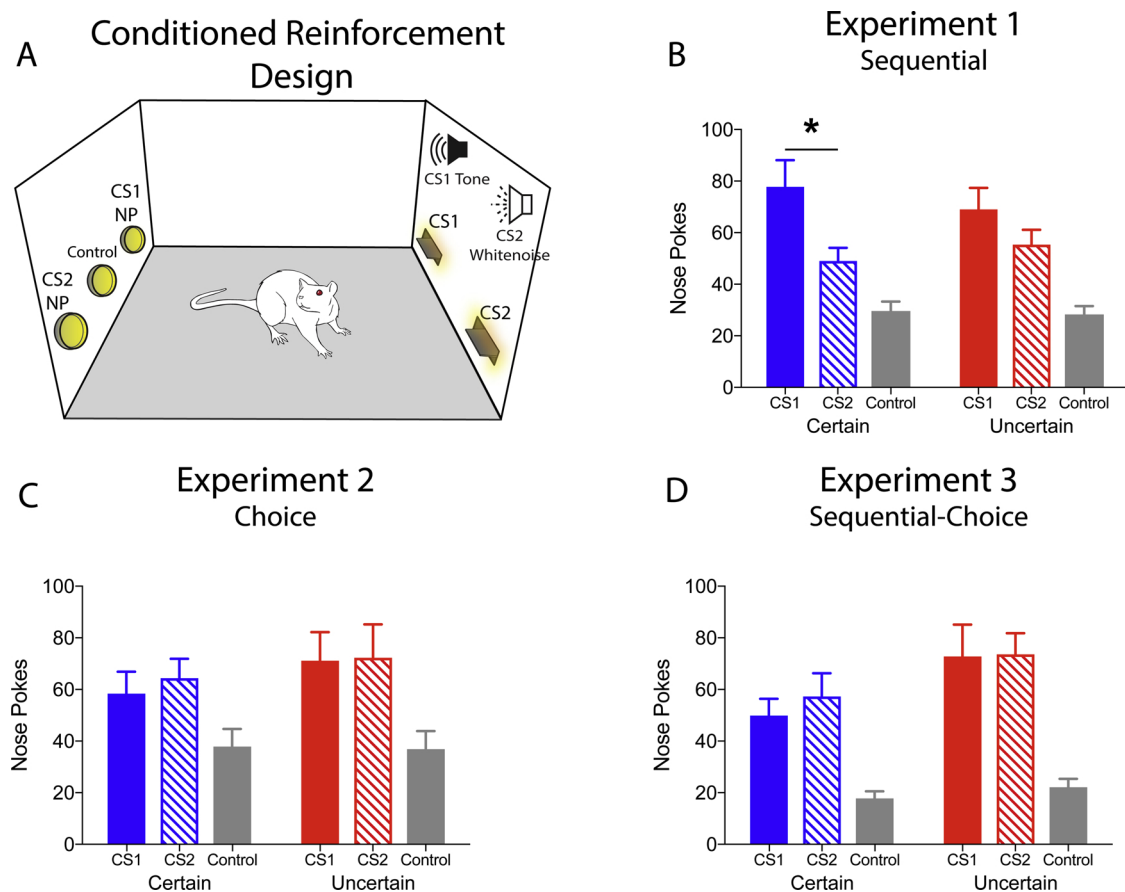
amphetamine or nicotine prior to their autoshaping session and their interaction with the CS1, CS2 and magazine dish was measured.

Approach behavior in animals in Experiment 1 was analyzed separately during the first and last 4 s of each CS trial in order to independently examine the effects of amphetamine and nicotine on either the CS1 or CS2 separately. During the first 4 s, when animals were presented with the CS1 and the magazine, amphetamine, but not nicotine, affected responding differentially across cues (Amphetamine x Cue Type:  $F_{(1,14)} = 5.982$ ,  $p = 0.028$ ; Nicotine x Cue Type:  $F_{(1,14)} = 0.133$ ,  $p = 0.721$ ; Fig. 7A). Specifically, amphetamine increased attraction towards the magazine (Magazine:  $F_{(1,14)} = 7.665$ ,  $p = 0.015$ ) and tended to decrease responding on the CS1 (CS1:  $F_{(1,14)} = 3.526$ ,  $p = 0.081$ ), and did so similarly across both Certain and Uncertain conditions (Group:  $F_{(1,14)} = 1.169$ ,  $p = 0.298$ ). During the last 4 s of the CS trial, the CS1 was retracted, and animals were instead presented with the CS2 and the magazine. In contrast to the first 4 s of the CS trial, neither amphetamine nor nicotine appeared to have any effect on responding for the CS2 and the magazine (Amphetamine x Cue Type:  $F_{(1,14)} = 1.051$ ,  $p = 0.323$ ; Nicotine x Cue Type:  $F_{(1,14)} = 0.044$ ,  $p = 0.837$ ; Fig. 7A), suggesting that responding to the CS2 was more resilient to drug manipulations than was responding for the CS1. Instead, similar to prior training, animals under Uncertain reward conditions displayed greater attraction for the CS2, and largely ignored the magazine when compared to their Certain counterparts (Amphetamine: Group x Cue Type:  $F_{(1,14)} = 5.232$ ,  $p = 0.038$ ; Nicotine: Group x Cue Type:  $F_{(1,14)} = 5.212$ ,  $p = 0.039$ ) in a manner that seemed impervious to drug treatment.

In Experiment 2, results were analyzed separately during the first and last 4 s of each CS trial in order to distinguish whether an impact of drug treatment was specific to a given cue (e.g. CS1 vs CS2) or the timing of when a cue was introduced within the CS trial (first vs last 4 s). Results suggest that during the first 4 s of the CS trial, amphetamine had opposing effects on responding for the CS1 and the magazine (Amphetamine x Cue Type:  $F_{(1,14)} = 11.788$ ,  $p = 0.004$ ; Fig. 7B). In particular, amphetamine significantly decreased responding on the CS1 (Amphetamine:  $F_{(1,14)} = 14.962$ ,  $p = 0.002$ ), while increasing attraction and responding in the magazine (Amphetamine:  $F_{(1,14)} = 6.672$ ,  $p = 0.022$ ). A similar yet weaker effect could be seen with nicotine (Nicotine x Cue Type:  $F_{(1,14)} = 5.457$ ,  $p = 0.035$ ; Fig. 7B), which only significantly increased magazine responding relative to saline (Nicotine:  $F_{(1,14)} = 8.317$ ,  $p = 0.012$ ), but had no impact on CS1 responding (Nicotine:  $F_{(1,14)} = 1.78$ ,  $p = 0.203$ ).

Finally, when the CS2 was also presented during the last 4 s, there was a trend towards a selective effect of amphetamine across cues, but no effect of nicotine (Amphetamine x Cue Type:  $F_{(1,14)} = 2.798$ ,  $p = 0.078$ ; Nicotine x Cue Type:  $F_{(1,14)} = 1.066$ ,  $p = 0.358$ ; Fig. 7B). Again, amphetamine reduced the attraction of the CS1 in both groups (Amphetamine:  $F_{(1,14)} = 4.768$ ,  $p = 0.047$ ), but this time without significantly increasing behavior in the magazine (Amphetamine:  $F_{(1,14)} = 1.971$ ,  $p = 0.182$ ) or having any effect on the CS2 (Amphetamine:  $F_{(1,14)} = 0.134$ ,  $p = 0.720$ ) for which responding still remained greater under Uncertain reward conditions (Group:  $F_{(1,14)} = 5.106$ ,  $p = 0.040$ ). This suggests that the effect of amphetamine was to specifically decrease responding on the CS1 and not the CS2, and redirecting it towards the magazine, and that this effect was consistent throughout the CS trial and not restricted only to the first 4 s of the trial.





**Fig. 6. Conditioned Reinforcement design and analysis across experiments.** A) An illustration of the operant chamber design used for conditioned reinforcement. For this task, the boxes were equipped with three illuminated nose pokes on the back wall of the chamber. There were two active nose pokes (one for CS1, one for CS2, location counterbalanced) on either side of an inactive control nose poke in the middle. Responses on each active nosepoke resulted in a 3-second cue presentation (lever + auditory cue) of either the CS1 or CS2 (same location as during autoshaping) on the front wall, while entry into the inactive nose poke had no programmed consequence. B) In Experiment 1 (Sequential Design), rats in both Certain and Uncertain conditions showed more interest for the nosepokes associated with the presentation of the CS1 or CS2 over the control nosepoke port. However, rats in the Certain, but not the Uncertain group, showed a significant preference for the nosepoke associated with the presentation of the CS1. C) In Experiment 2 (Choice Design), both Certain and Uncertain groups developed a strong preference for responding in the active nosepoke ports over the control port. However, under both conditions there was no significant preference for one nose poke over the other, suggesting that both developed similar reinforcing properties, despite the Certain group mostly ignoring the CS2 during autoshaping. D) In Experiment 3 (Switch Design), both animals in the Certain and Uncertain groups displayed conditioned reinforcement for the CS1 and CS2 compared to the control nosepoke, and the Uncertain group showed a trend towards performing more nosepokes for the CS1 and CS2 than their Certain counterparts.

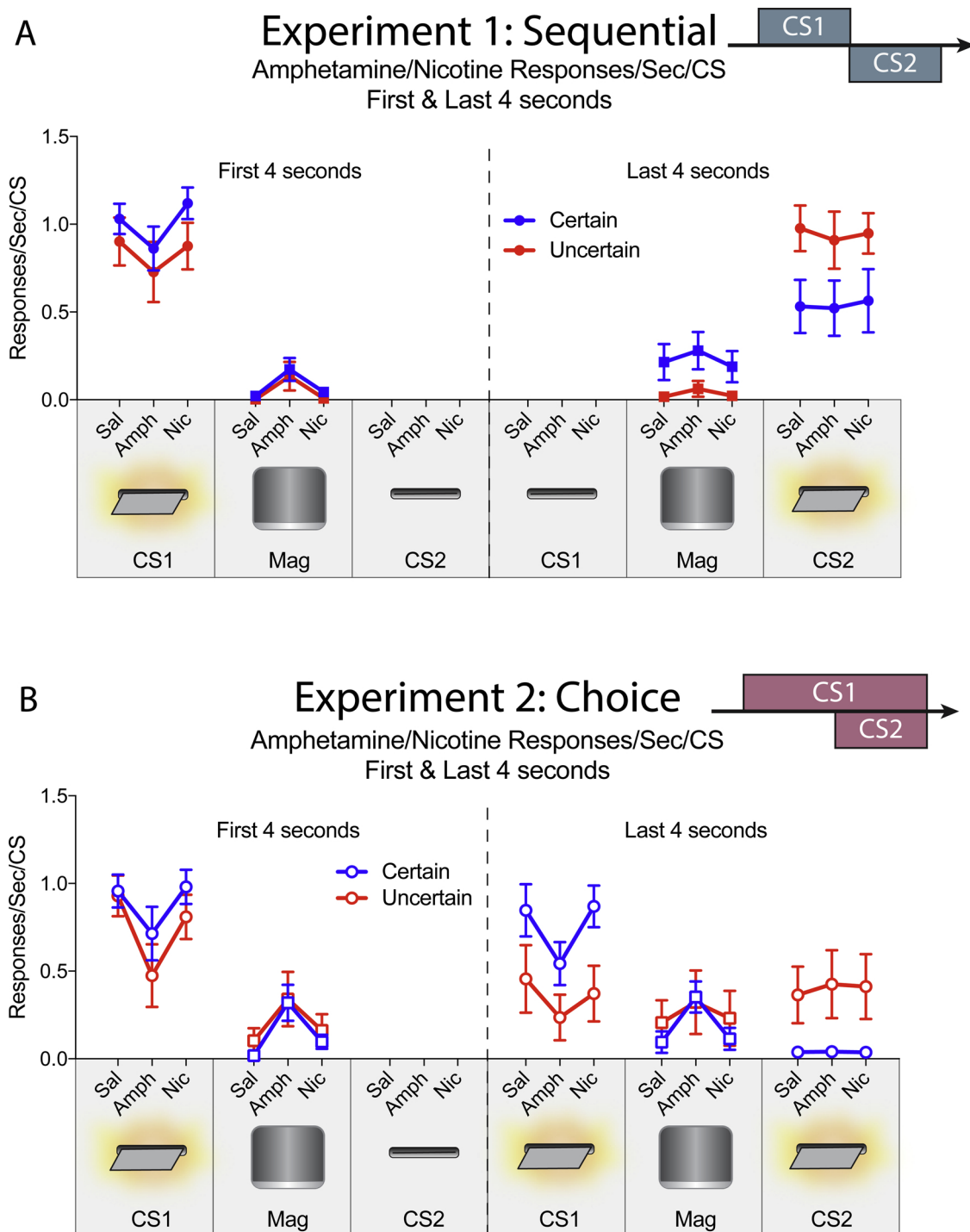
### 3.5. Operant conditioning and progressive ratio

Animals in Experiment 1 and 2 were then trained to acquire operant responding, before being exposed to a progressive ratio in order to examine the effect of incentive salience attribution and reward uncertainty on the ability of either lever to promote reward seeking. Both CS1 and CS2 levers were extended during the entire session and associated with reward delivery of a single sucrose pellet first on a fixed ratio of 1 (FR1) and then on a progressive ratio (PR).

In Experiment 1, animals under Certain or Uncertain reward conditions had been previously exposed to the CS1 and CS2 lever sequentially during autoshaping, and thus received equal amount of exposure to both levers (4 s each). Here, both groups rapidly acquired operant responding when the levers were extended throughout the session and each response was paired with the delivery of a single sucrose pellet. Although there was no difference in the animals' preference for either the CS1 or CS2 lever (Lever:  $F_{(1,14)} = 0.043$ ,  $p = 0.839$ ), animals exposed to reward uncertainty performed significantly more reward trials than their Certain counterparts (Group:  $F_{(1,14)} = 6.674$ ,  $p = 0.022$ ; Group x Lever:  $F_{(1,14)} = 0.696$ ,  $p = 0.418$ ; Fig. 8A). There was however no effect of reward uncertainty when

animals were subsequently exposed to a progressive ratio (Group:  $F_{(1,14)} = 0.024$ ,  $p = 0.878$ ; Lever:  $F_{(1,14)} = 0.494$ ,  $p = 0.494$ ; Group x Lever:  $F_{(1,14)} = 0.575$ ,  $p = 0.461$ ; Fig. 8B).

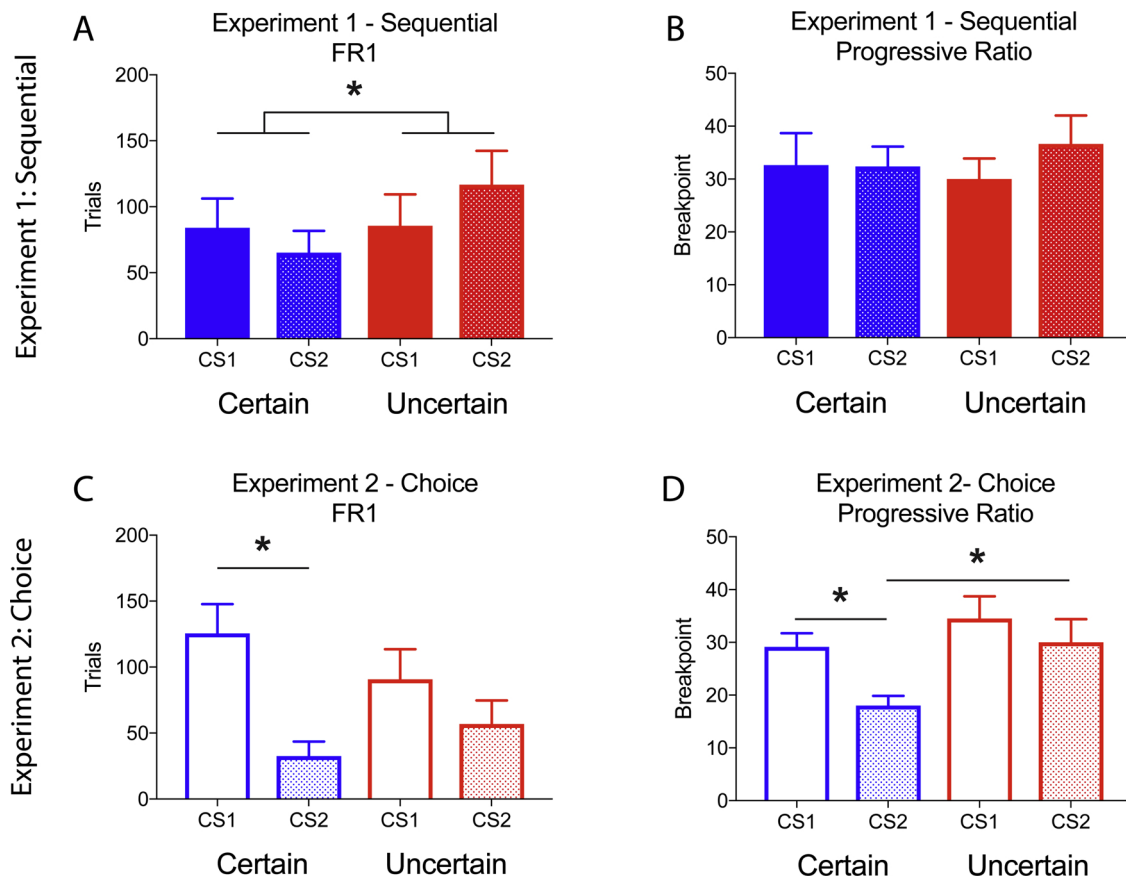
In Experiment 2, animals were presented first with the CS1 and then to both CS1 and CS2 during autoshaping, meaning that they were exposed to the CS1 for twice as much time as the CS2. As a likely result, animals showed a strong preference for responding on the CS1 lever during FR1 conditions (Group:  $F_{(1,14)} = 0.171$ ,  $p = 0.686$ ; Lever:  $F_{(1,14)} = 7.12$ ,  $p = 0.018$ ; Group x Lever:  $F_{(1,14)} = 1.543$ ,  $p = 0.235$ ; Fig. 8C). However, further examination showed that whereas animals exposed to Certain reward conditions during autoshaping acquired reward seeking more readily on the CS1 lever (CS1-CS2:  $t_{(7)} = 3.323$ ,  $p = 0.013$ ), this was not the case for animals exposed to Uncertain reward conditions (CS1-CS2:  $t_{(7)} = 0.882$ ,  $p = 0.407$ ). When motivation and reward seeking was examined using a progressive ratio, animals exposed to Uncertain reward conditions reached higher breakpoints for reward (Group:  $F_{(1,14)} = 17.2$ ,  $p = 0.001$ ; Lever:  $F_{(1,14)} = 3.154$ ,  $p = 0.097$ ; Group x Lever:  $F_{(1,14)} = 0.567$ ,  $p = 0.464$ ; Fig. 8D), most notably, uncertainty resulted in specifically greater breakpoints on the CS2 (CS2:  $t_{(14)} = 2.516$ ,  $p = 0.025$ ), but not the CS1 lever (CS1:  $t_{(14)} = 1.081$ ,  $p = 0.298$ ) than their Certain counterparts. In particular,



**Fig. 7.** Effects of acute amphetamine and nicotine on Lever Presses and Magazine Entries. A) In Experiment 1 (Sequential Design), during the first four seconds of the trial (left panel), amphetamine increased attraction to the magazine and decreased attraction to the CS1 in both the Certain and Uncertain group, while nicotine had no significant effect on responding. However, during the last four seconds (right panel), animals under Uncertain reward conditions maintained their greater attraction for the CS2 and ignored the magazine, but neither nicotine or amphetamine had any effect on responding for either the CS2 or the magazine, suggesting that the CS2 was resilient to drug manipulations. B) In Experiment 2 (Choice Design), during the first four seconds of the trial, amphetamine decreased responding on the CS1, while increasing attraction for the magazine for both Certain and Uncertain conditions. Nicotine in contrast, only significantly increasing magazine attraction, but had no effect on CS1 attraction. During the last four seconds, when both the CS1 and CS2 were presented together, amphetamine reduced attraction to the CS1 in both conditions, however it did not significantly affect magazine or CS2 attraction. Nicotine had no significant effect on CS1, CS2, or magazine attraction. Similar to autoshaping, responding for the CS2 still remained greater in the Uncertain reward condition.

Certain but not Uncertain animals displayed a greater breakpoint and thus greater motivation to respond on the CS1 rather than the CS2 lever (CS1 vs CS2: Certain:  $t_{(7)} = 2.851$ ,  $p = 0.025$ ; Uncertain:  $t_{(7)} = 0.571$ ,  $p = 0.586$ ; Fig. 8D).

Overall, although both CS1 and CS2 levers were presented simultaneously during the Progressive Ratio session, the average total number of sucrose rewards attained by the animals in either experiment averaged approximately 20 rewards, arguing against possible satiation effects.



**Fig. 8. Operant conditioning and progressive ratio for CS1 vs CS2.** A) In order to examine the effects of incentive salience attribution and reward uncertainty on the ability of CS1 and CS2 levers to promote reward seeking, animals were initially run on a fixed ratio of 1 (FR1), where the CS1 and CS2 levers were extended during the entire session. Animals from Experiment 1 (Sequential), showed no preference for either CS1 or CS2, although animals in the Uncertain reward condition performed significantly more trials. B) The same animals showed no difference in breakpoint when subsequently run on a progressive ratio that was independently applied to both CS1 and CS2 levers. C) In Experiment 2 (Choice), animals in the Certain reward condition responded significantly more on the CS1 during initial acquisition of operant conditioning (FR1). However, the animals in the Uncertain reward condition, displayed equivalent motivation to respond on both CS1 and CS2. D) Under a progressive ratio, animals in the Uncertain reward condition showed similar breakpoints for the CS1 and CS2. In contrast, animals exposed to Certain reward conditions showed a higher breakpoint for CS1 than CS2, and had a lower CS2 breakpoint than the Uncertain condition.

### 3.6. Post-conditioning anxiety

Following all conditioning procedures, animals were once again exposed to the elevated plus maze in order to assess the impact of conditioning and reward uncertainty on anxiety.

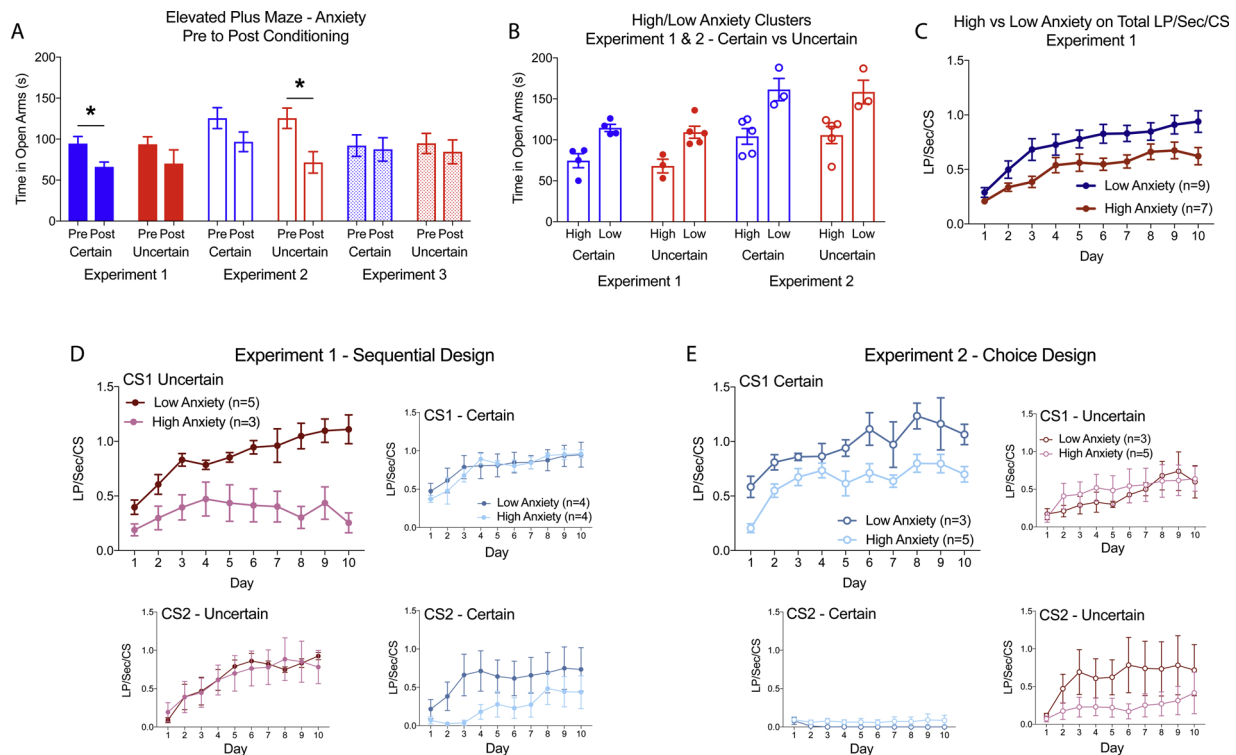
There were no overall differences in anxiety between Certain and Uncertain animals or across experiments following conditioning (Post-EPM: Group:  $F_{(1,42)} = 0.558$ ,  $p = 0.459$ ; Experiment:  $F_{(2,42)} = 1.097$ ,  $p = 0.343$ ; Group  $\times$  Experiment:  $F_{(2,42)} = 0.664$ ,  $p = 0.520$ ; Fig. 9A). There was however an effect of conditioning on anxiety when the time spent in the open arm was compared between pre- and post-conditioning (Pre-Post:  $F_{(1,42)} = 14.398$ ,  $p = 0.000$ ). In particular, anxiety was increased (seen as a decrease in time spent in the open arms) in both Experiment 1 & 2 (Pre-Post: Experiment 1:  $F_{(1,14)} = 7.384$ ,  $p = 0.017$ ; Experiment 2:  $F_{(1,14)} = 13.337$ ,  $p = 0.003$ ; Experiment 3:  $F_{(1,14)} = 0.333$ ,  $p = 0.573$ ). In Experiment 1 this was largely due to an increase in anxiety under Certain reward conditions (Certain:  $t_{(7)} = 4.654$ ,  $p = 0.002$ ; Uncertain:  $t_{(7)} = 1.302$ ,  $p = 0.234$ ), and the reverse was true in Experiment 2 (Certain:  $t_{(7)} = 1.826$ ,  $p = 0.111$ ; Uncertain:  $t_{(7)} = 3.32$ ,  $p = 0.013$ ; Fig. 9A).

### 3.7. Anxiety clusters - high/low anxiety

In order to investigate the effects of anxiety levels on sensitivity to reward-related cues, animals were separated into high and low anxiety

groups in Experiment 1 (Sequential Design) and Experiment 2 (Choice Design). K-means clustering was performed separately for each experiment based on the amount of time spent in the open arms of the elevated plus maze during initial EPM testing regardless of certainty condition. As shown in Fig. 9B, clustering effectively produced high and low anxiety groups with significantly different levels of anxiety that were similar across reward conditions for both Experiment 1 (High/Low Anxiety:  $F_{(1,12)} = 29.238$ ,  $p = 0.000$ ; Group:  $F_{(1,12)} = 0.617$ ,  $p = 0.447$ ) and Experiment 2 (High/Low Anxiety:  $F_{(1,12)} = 21.311$ ,  $p = 0.001$ ; Group:  $F_{(1,12)} = 0.003$ ,  $p = 0.954$ ; Fig. 9B).

The possible impact of high and low anxiety clusters on cue attraction was then examined across Certain and Uncertain reward conditions for each experiment. For Experiment 1, anxiety decreased the degree of CS attraction across all animals (Anxiety:  $F_{(1,12)} = 4.991$ ,  $p = 0.045$ ; Fig. 9C). More importantly, anxiety differentially impacted responding on the CS1 and CS2 depending on Certain or Uncertain reward conditions (CS Type  $\times$  Anxiety  $\times$  Group:  $F_{(1,12)} = 8.797$ ,  $p = 0.012$ ). In particular, under Uncertain reward conditions, animals with lower levels of anxiety showed greater attraction for the CS1 and increased their responding across days (Anxiety:  $F_{(1,6)} = 15.543$ ,  $p = 0.008$ ; Anxiety  $\times$  Day:  $F_{(9,54)} = 4.701$ ,  $p = 0.000$ ; Fig. 9D), whereas attraction for the CS2 was similar for both high and low anxiety animals (Anxiety:  $F_{(1,6)} = 0.007$ ,  $p = 0.936$ ). In contrast, there was no effect of anxiety under Certain reward conditions (CS1 - Anxiety:  $F_{(1,6)} = 0.023$ ,  $p = 0.884$ ; CS2 - Anxiety:  $F_{(1,6)} = 2.203$ ,  $p = 0.188$ ), although 3 out of



**Fig. 9.** Anxiety pre- and post-conditioning and the impact of anxiety on CS1 and CS2 cue attraction during autoshaping. A) Anxiety levels were measured again after conditioning and compared to baseline anxiety levels prior to conditioning procedures. Anxiety levels showed an increase (decreased open arm time) in the Certain condition of Experiment 1, and the Uncertain condition of Experiment 2. B) For each experiment, animals were divided into high and low anxiety groups through K-means clustering based on their open arm time pre-conditioning. C) In Experiment 1, across all conditions, animals in the low anxiety cluster performed a significantly greater rate of lever presses than the high anxiety cluster. D) In Experiment 1, Uncertain animals in the low anxiety group were more attracted to the CS1 over the ten days of autoshaping. E) In Experiment 2, low anxiety animals in the Certain condition displayed greater attraction to the CS1.

4 low anxiety animals tended to show greater attraction for the incentive CS2 cue.

In Experiment 2, when animals were given a choice between the CS1 and CS2 during the last 4 s, there was a trend towards a general effect of anxiety and an interaction of anxiety on the CS1 and CS2 cue depending on the reward condition (Anxiety:  $F_{(1,12)} = 4.052$ ,  $p = 0.067$ ; CS Type  $\times$  Anxiety  $\times$  Group:  $F_{(1,12)} = 3.592$ ,  $p = 0.082$ ). This effect was driven by greater CS1 cue attraction in low anxiety animals under Certain reward conditions (Anxiety:  $F_{(1,6)} = 12.191$ ,  $p = 0.013$ ; Fig. 9E). There was otherwise no other effect of anxiety ( $F$ 's  $< 2.097$ ,  $p$ 's  $> 0.198$ ).

## 4. Discussion

### 4.1. Reward uncertainty recruits and ascribes incentive value to a CS2

In the present study we examined the impact of reward uncertainty on the attraction to cues bearing predominantly either predictive or incentive value. In the current design, the CS1 carries the most predictive value as it signals the initiation of each CS trial. Overall we found that although reward uncertainty degrades the predictive value of the CS1, this does not appear to noticeably decrease its attraction, as measured by similar levels of CS1 sign-tracking during the first four seconds of each trial. However, reward uncertainty increased the attraction and interaction with a secondary lever cue (CS2) that was more proximal to reward delivery, and which carried little to no additional predictive information. This was in contrast to animals exposed to Certain reward conditions who for example, in Experiment 1, developed a strong preference and attraction towards the predictive CS1 during the initial 4 s of each CS trial and showed diminished attraction towards the CS2 during the last 4 s of the trial. This is in line with previous studies suggesting that the CS1, which in this case is the cue

that is more distal in time to the reward, develops the most attraction over time [21,27,36]. However, this was not the case for animals exposed to reward uncertainty in Experiment 1. Under Uncertain conditions, animals developed equal attraction to both the CS1 and CS2. This suggests that despite the reduced predictive information carried by the CS1, animals were still strongly attracted to it, and in addition also attributed high levels of incentive value towards the CS2.

In the present experiments we did not find a significantly greater amount of sign-tracking under conditions of reward uncertainty as previously reported [13]. However, this is likely due to the interaction of reward uncertainty with the design of the task. Most notably, the presentation of the CS2 during each CS trial drew animals under Uncertain reward conditions away from the CS1 and thus reduced the time each animal spent in contact with either lever. It is also worth noting that the current study comprised a high proportion of sign-trackers (87.5%), which could provide a ceiling effect making it hard for reward uncertainty to further increase the rate of sign-tracking or number of sign-trackers. However the high proportion of sign-trackers seen here is similar to previous studies we have published [13,16,17], and can possibly be explained in part by several factors, notably the vendor from which the animals initially came from [37], the use of multiple CS levers [14] and of a compound (lever + tone) cue [23,38].

### 4.2. Under Certain conditions, the CS1 and CS2 elicit distinct patterns of approach behavior

Our results for Experiment 1 show that starting on Day 1 of autoshaping, both Certain and Uncertain groups display an initial preference for the CS1 over the CS2 in terms of lever responses and their response bias. In particular, animals in both groups display an intermediate response (equivalent amounts of sign- and goal-tracking) when



the CS1 is presented, but primarily goal-track in response to the CS2 presentation during the last 4 s of the CS trial. This suggests that the predictive value of the CS1 initially draws the most attention and attraction, while the CS2 is virtually ignored, and highlights the fact the these two cues do not share the same psychological properties. However, across training, the CS1 and CS2 acquire more incentive value, yet only under Uncertain reward conditions does this initial bias towards the CS1 fully disappear overtime, resulting in similar levels of sign-tracking to both cues. In contrast, Certain animals sign-track in response to the CS1, but show an intermediate response to the CS2. This suggests that animals can exhibit both sign-tracking and goal-tracking phenotypes for similar cues with close temporal relationships, but that reward uncertainty ascribes more incentive value to cues that might otherwise be partially ignored. This is in line with previous findings showing that reward uncertainty would produce greater approach and interaction with a spatially distal lever cue placed on the back wall of a testing chamber, away from the magazine dish [14].

#### 4.3. Reward uncertainty increases the conditioned reinforcing properties of incentive cues, despite reduced predictive value

Animals under Certain reward conditions ascribed high levels of both predictive and incentive value to the CS1, and only ascribed reduced incentive value to the CS2. This difference in incentive salience translated into the CS1 displaying more potent conditioned reinforcer properties than the CS2, as measured in the conditioned reinforcement test for Certain animals. In contrast, Uncertain animals ascribed similar levels of incentive value to both CS1 and CS2, and as a result worked equivalently hard to gain access to both cues in conditioned reinforcement. The finding that uncertain Pavlovian cues still develop conditioned reinforcing properties despite reduced predictive value is in line with previous studies [16,17], but also suggests that sequential cues under Uncertain reward conditions, like in slot machine gambling, acquire rewarding properties that may promote further gambling.

#### 4.4. Under reward uncertainty, the CS2 can become more attractive than the CS1

Our results from Experiment 1 implied that the CS2 acquires more incentive value and is attractive to all animals under Uncertain reward conditions. However, it remained unclear whether the incentive value given to the CS2 was greater than that of a predictive CS1 with diminished predictive value in the Uncertain conditions. Experiment 2 examined this question by giving animals a choice to show their attraction to the CS2 during the last 4 s of the CS trial, while the CS1 was still present. Unsurprisingly, animals under Certain reward conditions exclusively engaged with the CS1 and consistently ignored the CS2 during the last 4 s. This supports the idea that the CS2 does not provide any further information to these animals and is largely overshadowed by the presence of the CS1 [39]. In contrast, animals under Uncertain reward conditions were drawn away from the CS1 and directed their attention towards the CS2. This suggests that for them, the CS2 had acquired greater levels of incentive value than even the CS1, although this effect was prominent in only half (4 out of 8) the animals exposed to uncertainty.

For animals exposed to reward uncertainty, this translated again into strong conditioned reinforcing properties for both the CS1 and CS2. However, surprisingly, animals exposed to Certain conditions ascribed conditioned reinforcing properties to the CS2 despite virtually ignoring it during autoshaping [7,40]. One possible explanation for this finding, is that under Certain reward conditions, animals still attributed incentive value to the CS2, making it a conditioned reinforcer, but to a far lesser extent than the CS1, making the CS1 consistently preferred and chosen over the CS2 during autoshaping. Previous studies by Meyer and colleagues have shown that when the form of the CS limits approach

behavior, in their case using a diffuse auditory CS as opposed to an extended lever, the cue can still acquire reinforcing efficacy [23]. Their results also raise the possibility that given the compound nature of the CSs used in the current experiments (lever + auditory cue), that it is the auditory cue that is driving conditioned reinforcement (although see [38]). In either case, our findings shed further light on the manner with which cues acquire reinforcing properties. They suggest that even two cues sharing similar sensory properties can be simultaneously ascribed with incentive value and become conditioned reinforcers, even when one of these cues garners little to no attention [41].

#### 4.5. Similar rates of sign-tracking may hide underlying differences in the degree of incentive value attributed to cues

The amount to which a particular cue garners attention, as measured by rates of lever pressing and sign-tracking, may not always fully convey the underlying, and at times relative degree, of incentive value carried by that cue. This is best exemplified by Experiment 3, where animals were initially exposed to the CS1 and CS2 sequentially for the first 5 days of training. In this case, both groups developed similar levels of lever presses and sign-tracking for both the CS1 and CS2, suggesting similar amounts of incentive salience placed on both cues. However, underlying differences in the value attributed to each cue only became apparent on Day 6 when the design of cue presentation was suddenly changed, and the CS1 presentation was extended to 8 s so that it was simultaneously present with the CS2 during the last 4 s. Certain animals immediately changed their behavior and focused exclusively on the CS1, and consequently ignored the CS2, as they had in Experiment 2. In contrast, this was not the case for the Uncertain group, as they largely maintained their behavior directed towards the CS2, spending their first 4 s on the CS1, before directing their attention to the CS2 during the last 4 s. Therefore, although the amount of lever pressing and sign-tracking for the CS2 was initially identical for the two groups during the first 5 days, the underlying differences only became apparent when a choice was available.

Again, despite these differences in lever pressing and sign-tracking to the CS1 and CS2, both cues acquired conditioned reinforcing properties and were sought after when rats were given the chance to learn a new behavior for access to them. However it should be noted that in this case, animals under Uncertain reward conditions tended to work harder for these CSs, suggesting a tendency towards greater motivation for these cues.

Overall, the results from autoshaping suggest that reward uncertainty ascribes a greater amount of incentive value to the CS2, without diminishing the attraction of the CS1, despite decrements to its predictive value.

#### 4.6. Acute amphetamine shifts behavior from the predictive CS1 towards the goal dish in Certain conditions, but spares Uncertain animals' attraction for the CS2

It has been suggested that reward uncertainty modulates motivation through effects on the mesolimbic dopamine system [42,43]. In some cases chronic exposure to uncertainty has even been shown to sensitize these dopaminergic systems [44,45] and result in greater acquisition of drug self-administration [46] and risky decision-making [47]. In addition, exposure to acute amphetamine or amphetamine sensitization has been shown to shift firing patterns of the ventral pallidum, away from the CS1 and towards the CS2, presumably increasing the incentive salience of the cue [20]. Here we found that acute injections of amphetamine shifted animals away from showing cue attraction towards the CS1. However, rather than redirecting their behavior towards the CS2, animals displayed an increased interaction with the magazine dish. This appeared to be the case whether the CS1 was presented for either 4 (Experiment 1) or 8 s (Experiment 2), and impacted animals under either Certain or Uncertain conditions similarly. However,

amphetamine had no effect on attraction to the CS2 during the last 4 s. As a result, Uncertain animals continued to show greater attraction to the CS2, while Certain animals were more drawn to the food cup. This suggests that the effects of amphetamine primarily targeted the predictive CS1, but that the CS2, which is primarily attractive under conditions of reward uncertainty, was largely resistant to its effects. This proposes a complex interaction between amphetamine, reward uncertainty and the value attributed to the cue. Under Certain reward conditions, amphetamine appears to shift behavior away from the predictive CS1 and towards more proximal cues such as the goal dish. This is in line with previous studies showing that acute or prior amphetamine administration increases goal-tracking while simultaneously decreasing sign-tracking [48,49], resulting in a shift in responding away from distant cues, and towards cues closest to the reward. However, this transition from sign- to goal-tracking goes against the idea that amphetamine might increase an animal's approach towards their prepotent cue, producing respective increases in either sign- or goal-tracking [50]. The current findings are nonetheless at least partially supported by the results of Tindell and colleagues, who showed that acute amphetamine and amphetamine sensitization increases the rate code of ventral pallidal neurons in response to both the CS2 and the more proximal UCS [20]. The changes in cue attraction generated by the exposure to reward uncertainty in the present study may instead be somewhat impervious to the effects of amphetamine. This is in part supported by previous studies showing that while amphetamine sensitization increases cue attraction in an autoshaping paradigm, this effect does not summate with the effects of reward uncertainty [15]. Together these findings suggest that amphetamine and reward uncertainty may act on cue attraction in slightly different ways.

Similar to amphetamine, nicotine has been shown to be particularly effective in establishing or enhancing the incentive-motivational properties of reward-associated conditioned cues [51,52]. For example, several studies report that nicotine enhances cue attraction in the form of greater sign-tracking when the brief extension of a retractable lever acts as a CS [53,54]. However, these effects seem to be dependent on the sensory properties of the CS. If the CS consists of an auditory or visual stimulus rather than an extendable lever, nicotine instead enhances approach behavior to the location of primary reward delivery in the form of goal-tracking [55,56]. In gambling, cues tend to take on various forms and involve multiple sensory properties, and recent findings where the CS is a compound cue, consisting of a lever presentation and an auditory cue, suggest that nicotine tends to increase goal-tracking, but that this effect is countered under conditions of reward uncertainty [17]. In the present study, the CS consisted of a compound (lever + sound) cue, nevertheless, here we found no effect of acute nicotine administration on sign- or goal-tracking. This might be explained by the fact that most studies examining nicotine's effects on autoshaping involve repeated administration over several days [53,54,56,57]. Here nicotine was injected only once, and it is possible that its effects develop across repeated exposure, or that nicotine's initial aversive reactions (that diminish over repeated injections) interfered with the expression of cue attraction [58,59].

#### *4.7. Under operant conditions, prior reward uncertainty triggers greater motivation and reward seeking directed at the CS1 and CS2 levers*

The present study also aimed to examine the ability of reward uncertainty and cues carrying different degrees of predictive and incentive value to trigger reward-seeking under operant conditions. Similar to recent findings [17], animals previously exposed to Uncertain reward conditions acquired initial reward-seeking at higher rates than Certain animals, as measured by a greater number of trials completed (Experiment 1). In addition, Uncertain animals in Experiment 2 performed similar levels of operant responding on both CS1 and CS2 levers despite having been previously exposed to the CS1 lever for twice the amount of time as the CS2 during autoshaping. This was not the case for animals

under Certain conditions who showed a strong preference for the CS1 and mostly ignored the CS2. Similarly, under a progressive ratio, animals previously exposed to Certain conditions were willing to pay almost twice the price for a reward from the CS1 lever over the CS2 lever, which was not the case for animals previously exposed to Uncertain conditions. Taken with our previous results, this suggests a further dichotomy regarding proximal and distal cues and their ability to influence behavior. Notably, animals under Certain reward conditions will ignore a more proximal CS2 if the initial predictive CS1 is still present (Experiment 2). However, despite being ignored, the CS2 will still become imbued with reinforcing properties, as seen in conditioned reinforcement. Yet it will only trigger diminished levels of reward seeking, as seen in operant conditioning (both FR1 and PR). This is in contrast with reward uncertainty, which appears to rectify these imbalances by attributing high levels of incentive salience, conditioned reinforcement and reward-seeking to the CS2. Reward uncertainty may therefore assign value to a greater number of cues (both CS1 and CS2) and instill them with the ability to generate motivation in a greater variety of ways.

#### *4.8. Initial low anxiety produces a greater attraction to cues*

Finally, we found overall that conditioning appeared to increase anxiety, which is in line with previous results in females, but not males [17]. In addition, we found that animals with initially low levels of anxiety tended to show higher levels of cue interaction. Specifically, animals exposed to Uncertain reward conditions in Experiment 1 displayed greater interaction with CS1, although responding to the CS2 was high for both high and low anxiety groups. In contrast, in Experiment 2, low anxiety animals exposed to Certain reward conditions showed higher levels of cue attraction to the CS1. These results suggest that anxiety levels seemed to primarily impact responding towards the predictive CS1 rather than the CS2. Our present results are in contrast to previous findings that suggested that high anxiety produced a greater ratio of lever presses to magazine entries [16]. However, unlike our current results that center around lever responding in females, these previous results measured the relative focus on the lever as opposed to the magazine dish in both males and females. Our current results also suggest that the effects may be different depending on the primarily predictive or incentive nature of a cue. In all, this suggests that further studies are likely necessary to unravel the complex relationship that anxiety possesses with cue attraction under Certain and Uncertain reward conditions, and whether the effect is specific to cues carrying primarily predictive or incentive value.

## **5. Conclusion**

The goal of the present study was to probe the effect of reward uncertainty on the predictive and incentive value of Pavlovian reward cues. Our findings suggest that through various serial cue designs, the incentive and predictive value of cues can be teased apart and that reward uncertainty results in robust attraction to a predictive cue, despite the cue's degraded predictive value. More importantly, reward uncertainty imbues more incentive value and produces a heightened attraction to reward-proximal cues, despite their limited predictive value. As a result, under conditions of reward uncertainty akin to that of slot machines, these reward-proximal cues, that are otherwise largely ignored under Certain reward conditions, become powerful incentives that can promote reward-seeking and high levels of engagement, and could be strong triggers for relapse.

## **Conflicts of interest**

None.

## Author Contributions

Experiments were designed by TIR, ASK, MJFR, data was collected by CMF, CC, ASK, JRC, analyzed, interpreted by ASK, CC, JRC, MJFR, and written and edited by CC, ASK, JRC, CMF, TIR, MJFR.

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