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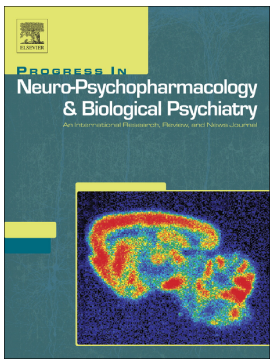

Reward uncertainty attributes incentive value to reward proximal cues, while amphetamine sensitization reverts attention to more predictive reward distal cues

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Reward uncertainty attributes incentive value to reward proximal cues, while amphetamine sensitization reverts attention to more predictive reward distal cues

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Abstract

Slot-machine gambling incorporates numerous audiovisual cues prior to and during reward delivery (e.g. spinning wheels, flashing lights, celebratory sounds). Over time, these cues may motivate playing and even elicit cravings and relapse in those affected by gambling disorder. Animal studies suggest a heightened attraction to these cues despite diminished predictive ability under reward uncertainty, as evidenced by sign-tracking behavior in rats. Repeated amphetamine administration may also enhance the incentive value attributed to cues. Here, we explored the impact of reward uncertainty and prior amphetamine sensitization on the relative attractiveness and conditioned reinforcing properties of serial Pavlovian cues with different degrees of predictive and incentive value in rats. Animals were sensitized through repeated injections of amphetamine (1-4 mg/kg) or saline and then trained in a Pavlovian autoshaping task involving two sequential lever-auditory cue combinations (CS1, CS2) under Certain (100%-1) or Uncertain (50%-1-2-3) reward conditions. Subsequently, we evaluated the impact of acute amphetamine exposure on cue attraction. Our results suggest that Uncertainty alone enhanced attraction toward the reward-proximal CS2. However, combined sensitization and Uncertainty reversed cue preference relative to Uncertainty alone, enhancing attraction toward the more predictive reward-distal CS1. Both cues acquired conditioned reinforcing properties, despite the CS2 being otherwise ignored in all groups besides Uncertainty. However, combined sensitization and Uncertainty increased the reinforcing value of both cues and doubled the amount of interaction with the CS1 lever per presentation. Our results imply competitive mechanisms for attributing incentive value to gambling-related cues between reward uncertainty, prior amphetamine sensitization, and acute amphetamine administration.

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Introduction

Gambling disorder is a behavioral addiction characterized by repeated and problematic gambling, affecting 1% to 2% of adults in the United States each year (American Psychiatric Association, 2013; Kessler et al., 2008). In gambling games like slot machines, cues such as flashing lights and celebratory sounds are repeatedly presented leading up to and during reward delivery. Over time, these cues can become excessively attractive and motivate individuals to continue playing (Barrus et al., 2015; Brevers et al., 2014; Dixon et al., 2013; Griffiths, 1993). These cues can also trigger craving during periods of abstinence and are often responsible for relapse in individuals recovering from gambling disorder (Hellberg et al., 2019; M. J. F. Robinson et al., 2015b). However, gambling cues are not reliable predictors of reward as they are presented in both the presence and absence of reward, which can itself often vary in magnitude.

While such reward uncertainty degrades the predictive value of these cues, previous studies in rats using a Pavlovian Conditioned Approach paradigm have suggested an overall increase in attraction and approach to these cues (Anselme et al., 2013; Hellberg et al., 2018a; M. J. F. Robinson et al., 2014). This increase in cue attraction can be observed as an increase in sign-tracking (approach and response to a lever cue) and a simultaneous reduction in goal-tracking (approach and interaction with the location of reward delivery), as well as an increase in the overall number of sign-tracking animals (M. J. F. Robinson et al., 2015a). This suggests that reward uncertainty amplifies the attribution of incentive value to cues despite them having little to no predictive value. This was evidenced in a recently published study using serial cues where the initial presentation of a CS1 (lever + sound) followed by the presentation of a CS2 (different lever + sound) preceded the delivery of a certain or uncertain sucrose reward (M. J. F. Robinson et al., 2019). Translated into a gambling context, the reward-distal CS1 can be thought of as representing the cues that predict the onset of a gambling event, such as a those

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surrounding the initiation of a spin on a slot machine. In contrast, the CS2 represents the cues most temporally proximal to reward delivery, which would coincide with the cues that occur during the anticipation of a possible reward outcome. Here the CS1 possesses the greatest predictive value as it predicts both the onset of a CS trial and the impending reward outcome. In contrast, the CS2 provides little to no additional information, but has the potential to be assigned with incentive value due to its proximity to reward. As a result, the CS2 is largely ignored under certain reward conditions, yet suddenly becomes attractive under uncertain reward conditions, suggesting that reward uncertainty can promote the attribution of incentive value to otherwise ignored or overshadowed cues (M. J. F. Robinson et al., 2019). The incentive value then attributed to cues is thought to be responsible for their subsequent transformation into motivational magnets that produce craving and relapse (M. J. F. Robinson et al., 2013; T. E. Robinson and Berridge, 1993). Besides reward uncertainty, increases in mesolimbic dopamine activity are also believed to further heighten incentive value attribution to cues (Mead et al., 2004; Peciña and Berridge, 2013; M. J. F. Robinson et al., 2015a). For example, research has found that amphetamine sensitization, as well as acute amphetamine administration, can amplify the incentive value coding of reward-proximal cues by increasing neuronal firing in the ventral pallidum to both a CS2 and reward delivery (Tindell et al., 2005). However, the impact of acute amphetamine and amphetamine sensitization on Pavlovian conditioned approach with a single cue has been equivocal. While some studies have reported an increase in sign-tracking (Doremus-Fitzwater and Spear, 2011; M. J. F. Robinson et al., 2015a), others report increases in goal-tracking accompanied by simultaneous decreases in sign-tracking (Holden and Peoples, 2010; Simon et al., 2009). Alternative methods for measuring the incentive value of a cue involve conditioned reinforcement tests that assess the ability of a cue to support the acquisition of a novel instrumental action. Previous studies have shown that administration of acute amphetamine enhances the conditioned reinforcing properties of an auditory or lever cue, and specifically increases the occurrence of contact with a lever cue only in sign-trackers (Meyer et

al., 2014). In the same vein, incentive sensitization through repeated injections of either amphetamine or cocaine has also been shown to enhance the conditioned reinforcing properties of a cue (Mead et al., 2004; Taylor and Horger, 1999), suggesting that prior sensitization can amplify the incentive value of cues. This type of stimulant-induced incentive sensitization can be assessed through changes in psychomotor activity, primarily by changes in the amount of stereotypical behavior enacted by animals (Mead et al., 2004; T. E. Robinson et al., 1998; Tindell et al., 2005).

Although gambling is primarily an instrumental task centered around risky decision-making, the preponderance of Pavlovian cues with varying degrees of predictive value that surround the slot machine experience is thought to play a critical role in drawing and holding the attention of players, while simultaneously promoting reward seeking and risky choices (Barrus et al., 2015). Under circumstances where cues reliably predict rewarded outcomes, many extraneous cues that are more proximal to reward tend to be overshadowed and largely ignored, meaning that only the most predictive cues can manage to hold an individual's attention. However our previous study found that gambling-like reward uncertainty can cause otherwise ignored cues with little to no predictive value to capture an individual's attention and become highly attractive, potentially making these cues able to trigger craving and more sustained gambling behavior (M. J. F. Robinson et al., 2019). Cue attraction can be further modulated by the consumption of drugs such as alcohol and nicotine which often accompanies gambling behavior (Conway et al., 2006; Hellberg et al., 2018a; 2018b; McGrath and Barrett, 2009; Petry et al., 2005; Russell and M. J. F. Robinson, 2019). Studies also show a high level of comorbidity between substance use disorders and gambling disorder (57.5%) (Chou and Afifi, 2011; Lorains et al., 2011; Walther et al., 2012), which may sensitize reward pathways and produce incentive sensitization (M. J. F. Robinson et al., 2013; T. E. Robinson and Berridge, 1993). However, the behavioral impact of prior drug sensitization on the attraction of cues with varying degrees of predictive and incentive value has yet to be determined. Similarly, it is

unclear whether prior sensitization affects the conditioned reinforcing properties of gambling-like cues and ascribes these cues with a greater ability to engage and reinforce gambling behavior.

In the present study, we sought to investigate the effects of both reward uncertainty and prior sensitization (using repeated amphetamine administration) on the attribution of incentive and predictive value by rats to serial Pavlovian cues. Here we used a previously established design consisting of two serial yet overlapping cues (M. J. F. Robinson et al., 2019), except that prior to any conditioning, animals were administered daily injections of either saline or incrementally increasing doses of amphetamine (1-4 mg/kg) across 14 days, followed by a 14-day rest period. Following sensitization treatment, rats underwent 10 days of autoshaping consisting of a series of CS trials, each lasting a total of 8 seconds. Each CS trial began with the presentation of a CS1 (lever + auditory cue) that predicted the onset of the CS trial, followed by the additional presentation of a more reward-proximal CS2 (different lever + different auditory cue) (Fig 1A). This experimental design imbues the CS1 with the majority of the predictive value for the reward. In contrast, the CS2 carries very little predictive value as it provides no additional information regarding the reward outcome and is largely overshadowed by the presence of the CS1. Instead, it has been suggested and we have recently shown that the CS2 has the potential to possess greater incentive value due to its greater temporal proximity to reward delivery (Matthews and Lerer, 1987; Meyer et al., 2014; M. J. F. Robinson et al., 2019; Tindell et al., 2005). After 8 seconds, each CS trial ended in a UCS outcome under either Certain or Uncertain reward conditions. Certain reward conditions consisted of the delivery of a single sucrose pellet on 100% of CS trials (100%-1), 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% of trials (50%-1-2-3). During autoshaping, cue attraction for each rat was measured as the amount of either CS1 or CS2 lever presses, or magazine entries into the food dish during each 8-second CS trial. The ability of either CS1 or CS2 to act as a conditioned reinforcer was then measured using a one-day conditioned reinforcement task. Finally, the impact of acute

amphetamine administration on cue attraction was then tested under autoshaping conditions. Video recordings of the animals from this day of autoshaping were then scored to assess psychomotor sensitization across groups as an indication of stimulant-induced incentive sensitization.

Methods

Animals

Thirty two male Sprague-Dawley rats (3-6 months old) purchased from Envigo and bred in-house were socially housed in groups of two on 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 sensitization, rats were handled and habituated for 2-3 days. Nearing the end of the sensitization protocol and before autoshaping began, all animals were food-restricted to 85-90% of initial body weight with water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at Wesleyan University.

Apparatus

All testing (sensitization, training) was conducted in Med Associates Inc. modular test chambers (25.8 x 32.2 x 33.2 cm) with metal bar floors, two modular front and back walls and two plexiglass walls, as previously described (Lesser et al., 2017). 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, location counterbalanced, and one inactive in the center). During this time,

the food cup on the front wall was covered with a custom metal plate. MedPC® 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.

Amphetamine Sensitization

Rats were initially assigned to receive repeated intraperitoneal injections of either amphetamine (N = 16) or saline (N = 16). Sensitization procedures were performed in the same test chambers as later training and testing, so as to retain any effects of context during subsequent tests. Rats were administered a single daily injection of saline (1 mg/kg, IP) or escalating doses of amphetamine (1-4 mg/kg, IP; dose order: 1, 1, 1, 2, 2, 2, 3, 3, 3, 3, 4, 4, 4, 4) for 14 consecutive days. After each injection, rats were placed in the conditioning chamber and left for 36 minutes (to match the time of the autoshaping program) undisturbed. Behavior was recorded using an overhead infrared video camera (Advidia™) and magazine entries were automatically recorded. After 14 days of injections, all rats spent 14 days in their home cages undisturbed.

Groups and Conditions

The two groups of 16 rats initially assigned to either amphetamine or saline injections were further divided into two groups (N = 8 for each group) that differed by reward condition (Certain: 100%-1 or Uncertain: 50%-1-2-3) according to the probability and magnitude of reward delivery per trial during autoshaping. In the Certain reward condition, each of the 36 CS trials, resulted in the delivery of 1 sucrose pellet to the magazine dish. In the Uncertain 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 Uncertain reward condition created uncertainty in the probability and magnitude of reward delivery. However, despite the reward condition, 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.

Magazine Training and Autoshaping

Two days prior to autoshaping, all animals were exposed to sucrose pellets in their homecage in order to reduce neophobia. Rats then underwent one day of magazine training which consisted of a 30 minute session where rats received 30 sucrose pellets from the magazine dish on a 45 second variable intertrial-interval (VI-45; 15-75 sec). Rats in all experiments were then exposed to 10 consecutive days of autoshaping, with each session consisting of 36 conditioned stimulus (CS) trial presentations, on a variable intertrial-interval (VI-45), and lasting approximately 36 minutes. Each CS trial lasted 8 seconds 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), as described above. Throughout each session, lever presses (LP) on either lever and magazine entries (ME) were recorded but had no programmed consequence.

In order to examine the impact of uncertainty on cue attraction during every CS trial, the predictive and incentive value of the Pavlovian CS was dissected by presenting two separate cues (lever + sound) prior to the UCS. At the beginning of every 8 second CS trial, an initial CS1 (left or right illuminated lever + tone or white noise) predicted the onset of the CS trial and bore the majority of UCS predictive value. Halfway into the CS trial, after four seconds, and while the CS1 was still present, the CS2 (right or left illuminated lever + whitenoise or tone; assignment counterbalanced), was presented for 4 seconds. After the 8 seconds of cue presentation, both CS1 and CS2 levers were retracted and the UCS was delivered. The CS1 therefore initially predicted the onset of the CS trial and was present alone for 4 seconds. Then for the last 4 seconds of the 8-second CS trial, both CS1 and CS2 were presented concurrently.

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: 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 (T. E. Robinson et al., 2014). 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 with scores ranging from -1 to 1 (Meyer et al., 2012). Animals with a strong preference for the lever cues had a response bias between 0.5 and 1 and were classified as sign-trackers, whereas goal-trackers had a response bias between -1 and -0.5. 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 tendency to sign- or goal-track was based on responses during the CS presentations of the last day (Day 10) of Pavlovian autoshaping. 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 seconds of the CS trial when the animal can direct responses towards either the CS1, CS2 or magazine. Instead, the response bias is best calculated using a previously published equation (M. J. F. Robinson et al., 2019; Tindell et al., 2005) that simultaneously includes all three factors (CS1, CS2 and ME).

This novel 'profile analysis' compares the attraction and interaction animals performed with three major targets during each CS trial, notably the CS1, CS2 and magazine dish. It 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 (last 4 seconds of CS trial), an animal might principally focus on the CS1 lever, thereby performing no CS2 lever responses or magazine entries during the last 4 seconds 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. However, the novel behavioral profile analysis vector accounts for CS1, CS2 and magazine responses, and allows for 0 values in each and any factor. 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 seconds of cue presentation (e.g. first 4 vs. last 4 seconds 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 (Tindell et al., 2005), we created a two dimensional vector (α, β) 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 $\theta = 0°$, this would imply that $y > x = z$ suggesting that attraction and responding was greatest for CS2. Similarly, $\theta = 120°$ would imply primary attraction to the CS1, whereas $\theta = 240°$ would suggest a principal attraction towards the magazine. Therefore while a $\theta = 0°$ or $120°$ would suggest an animal is sign-tracking, $\theta = 240°$ would designate primarily goal-tracking behavior. Consequently, an animal with $\theta = 60°$ would be expressing sign-tracking behavior with a split attraction between CS1 and CS2. 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°- 60°), with CS1 > CS2 > magazine, dominant attraction towards a reward-proximal cue would predict Group Profile Vectors predominantly directed towards the CS2 (60°- 300°) where CS2 > CS1 > magazine. Of particular interest here, is the ability of reward uncertainty and sensitization to shift Group Profile Vectors away from CS1 attraction and towards greater CS2 attraction.

Conditioned Reinforcement

Following 10 days of autoshaping, rats completed a single 30 minute session of conditioned reinforcement to assess the relative incentive value of both 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. 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 seconds, with nose port-lever pairing counterbalanced across subjects. The location of the CS1 and CS2 was matched to its training location for each animal. The center nose port served as a control and had no programmed consequence. All nose ports became inactive at the end of the session. Med-PC software automatically recorded the number of nose pokes per port and lever presses.

Acute Amphetamine Effects on Autoshaping

Following the single conditioned reinforcement session, the impact of acute amphetamine on the attraction to the CS1 and CS2 was measured during autoshaping. Rats underwent two additional days of autoshaping, in which each session was preceded by a single injection, 15 minutes prior to the start of the autoshaping session. On the first day, rats received injections of saline (1 ml/kg, IP) to habituate animals to injections and establish baseline behavior. On the second day, rats received an amphetamine (0.5 mg/kg, IP) injection 15-20 minutes prior to the autoshaping session.

Sensitization and video scoring

Video recordings from each rat on the day of acute amphetamine exposure were randomly assigned to experimenters blind to experimental conditions. Videos were scored for both the number and duration (to the nearest whole second) of rearing, grooming, and stereotypy behavioral events. The scoring process consisted of a detailed analysis of seven 60-

second periods, examining the 1st, 5th, 10th, 15th, 20th, 25th, and 30th minute of each session (Doremus-Fitzwater and Spear, 2011; Silverman et al., 2016; Souza et al., 2014). A rearing event began when both front paws left the floor of the operant chamber, with or without wall-climbing behavior, unless it involved orientation change (turning), grooming, lever interaction, magazine entry, or the consumption of a sucrose pellet. Once both front paws came into contact with the floor again, the duration of each rearing event ended. Grooming was qualified as any moment when a part of the body was licked or when a paw(s) was used to rub and/or scratch a part of the body (Fan et al., 2011; Silverman et al., 2016). Stereotypy was scored as any moment when the animal displayed repetitive movements of the head from side to side or in a circular motion, with or without sniffing behavior, that were directed at the wall, floor, or corner of the operant chamber (Doremus-Fitzwater and Spear, 2011; Hadamitzky et al., 2012; Kuczenski and Segal, 1999; Silverman et al., 2016; Wolgin, 2012). However, behavior was not considered stereotypy if these criteria were met when the head of the animal was inside the magazine or during lever interaction in the CS trials. The number and duration of rearing, grooming, and stereotypy events were used to determine the average percentage of the total scoring period during which the behavior occurred (Fan et al., 2011; Kuczenski and Segal, 1999). These calculated values were then compared between experimental groups to assess psychomotor sensitization.

Statistics and analysis

All data were analyzed using one-way/repeated measures ANOVAs or paired/unpaired t-tests (IBM SPSS 25 and Graphpad PRISM 8), 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. All analyses were two-tailed and performed at a level of significance of $p < 0.05$.

Results

Autoshaping

Sign-trackers and Goal-trackers and overall conditioned approach

Following 14 days of repeated amphetamine or saline treatment, animals were returned to their home cage for 14 days. Rats were then exposed to 10 consecutive days of autoshaping consisting of repeated CS-UCS pairings. Here, each CS trial lasted 8 seconds and began with the presentation of a CS1 (lever + auditory cue), followed by the additional presentation of a CS2 (different lever + auditory cue) under either Certain or Uncertain reward conditions (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.

Across ten days of autoshaping, animals showed acquisition of Pavlovian conditioning by increasing their total responses across days (Day: $F_{(9,252)} = 21.366$, $p = 0.000$; Fig 1C). This consisted of a progressive decrease in the total number of magazine entries, in favor of increasing total lever presses (Response Type: $F_{(1,28)} = 143.740$, $p = 0.000$; Day x Response Type: $F_{(9,252)} = 55.177$, $p = 0.000$). However, there was no significant effect of prior amphetamine sensitization or reward uncertainty on total behaviors (Group: $F_{(3,28)} = 0.027$, $p = 0.994$). Examination of the animals' response bias (LP-ME/LP+ME) indicates that, across the ten autoshaping days, all four groups began with similar intermediate behavior on the first day of training and progressively developed similar strong sign-tracking behavior (Day: $F_{(9,252)} = 61.354$, $p = 0.000$; Group: $F_{(3,28)} = 0.215$, $p = 0.885$; Day x Group: $F_{(9,252)} = 0.720$, $p = 0.846$; Fig 1D). By day

ten of autoshaping, all 32 animals displayed predominantly sign-tracking behavior which is in line with previous studies from our lab (Hellberg et al., 2018a; M. J. F. Robinson et al., 2019; Russell and M. J. F. Robinson, 2019).

Attraction to CS1 and CS2 under Certain or Uncertain reward conditions

Lever presses and magazine entries during the CS1 and CS2 cue presentations were recorded separately to measure cue-specific behavior. Responses per session were transformed into lever presses and magazine entries per second per CS presentation (LP/Sec/CS or ME/Sec/CS), to allow for a more standardized comparison of lever interaction regardless of the duration of each cue presentation.

During the first 4 seconds of each CS trial when the CS1 was initially presented alone, animals across all four groups showed similar responses (Group: $F_{(3,28)} = 0.461$, $p = 0.712$) and primarily directed their attention towards the CS1, largely ignoring the magazine (Day: $F_{(9,252)} = 21.648$, $p = 0.000$; Response Type: $F_{(1,28)} = 100.857$, $p = 0.000$; Day x Response Type: $F_{(9,252)} = 55.170$, $p = 0.000$; Fig 2A).

After 4 seconds of CS1 presentation, the CS2 was presented concurrently, giving animals the option of directing their attention towards the CS1, CS2 or magazine dish during the last 4 seconds of the CS trial. Although animals increased responding on the CS1 in the last 4 seconds of the CS trial across the 10 days of training (Day: $F_{(9,252)} = 15.151$, $p = 0.000$), there was no difference in responding between groups (Group: $F_{(3,28)} = 1.461$, $p = 0.246$; Day x Group: $F_{(27,252)} = 1.206$, $p = 0.228$; Fig 2B). However, in contrast to the first 4 seconds of CS1 presentation, there was a notable decrease in responding on the CS1 across all groups, an effect likely attributable to the introduction of the CS2 (First vs Last 4 seconds: $F_{(1,28)} = 17.720$, $p = 0.000$; First vs Last x Group: $F_{(1,28)} = 0.292$, $p = 0.831$). Contrary to CS1 responding, responses on the CS2 were distinct between groups (Group: $F_{(3,28)} = 6.333$, $p = 0.002$; Day: $F_{(9,252)} = 3.109$, $p = 0.001$; Day x Group: $F_{(27,252)} = 2.392$, $p = 0.000$; Fig 2C). Specifically, animals in the Saline Uncertain

condition performed significantly more CS2 responses than all three other groups (Tukey HSD: p's < 0.025), suggesting that under uncertain reward conditions, the CS2 had acquired considerable incentive value. However, all four groups largely ignored the magazine during the last 4 seconds of the CS trial, showing a progressive decrease in magazine entries across training (Group: $F_{(3,28)} = 0.300$, $p = 0.825$; Day: $F_{(9,252)} = 21.499$, $p = 0.000$; Day x Group: $F_{(27,252)} = 0.804$, $p = 0.745$; Fig 2C).

Vector profiles were then created to more closely examine how individuals and overall groups directed attention and responded to either the CS1, CS2 or magazine during the last 4 seconds of CS trials on day 10. Overall, animals across all groups showed little interest in the magazine and instead directed their attention almost exclusively towards the two CS levers (Response Type: $F_{(2,56)} = 21.768$, $p = 0.000$; Fig 3A-D). However, not all groups showed the same cue preference (Response Type x Group: $F_{(6,56)} = 2.738$, $p = 0.021$). Notably, the Saline Certain group directed its behavior primarily towards the CS1, with all animals (8 out of 8; Fig 3A) showing an exclusive preference for the CS1 (see Video 1). In contrast, exposure to reward uncertainty alone (Saline Uncertain), shifted behavior towards the CS2, with the majority of animals (5 out of 8; Fig 3C) displaying a strong attraction to the CS2. Surprisingly, prior exposure to amphetamine resulted in behavior favoring the CS1. This was the case under both reward conditions (Amphetamine Certain & Amphetamine Uncertain; Fig 3B & 3D), although there may be a mild shift in interest towards the CS2, with at least one animal in each of the two groups showing a marked preference for the CS2.

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 even uncertain reward cues that possess limited

predictive value were capable of supporting acquisition a novel nosepoking task. The back wall of the chamber was equipped with three nosepoke ports, one which triggered a brief 4 sec presentation of the CS1 (lever + auditory cue), while another triggered a presentation of the CS2 (Fig 4A), 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.

As expected, animals across all four groups similarly showed more interest for the noseports associated with the presentation of the CS1 and CS2 over the control noseport (Nosepoke: $F_{(2,56)} = 29.382$, $p = 0.000$; Group: $F_{(3,28)} = 1.388$, $p = 0.267$; Nosepoke x Group: $F_{(6,56)} = 1.396$, $p = 0.232$; Fig 4B). In particular, all groups responded more on the noseport that delivered CS1 than the control noseport (CS1-Control: $t_{(7)}$'s > 2.648, *p's* < 0.034), suggesting that the CS1 had become a conditioned reinforcer, despite lower predictive value under Uncertain conditions. The same was true for the noseport that delivered a brief presentation of the CS2 (CS2-Control: $t_{(7)}$'s > 3.746, *p's* < 0.008), except for the Saline Uncertain group which did not reach significance (CS2-Control: $t_{(7)} = 1.810$, $p = 0.113$) despite eliciting a 159% greater level of responding than the control noseport. Finally, there was no difference in nosepoke responses between CS1 and CS2 (CS1-CS2: $t_{(7)}$'s < 1.494, *p's* > 0.178). There was however an interaction between reward conditions and prior sensitization. Specifically, animals treated with saline tended to work harder for cues under Certain rather than Uncertain conditions, whereas prior exposure to amphetamine sensitization reversed this tendency and gave more value to and made animals work harder for cues under Uncertain rather Certain reward conditions (Sensitization x Reward Condition: $F_{(1,28)} = 4.825$, $p = 0.036$; Fig 4B).

Across all four groups, animals consistently responded on either the CS1 or CS2 lever when it was presented, suggesting that they were specifically nosepoking to gain access to the cues. In addition, rats tended to respond more on the CS1 than the CS2, although there was no one group showing greater responding overall (Lever Press: $F_{(1,28)} = 4.794$, $p = 0.037$; Lever Press x Group: $F_{(3,28)} = 1.475$, $p = 0.243$; Group: $F_{(3,28)} = 1.374$, $p = 0.271$; Fig 4C). It should be

noted however that although the effect did not reach significance (CS1-CS2: $t_{(7)}$ = 1.825, p = 0.111), on average, animals exposed to prior amphetamine and to reward uncertainty (Amphetamine Uncertain) responded on the CS1 almost 3 times more than on the CS2, and about two to three times more on the CS1 than any other group on either cue.

The effect of acute amphetamine on CS1 and CS2 attraction

Following conditioned reinforcement, animals received one day of autoshaping to re-establish conditioned approach behavior, preceded by a saline (1 ml/kg, IP) injection. The following day, animals in all experimental groups received an injection of amphetamine (0.5 mg/kg, IP) prior to their autoshaping session and interaction with the CS1, CS2 and magazine dish was measured throughout the session.

Approach behavior was analyzed separately during the first and last 4 seconds of each CS trial in order to distinguish whether the impact of acute amphetamine 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 seconds). During the first 4 seconds, when animals were presented with the CS1 and the magazine, amphetamine affected responding differentially across cues (Drug: $F_{(1,28)}$= 55.232, p = 0.000; Cue Type: $F_{(1,28)}$= 98.169, p = 0.000; Drug x Cue Type: $F_{(1,28)}$= 79.664, p = 0.000; Fig 5A left panel). Specifically, amphetamine decreased responding on the CS1 (CS1: $F_{(1,28)}$= 74.051, p = 0.000) and conversely increased attraction towards the magazine (Magazine: $F_{(1,28)}$= 25.848, p = 0.000), and did so similarly across all groups (Group: $F_{(3,28)}$= 0.792, p = 0.509).

During the last 4 seconds of the CS trial, when the CS2 was introduced, amphetamine had a pronounced effect on responding that was specific to each cue (Drug: $F_{(1,28)}$= 6.931, p = 0.014; Cue Type: $F_{(2,56)}$= 10.344, p = 0.000; Drug x Cue Type: $F_{(2,56)}$= 18.833, p = 0.000; Fig 5A right panel), and that did not appear to be uniform across groups (Group: $F_{(3,28)}$= 3.756, p = 0.022; Group x Drug x Cue Type: $F_{(6,56)}$= 2.322, p = 0.045). Similar to during the first 4 seconds,

amphetamine again produced a significant decrease in responding on the CS1 (CS1: $F_{(1,28)}$= 21.037, p = 0.000), and increased responding directed at the magazine (Magazine: $F_{(1,28)}$= 22.305, p = 0.000). However, in contrast to the CS1 and the magazine, amphetamine did not appear to change responding directed at the CS2 (CS2: $F_{(1,28)}$= 0.872, p = 0.358), suggesting CS2 attraction was impervious to the effects of acute amphetamine exposure. Nonetheless, animals under Saline Uncertain conditions still showed a stronger preference for the CS2 compared to all other groups (Group: $F_{(3,28)}$= 8.084, p = 0.000; Tukey's HSD: p's < 0.007).

Closer examination of the impact of acute amphetamine using vector profile analysis revealed an increase in the number of goal-trackers, based on their predominant cue preference. This was specifically the case for groups exposed to Certain reward conditions and could be seen as an increase in the amount of magazine entries during the last 4 seconds. For example, administration of amphetamine to Saline Certain animals increased the number of goal-trackers from 0 to 4 (Saline: 8 CS1-preferring; Amphetamine: 4 CS1-preferring, 4 Magazine-preferring; Fig 5B). The same was true of animals under Amphetamine Certain conditions, producing 4 goal-trackers when previously there was none (Saline: N = 7 CS1-preferring, 1 CS2-preferring; Amphetamine: N = 2 Mixed CS1/Magazine-preferring, 2 CS2-preferring, 4 Magazine-preferring; Fig 5C). In contrast, prior exposure to reward uncertainty tended to make animals resistant to these effects of acute amphetamine (see Video 1). Animals under Saline Uncertain conditions were largely unchanged (Saline: N = 2 CS1-preferring, 6 CS2-preferring; Amphetamine: N = 2 CS1-preferring, 6 CS2-preferring; Fig 5D). This amphetamine-induced shift towards goal-tracking across the entire CS trial could be seen as an increase in magazine entries in response to drug that was specific to groups exposed to Certain reward conditions but not prior sensitization (Drug x Reward Condition: $F_{(1,28)}$= 6.165, p = 0.019; Drug x Sensitization: $F_{(1,28)}$= 0.555, p = 0.463). In particular, amphetamine increased attraction to the magazine as the CS trial progressed from the first to the last 4 seconds, specifically for animals exposed to Certain conditions (First vs Last 4 seconds x Drug x Reward Condition $F_{(1,28)}$=

5.307, $p = 0.029$; Amph-Saline Difference for First vs Last 4 sec: Certain: $t_{(15)} = 2.691$, $p = 0.017$; Uncertain: $t_{(15)} = 0.428$, $p = 0.675$; Fig 5F). This can be further exemplified by the ability of acute amphetamine to shift the group vector for Certain animals towards the magazine (see blue circular arrow in Fig 5G).

Locomotor sensitization

The effects of prior amphetamine treatment and acute amphetamine treatment on behavior were examined on the last day of autoshaping when all animals were given an acute injection of amphetamine. Behavioral analysis was performed using video scoring of grooming, rearing, and stereotypy during 1 minute bins across the entire session. Overall, animals spent very little time grooming (< 5% on average), and instead spent a significantly higher proportion of their time rearing and showing stereotypy (Behavior Type: $F_{(2,56)} = 29.388$, $p = 0.000$). Furthermore, repeated amphetamine pre-treatment increased the percentage of time spent performing these behaviors (Sensitization: $F_{(1,28)} = 4.577$, $p = 0.041$; Behavior Type x Sensitization: $F_{(2,56)} = 3.335$, $p = 0.043$; Fig 6A). Analysis by group revealed that the combination of prior sensitization with amphetamine and repeated exposure to reward uncertainty resulted in a significantly greater proportion of time spent showing stereotypy (Group: $F_{(3,31)} = 3.758$, $p = 0.022$; Fig 6B), specifically in comparison to animals previously exposed to saline and uncertain reward conditions (Tukey's HSD: $p = 0.015$).

Discussion

In the present study, we examined the impact of prior repeated amphetamine administration and reward uncertainty on the attribution of incentive value to Pavlovian serial cues bearing different levels of predictive value. Animals were trained through repeated presentations of a CS1 (lever+tone) that lasted 8 seconds and was concurrently presented with

a CS2 (different lever+tone) during the last 4 seconds. In this design, the CS1 carries the majority of the predictive value as it predicts not only the ultimate delivery of reward, but also the presentation of the CS2. In contrast, the CS2 provides little to no additional information regarding each trial and is largely overshadowed by the presence of the CS1. We found that across the 10 days of autoshaping, animals predominantly developed sign-tracking behavior, which is in line with our previous studies using Sprague Dawley rats bred in-house and exposed to similar conditions (Hellberg et al., 2018a; M. J. F. Robinson et al., 2019; Russell and M. J. F. Robinson, 2019). Closer analysis of each CS trial found that all animals interacted almost exclusively with the CS1 during the first four seconds of each trial, regardless of reward condition or prior exposure to repeated amphetamine injections. In contrast, there were differences between groups during the last four seconds of each CS trial. While all animals under Certain (100%-1) reward conditions remained attracted to the more predictive CS1 during the last four seconds, animals under the Uncertain (50%-1-2-3) reward condition without prior exposure to amphetamine shifted approach behavior to the reward-proximal CS2, a finding also consistent with our previous studies (M. J. F. Robinson et al., 2019). Similar to those previous results, there also appeared to be a strong dichotomy in the focus of attraction for each individual animal in the Saline Uncertain group, with five out of eight animals displaying a strong preference for the CS2, while the remaining three animals preferred the CS1.

While the attractiveness of the CS2 seen in animals treated with saline and exposed to Uncertain reward conditions reflects greater levels of attributed incentive value, it could be argued that this motivation generated under uncertainty may have also been influenced by the role of prediction error in associative learning. Specifically, greater uncertainty about the predictive value of a cue, should result in greater prediction errors, which in turn would ascribe the CS2 with more attention (Holland and Schiffino, 2016; Ouden et al., 2012; Paskewitz and Jones, 2018; Schultz and Dickinson, 2000; Smout et al., 2019). While greater attention to the CS2 could result in more approach behavior, it is worth noting that reward uncertainty would

similarly increase prediction errors for the CS1 and may not explain the shift in approach towards the CS2 seen in the Saline Uncertain group. In addition, whereas the attention elicited by prediction errors under uncertainty appears critical to associative learning, evidence from Holland & Schiffino suggests that although uncertainty may heighten associative learning, it is certainty and prediction which promotes action and approach to a CS (Holland and Schiffino, 2016). This suggestion appears to be in contrast with the current findings where uncertainty increased approach towards the CS2. Furthermore, studies using autoshaping suggest that both sign-tracking and goal-tracking animals learn the association between the CS and the UCS to a similar degree, yet only some (sign-trackers) approach and interact with the CS. This suggests a possible dissociation between incentive and predictive value and implies that robust associative learning does not necessarily result in approach to the CS (Flagel et al., 2011; Mohebi et al., 2019; T. E. Robinson and Flagel, 2009).

One of the principal aims of the present study was to examine whether prior exposure to amphetamine could exacerbate the attraction and increase the incentive value directed towards the CS2 under uncertain reward conditions. Surprisingly, prior exposure to amphetamine under Uncertain reward conditions appeared to reverse rather than enhance the aforementioned increase in CS2 attraction. Notably, animals exposed to prior amphetamine and reward uncertainty showed greater attraction to the CS1, with only 2 animals showing mild preferences for the CS2. This suggests that prior sensitization of the mesolimbic system under Uncertain reward conditions may shift cue attraction toward reward-distal cues, which predict the onset of a reward event, and which may align best with the initiation of a gambling event, rather than further enhance the attraction to cues most temporally proximal to reward delivery, which would coincide with the anticipation of a possible reward outcome. This is contrary to what might have been expected based on reports from Tindell and her colleagues. When looking at firing patterns in the ventral pallidum following a similar pattern of amphetamine sensitization, they found an increase in firing to the CS2 and reward delivery, but not the CS1 (Tindell et al., 2005).

It is worth noting however that they did not measure approach behavior to accompany these changes in firing patterns, in part due to the fact that their cues were strictly auditory, making it hard for them to trigger any approach behavior.

Interestingly, prior amphetamine sensitization in animals exposed to Certain reward conditions produced only a very mild shift away from the CS1 and towards the CS2, as can be seen by the individual preferences and vector profile analysis. That same vector profile analysis examined at a group level suggests that prior sensitization shifted both Certain and Uncertain groups away from the extremes of being either predominantly CS1- or CS2-preferring. Instead, more animals seemed to show a more mixed approach profile consisting of both CS1- and CS2-directed behavior. It is possible that amphetamine sensitization had the effect of heightening the incentive value ascribed to both CS1 and CS2, causing animals to display more intermediate behavior rather than strictly preferring one of the two cues. However, in contrast to the current findings, previous studies using a shorter amphetamine sensitization regimen have shown that prior amphetamine sensitization increased Pavlovian conditioning to levels similar to those of reward uncertainty (Doremus-Fitzwater and Spear, 2011; Harmer and Phillips, 1998; M. J. F. Robinson et al., 2015a). However, the combination of reward uncertainty and prior amphetamine sensitization did not summate to evoke even greater responses (M. J. F. Robinson et al., 2015a), although this could be attributed to ceiling effects. It is worth noting, however, that similar studies have reported a decrease in sign-tracking and a corresponding increase in goal-tracking following a prior amphetamine sensitization regimen (Holden and Peoples, 2010; Simon et al., 2009).

Overall, despite a demonstrated cue preference for either the CS1 or CS2 across experimental groups during autoshaping, we found that all animals ascribed both CS1 and CS2 with strong incentive value when tested for conditioned reinforcement. This suggests that both cues developed rewarding properties even if they had been previously ignored during autoshaping.

Importantly, we found an interaction between reward uncertainty and amphetamine sensitization. Most notably, when animals were previously treated with saline, those exposed to Certain reward conditions displayed more overall conditioned reinforcement for cues than those under Uncertain reward conditions. In contrast, prior sensitization reversed this effect and made all cues more reinforcing under Uncertain rather than Certain conditions. This suggests that a crucial effect of sensitization is to make gambling-like uncertain reward cues more rewarding, with the potential impact of spurring on continued gambling, especially in contexts where uncertain cues are abundant, such as with electronic slot machines.

Previous findings have shown an increase in conditioned reinforcement with prior sensitization both in mice (Mead et al., 2004) and rats (Taylor and Horger, 1999), and following acute microinjections of amphetamine into the nucleus accumbens or ventral pallidum (Fletcher et al., 1998), although it is worth noting that at least one study found no effect of prior sensitization in rats (Harmer and Phillips, 1998). However in all cases, these results were found under conditions of reward certainty rather than under uncertainty, and were not replicated here when examining the impact of prior amphetamine sensitization in animals trained under Certain reward conditions.

Surprisingly, despite similar levels of responding for each cue within each group, it is worth noting that the number of CS1 lever responses was at least doubled in animals with prior amphetamine exposure under Uncertain reward conditions, although this effect did not reach significance. This lends further support to the idea that prior amphetamine sensitization may have countered the increased attraction to the CS2 under reward uncertainty by enhancing the value ascribed to the CS1. Conditioned reinforcement therefore highlights the fact that both CS1 and CS2 acquire incentive value, even when one of the two is ignored during the last four seconds of autoshaping. This is similar to previous studies using a diffuse auditory CS that fails to produce any sign-tracking and still becomes a conditioned reinforcer (Meyer et al., 2014). Yet, our findings suggest that autoshaping might be more sensitive to the relative amount of

incentive value attributed to either cue by placing them in competition for the animal's attention during the last four seconds of each CS trial.

In contrast to prior amphetamine sensitization, the acute administration of amphetamine prior to an autoshaping session had profound effects on behavior during the first four seconds of the CS trial. In particular, acute amphetamine cut the number of CS1 responses during the first four seconds to less than half, while simultaneously redirecting attention towards the magazine and increasing the number of magazine entries. This finding is in line with previous reports suggesting that acute amphetamine prior to autoshaping reduces sign-tracking and increases goal-tracking (Holden and Peoples, 2010). During the last four seconds of the CS trial, the primary effect of amphetamine again seemed to be to reduce attraction towards the CS1. However it appeared to affect animals under Certain conditions more than those under Uncertain reward conditions. The same was also true regarding magazine entries, which increased significantly only under Certain reward conditions. In several cases, closer analysis of this effect between individuals showed that under Certain conditions, acute amphetamine shifted behavior for some animals away from sign-tracking and towards goal-tracking (Holden and Peoples, 2010). In contrast, the attraction towards the CS2, which was mainly driven by the Uncertain Saline group, seemed largely unaffected by acute amphetamine administration. Similarly, the trend to produce animals with a primarily goal-tracking behavior was absent under Uncertainty, unless animals had been previously sensitized.

There seemed to be no synergistic effect of acute amphetamine and prior amphetamine sensitization as had been previously reported by Tindell and colleagues for firing patterns in the ventral pallidum (Tindell et al., 2005). Instead, prior amphetamine sensitization and the administration of acute amphetamine appeared to have differing roles on cue attraction. At times it seemed like the impact of amphetamine sensitization depended on the corresponding reward conditions, as it tended to move animals away from more exclusive CS1 or CS2 preferring profiles. In contrast, acute amphetamine had a strong and clear effect in reducing

CS1 lever interaction in favor of increasing goal-tracking and attraction towards the food dish. This effect was most prominent in animals exposed to Certain reward conditions, and suggests that reward uncertainty might confer some protection against the effects of acute amphetamine. This can be explained in part by the increased attraction that Uncertain Saline animals had for the CS2 during the last four seconds, however the same also seemed to be true for animals exposed to prior sensitization and uncertainty, despite only minimal attraction towards the CS2.

An alternative interpretation of the present findings argues that the current CS1-CS2 design produces a form of overshadowing, whereby the temporal order of compound cue presentations (CS1 being first) influences cue salience and reduces learning of any value for the CS2, making it largely ignored. This would be the case for animals exposed to Certain reward conditions, where attraction is primarily and almost exclusively directed towards the CS1. Previous studies from O'Tuathaigh and Moran have shown that in a more typical overshadowing design, involving lick suppression of aversive audiovisual compound cues, acute amphetamine exposure disrupts overshadowing and restores learning and attribution of value to the less salient cue (O'Tuathaigh and Moran, 2004; 2002). However, in the present study, acute amphetamine did not disrupt overshadowing of the CS2 (by increasing attraction towards it) in animals under Certain conditions (Saline/Amphetamine Certain), but rather it made them approach the magazine. Instead, our results would tend to suggest that exposure to reward uncertainty, rather than acute amphetamine, disrupts overshadowing and allows learning to occur for the CS2; as seen by greater approach behavior towards the CS2 in animals exposed to Saline and Uncertain reward conditions. This is in line with findings by Urushihara and Miller who reported that partial reinforcement (probability uncertainty), where the compound CS is presented with the UCS on only 10% of trials, reduces overshadowing, allowing the overshadowed CS to more robustly suppress licking (Urushihara and Miller, 2007). Interestingly, we find that prior amphetamine sensitization reinstates the supposed overshadowing effect in animals exposed to Uncertainty (Amphetamine Uncertain) and reduces attraction to the CS2.

This effect of amphetamine sensitization would be opposite to the effect of acute amphetamine reported by O'Tuathaigh and Moran (O'Tuathaigh and Moran, 2004; 2002). However, it is important to note when examining the current findings within an overshadowing framework, that there are several methodological differences between typical overshadowing studies and the current experiments. First, there are unequivocal differences in the experimental design. Notably, the compound cues (CS1 and CS2) used here share near-identical physical characteristics and are only distinguishable by their distinct auditory stimulus pairings (which are counterbalanced), temporal order, and duration rather than by the salience of their physical properties. Second, in the present study, acute amphetamine was only administered after repeated conditioning sessions, once learning had already occurred, rather than during initial learning, which may reduce its impact on stimulus selection. Finally, in contrast to regular overshadowing experiments, here the approach towards either CS is measured simultaneously, placing both cues in competition for attention, making it difficult to assess the true value of the overshadowed CS2 alone.

Finally, analysis of locomotor behavior during the acute amphetamine session suggests that prior sensitization increases stereotypy, which is in line with previous findings (Doremus-Fitzwater and Spear, 2011; Fowler et al., 2003; Hadamitzky et al., 2012; Kuczenski and Segal, 1999; Tindell et al., 2005; Wolgin, 2012). Yet, prior amphetamine sensitization did not seem to increase other non-specific effects such as grooming and rearing. Surprisingly, amphetamine sensitization had the greatest impact on stereotypy for animals also exposed to reward uncertainty. In contrast to many of the effects reported above, this suggests a form of synergy between uncertainty and amphetamine sensitization, whereby exposure to reward uncertainty in combination with prior amphetamine sensitization might result in even greater locomotor sensitization than either manipulation alone.

Conclusions

Overall, our findings suggest a complex interaction between reward uncertainty and stimulant-induced changes in dopaminergic activity, which may help elucidate the multiple effects that uncertain cues have on gambling. On the one hand, reward uncertainty may recruit and attribute more incentive value to cues that might otherwise be largely ignored, specifically for cues that are more proximal to reward and may be present after a trial has been initiated but during the anticipatory phase prior to reward delivery. In contrast, the presence of underlying sensitization of mesolimbic pathways, as could be expected from prior chronic drug use and abuse, may enhance the value ascribed to cues more distal to reward delivery which have more predictive power in signaling the onset of a given trial rather than the outcome of a reward event. Together, gambling-like reward uncertainty and prior drug sensitization of reward pathways, seem to increase the rewarding value of cues as a whole, which may heighten their ability to engage and sustain gambling behavior. Finally, acute increases in dopaminergic function which may result from drug use (e.g., alcohol, nicotine, etc.) whilst gambling, could draw attention closer to reward outcomes and events most proximal to reward delivery, without diminishing the impact of cues surrounding the anticipation of reward delivery.

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Declarations of interest:

None

Author Contributions:

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

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Figure Legends

Figure 1. Experimental timeline and overall responding and response bias during autoshaping. A) An overview of the experimental timeline describing the order and duration of the sensitization protocol and each individual task. B) Animals were trained in an autoshaping task involving two sequential lever + distinct auditory cue combinations (CS1 and CS2). In each of the 36 trials per daily session, the CS1 was presented for the full 8-second period while the

CS2 was only presented during the last 4 seconds. Each trial concluded with the retraction of both levers and the delivery of a sucrose pellet(s) or lack thereof depending on whether animals were exposed to the Certain (100%-1) or Uncertain (50%-1-2-3) reward condition. A diagram shows the location of the pellet-dispensing magazine (food dish) and relative position of the CS1 and CS2 (position and auditory cue counterbalanced). C) Regardless of reward condition and sensitization, animals performed a far greater number of lever presses than magazine entries across 10 days of autoshaping. D) This difference between lever interaction and magazine entries resulted in a strong response bias indicative of sign-tracking for all animals.

Figure 2. Cue attraction for the first vs. last 4 seconds of each CS trial. A) During the first 4 seconds of the CS trial when only the CS1 was presented, all animals responded to the CS1 lever at the expense of magazine entries. B) During the last four seconds when both CS1 and CS2 were presented concomitantly. There was an overall decrease in responding for the CS1, which was more pronounced in the Saline Uncertain group. C) Conversely, animals in the Saline Uncertain group significantly increased CS2 responding during the last 4 seconds. D) There was also a progressive decrease in magazine entries during the last 4 seconds across the 10 days of autoshaping.

Figure 3. Vector profile analysis of cue attraction during the last 4 seconds of each CS trial for day 10. Each vector plot contains three major poles representing the mean magnitude of responses per second per CS trial directed towards the CS1, CS2, and Magazine Dish across CS trials during the last 4 seconds. A) All animals in the Saline Certain group (8 out of 8) directed their behavior primarily towards the CS1. B) Animals in the Amphetamine Certain group

primarily directed behavior towards the CS1 as well (6 out of 8). However, one animal directed behavior towards both CS1 and CS2 equally, while one animal directed behavior primarily towards the incentive CS2. C) Animals in the Saline Uncertain group directed behavior exclusively towards either the CS2 (5 out of 8) or the CS1 (3 out of 8). D) Animals in the Amphetamine Uncertain group primarily directed behavior towards the CS1 (6 out of 8), while two animals directed behavior towards the CS2. E) A comparison of the overall vector profiles from each group shows that prior amphetamine exposure produces a pronounced shift in cue-directed behavior, from the CS2 to the CS1, for animals in the Uncertain reward condition.

Figure 4. Conditioned reinforcement design and responses for each cue. A) A diagram showing the relative positions of CS1, Control, and CS2 nose poke ports (side counterbalanced) on the chamber side opposite to the CS1 and CS2 levers. 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) All animals worked with similar levels of effort to gain access to both CS1 and CS2 levers relative to the control. Exposure to prior amphetamine sensitization and reward uncertainty resulted in relatively more conditioned reinforcement for both cues. C) Upon lever presentation, the Amphetamine Uncertain group interacted with the CS1 lever two-fold more than animals on all other cues, although this effect did not reach significance.

Figure 5. Effects of acute amphetamine on Lever Presses and Magazine Entries. A) During the first four seconds of the trial (left panel), acute amphetamine increased attraction to the magazine while simultaneously decreasing attraction to the CS1 across all four groups. During

the last four seconds (right panel), amphetamine once again decreased attraction to the CS1 while increasing attraction to the magazine. However acute amphetamine did not seem to impact CS2 attraction, with animals in the Saline Uncertain group maintaining their greater attraction for the CS2 and largely ignoring the magazine, suggesting that the CS2 was resilient to drug manipulations. B-E) Vector profile analysis during the last 4 seconds of each CS trial shows individual responses following saline (faded) versus acute amphetamine (full) administration. It suggests an increase in magazine approach and goal-tracking in both the B) Saline Certain and C) Amphetamine Certain groups. Although this effect was somewhat seen in both D) Saline Uncertain and E) Amphetamine Uncertain groups, these animals remained largely unaffected, suggesting that prior exposure to reward uncertainty may provide protective factors against the acute effects of amphetamine on goal-tracking. F) Closer analysis of the effect of amphetamine (Amphetamine day - Saline day) on magazine entries between Certain and Uncertain groups showed a greater effect of amphetamine under Certain conditions which increased closer to the time of reward delivery (first vs last 4 seconds). G) An overall vector analysis of autoshaping that compares average approach behavior during the last 4 seconds across groups before (faded arrow) and after (full arrow) acute amphetamine exposure, showed a shift from CS1 sign-tracking to goal-tracking in both the Saline Certain and Amphetamine Certain groups. Concentric blue arrows show the shift in behavior due to acute amphetamine. In contrast, animals in both the Saline Uncertain and Amphetamine Uncertain groups predominantly maintain sign-tracking behavior to the CS2 and CS1, respectively.

Figure 6. Assessment of locomotor sensitization in response to acute amphetamine exposure. A) Analysis of the percent time spent engaging in grooming, rearing, and stereotypy behaviors following acute amphetamine exposure during autoshaping showed a significant increase in stereotypy, but not grooming and rearing, for animals that underwent prior

amphetamine sensitization. B) Surprisingly, acute amphetamine exposure had the largest impact on percent time spent in stereotypy for animals with prior amphetamine sensitization in the Uncertain reward condition, suggesting that prior repeated exposure to amphetamine may act synergistically with uncertainty to enhance locomotor sensitization.

Video 1. Demonstration video of representative behavior across experimental groups during autoshaping. The first video series shows the typical behavior in response to a CS trial of an animal in Saline Certain, Amphetamine Certain, Saline Uncertain, and Amphetamine Uncertain groups on the final day (Day 10) of autoshaping. Animals in the Saline Certain, Amphetamine Certain, and Amphetamine Uncertain groups were primarily attracted to the more predictive, reward-distal CS1. However, animals in the Saline Uncertain group were primarily attracted to the more incentive, reward-proximal CS2. The second video series shows the typical behavior of an animal in Saline Certain, Amphetamine Certain, Saline Uncertain, and Amphetamine Uncertain groups on the day of autoshaping with acute amphetamine. Although all groups maintained cue preference under acute amphetamine exposure, animals in Certain reward conditions showed a significant increase in entries into the magazine (increased goal-tracking) at the expense of CS1 responding, regardless of prior sensitization. Surprisingly, however, sign-tracking for animals in Uncertain reward conditions remained largely unaffected by acute amphetamine exposure.

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Conflicts of interest:

The authors report no conflicts of interest.

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The National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) were followed for all animal research contained within this manuscript.

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Highlights for: Reward uncertainty attributes incentive value to reward proximal cues, while amphetamine sensitization reverts attention to more predictive reward distal cues

- Reward uncertainty increases attraction to otherwise ignored reward-proximal cues
- Prior sensitization counters this effect and shifts attraction to reward-distal cues
- Prior sensitization makes uncertain gambling-like cues become more rewarding
- It also enhances responding on reward-distal cues during conditioned reinforcement
- Acute amphetamine promotes goal-tracking under Certain but not Uncertain conditions

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