

Cocaine seeking and consumption are oppositely regulated by mesolimbic dopamine

Corresponding Author: Dr Paul Phillips

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript by Burgeno et al investigates changes in dopamine (DA) levels in the nucleus accumbens core (Nacc) in response to contingent or non-contingent presentation to drug-related cues as function of chronic voluntary exposure to cocaine. They report that while, as previously reported by the same group, DA release induced by contingent cues decreases over time (i.e. undergoes tolerance), DA release induced by non-contingent cues increases over time (i.e. undergoes sensitization). The authors also show that this effect is mostly driven a subpopulation of rats that initially do not take much cocaine but over time, develops strong escalation of cocaine taking, leading to high levels of cocaine self-administration. Finally, using optogenetics the authors show that the reported changes in DA neurotransmission have causal effects of behavior because stimulation or inhibition of DA release mimics the effects observed.

I think the data are compelling and they are of interest for a wide audience.

However, I think there are several points that the authors should consider to improve their manuscript.

Main points.

- 1) One of my main concerns is with the approach measure. First of all, it is not clear how it was obtained and there is not enough information in the methods to understand it. At page 3 (results), the authors states that the nose-poke was inaccessible but do not explain how. Moreover, according to the methods, the highest score (=5) included interaction with the nose-poke. How was this possible if the nose-poke was inaccessible?
Also, in figure 2, both extinction responses (I guess nose-pokes) and approach scores are reported in this order. However, it appears (page 7) that approach responses were measured the day before extinction. Why inverting the order of presentation in the manuscript? Were these measures correlated? If they are correlated, are the authors sure that they measure different processes (in their words, CS-reinforced and CS-elicited responses) or simply rats were prevented from nose-poking because the nose-poke was inaccessible? If they are not correlated, how the authors interpret this?
Finally, in this figure, the mean value in the highest group was 3, which consists in "animal orients body towards nose-poke port" which makes me wonder about the interpretation of this measure as indicator of strong drug seeking.
- 2) Studies aiming to dissect seeking and taking behavior normally use a "seek" operandum and a "take" operandum. Given my previous comment, I wonder whether the authors should revise the text (and the title) to better frame their methods and results. For the title, I suggest focusing on the difference in contingent vs non-contingent cue-induced responses.
- 3) Introduction. One interesting aspect of this manuscript is that reconciles two sets of results in the literature and two theoretical frameworks about the role of DA in addiction. The first is the incentive salience framework that suggest that DA release increases over time in the course of addiction. As the authors clearly explains this hypothesis is mostly related to Pavlovian learning and therefore it is logical that this should be related to non-contingent presentation. The second framework, based on results by the group of Roy Wise in the context of contingent presentation of cues during active self-administration, proposes that levels of dopamine provide a sort of satiation signal and therefore, when levels are low animals should work more to obtain cocaine injections. This is explained in the discussion, but I find it surprising that this second framework is not presented in the introduction. I think that both hypotheses should be presented in the introduction to allow the reader to fully appreciate the interest of the paper.
- 4) A third theoretical framework about the role of DA in addiction that appears relevant to this work is the reward prediction error. Can the authors discuss how these results fit with this framework?

Minor points:

- 1) Abstract. The abstract states “Drug-cue reactivity, such as craving and automatized drug use” suggesting that these terms are synonyms. In contrast, drug-cue reactivity, craving and automatized drug use are conceptually very different processes and concepts and should not be confused. Elsewhere in the manuscript, the authors use the term “craving” for drug seeking. I suggest using the term drug seeking in all the descriptions in the results and limit use of the term “craving” to the instances in which they speculated about underlying behavioral processes.
- 2) Introduction. The sentence “We observed increasing dopamine release, a trajectory that is diametrically opposed to changes in dopamine evoked by the same stimulus when presented in response to a drug-taking action, measured in the same subjects” is not clear. Can the authors rephrase?
- 3) Introduction. The sentence “decreased NAcc dopamine to behavior-contingent stimuli producing increased drug consumption, and increased NAcc dopamine to non-contingent cues producing craving” does not explain what the measures are to separate consumption and craving. A better description of the actual measure would be more informative for the reader. Moreover, as in my main point 2 above, I wonder whether the authors can distinguish these processes in their procedure.
- 4) A more detailed description of the methods including detailed description of the behavioral procedures and how data were analysed is needed (eventually as supplementary material). For examples, data in the bottom panel in supplementary figures are % of % and it is not clear how they were obtained and what they represent. Similarly, for escalation, linear regression was used but it is not clear what parameters were used for this analysis and what was the criterion for escalation. Also, how was the delta conditioned approach value (fig. 3d and fig.3g) calculated? What does it represent?
- 5) Results. Although the authors states that increase in DA release from week 1 to week 3 only occurs in LgA rats, it appears that an increase may also have occurred also in ShA and did not reach statistical difference mostly because of the low n in the 3 week group. Can the authors indicate the n at each time point? Why was a lower n used in this group? I understand that running additional rats may be very complicated, the authors should acknowledge this problem and present the results in a more nuanced way.
- 6) Escalation of cocaine intake implies increased number of injections. Why only nose-pokes were shown in supplementary figures?

Reviewer #2

(Remarks to the Author)

The manuscript by Burgeno and colleagues is an interesting and timely study on how long-term cocaine intake alters rapid dopamine signals in the nucleus accumbens, as well as these signals play in cocaine reinforcement (operant behavior) and incentive sensitization (Pavlovian conditioning). The authors discovered that dopamine signals increase following non-contingent exposure to cues that were previously associated with chronic cocaine, but decreased when these cues followed contingent administration of cocaine. Additionally, optogenetic experiments showed that the heightened signals were causal to conditioned approach in the Pavlovian / non-contingent condition and the decreased signals were causal to escalation of cocaine intake in the long-access cocaine rats. This is a difficult experiment to perform, but the experimental design is strong. The within subject nature of the design for both the non-contingent and contingent exposures made this study particularly compelling for the multifaceted role that dopamine plasticity plays in cocaine / drug reinforcement. The writing, figures, and discussion were exceptionally clear, perhaps due in part, to this being a revised manuscript in response to a prior review. Given the timeliness and clarity of the experiments / design / writing, most of my remaining questions could be classified as minor. I feel the authors did a good job revising the manuscript and responding according to the prior reviewers' comments.

Minor Comments:

1. prior reviewer commented on differences in nucleus accumbens core vs shell dopamine, and so brain region differences have been addressed to some degree. However, this group previously reported that in addition to the decreasing dopamine signals in the nucleus accumbens following chronic cocaine already discussed in the contingent condition, there is a corresponding dopamine signal increase in the dorsolateral striatum following contingent chronic cocaine (Willhuhn et al., 2012; PNAS). Some reference to this study, and possibly a sentence or two in the discussion, might help reader place this work in the context of broader understanding of concomitant dopamine signals in other regions that might be a neural substrate for habit formation, yet still play a role in this behavior.

Reviewer #3

(Remarks to the Author)

Burgeno et al. investigate how mesolimbic dopamine signaling to drug-associated cues changes over the course of cocaine self-administration in rats, and how these changes relate to addictive behaviors. Using fast-scan cyclic voltammetry (FSCV) in the nucleus accumbens (NAcc) core during cocaine self-administration, they show that dopamine responses to a cocaine-paired cue evolve in opposite directions depending on the context of the cue presentation. Specifically, when the cue was presented non-contingently, cue-evoked dopamine release increased with prolonged drug use. In contrast, when the same cue was presented contingent on an operant response during drug self-administration, the dopamine signal decreased over time in those animals that escalated their cocaine intake. Behaviorally, these neurochemical changes were linked to core addiction-like behaviors: the growing dopamine response to non-contingent cues was associated with enhanced cue-induced “craving” (operationalized as elevated approach behavior and lever pressing for the cue), whereas the diminishing dopamine response to the contingent cue correlated with escalating drug consumption. The authors causally tested these

links using optogenetics. Together, the results support a model in which “diametrically opposite” dopamine adaptations both promote addiction: increased phasic dopamine to passive drug cues promotes craving and relapse risk, whereas decreased phasic dopamine during drug-taking promotes greater consumption.

Surprisingly few studies have examined dopamine dynamics during protracted abstinence and during cue-evoked seeking / conditioned reinforcement. These are highly challenging experiments to perform, and the authors should be commended for completing them in a highly rigorous fashion (e.g. well-powered, within-subject design, appropriate statistics, etc.). There are a few issues, which are readily addressable, detailed below.

Major:

1. Was contingent cue-evoked dopamine measured during abstinence? The diagram and behavioral data in figure 2 suggest that it was measured, but not reported. These data would be useful in determining whether abstinence-induced changes reflect an interaction with the cue presentation type, as the authors demonstrate for the changes occurring during ongoing self-administration, or if abstinence-induced changes reflect non-selective plasticity similar to PMID: 29175958.
2. The framing of the findings within the human literature take an overly narrow / somewhat cherry-picked view. For example, it is stated in the introduction that evidence corroborating the incentive sensitization theory has accumulated over the years, and this concept is revisited in the discussion. While a few studies can be found to support this, it ignores a much larger literature that has found null or opposite effects in humans (for review see PMID: 18720516). While it may not be necessary to fully flesh out the human literature, as currently written there are several broad statements made that are not well supported; the authors should either provide a more nuanced, well-cited argument or remove the statements entirely.
3. Along these same lines, statements throughout the introduction and discussion regarding clinical findings are supported by references studying amphetamine, alcohol, and opioids, though the clinical data for these drugs often differs from cocaine. The references should be replaced by cocaine studies, and/or should be qualified in the text.

Minor:

4. The sessions in which animals respond for presentation of the cue should be labeled as conditioned reinforcement (the contingent occurrence of a conditioned stimulus) rather than extinction (the complete discontinuation of an operant contingency). See APA dictionary and Ferster & Skinner, 1957.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Burgeno et al did a good job at addressing most of my concerns. The only doubt I still have is the relationship between CS-reinforced and CS-elicited responses. The authors now clearly explain that CS-elicited responses were obtained by blocking physical access to the nose-pokes but they do not address the question of whether CS-reinforced and CS-elicited are related phenomena (as it is expected) or not. As suggested in my previous review, they could perform correlation analysis between individual subjects' CS-reinforced and CS-elicited response measures. If the two measures are correlated, this would indicate that they represent related phenomena; if they don't, this may indicate that they are somewhat different processes. In my opinion, this analysis and clarifying the relationship between these two measures will further improve the manuscript.

Also, I still find somewhat confusing the sentence "We then assayed whether behavioral cue reactivity also underwent incubation" given that this was tested BEFORE CS-reinforced responses according to the experimental sequence described in the methods.

These points are minor and should not delay the publication of this excellent study.

Reviewer #2

(Remarks to the Author)

The authors did a good job responding to comments. I have no additional comments.

Reviewer #3

(Remarks to the Author)

The authors have addressed all of my previous concerns. The revised manuscript represents a significant advance for the field.

Open Access This Peer Review File is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made.

In cases where reviewers are anonymous, credit should be given to 'Anonymous Referee' and the source.

The images or other third party material in this Peer Review File are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder.

To view a copy of this license, visit <https://creativecommons.org/licenses/by/4.0/>

We appreciate the time the referees took to provide careful and constructive feedback on our original manuscript. We were encouraged by their positive evaluations of the impact and quality of the work and the manuscript itself. We have prudently addressed all the points made by the referees, resulting in the addition of new data, new analyses, new figures, and revisions to the previous figures and text.

Referee 1 made an important leading point, noting that the work was conducted exclusively in male subjects. The work within the manuscript represents more than a decade of research and, as such, began at a time when studying only males was standard practice. We are in complete agreement with the reviewer that the impact of the work would be appreciably greater if we had included female subjects. However, even though the *relative* impact of the work is lower than a hypothetical study with the same data in both sexes, we still believe that the work has high significance and broad appeal. We make this statement without in any way diminishing the importance of studying females—indeed, all of our ongoing studies include females—but we objectively note that males living with cocaine use disorder represent a massive global population and this condition is consequent to a large number of deaths each year. In the revised manuscript we have stated this limitation, constrained our conclusions to the male population, and expressed caution over extrapolation to the entire population. This referee also asked several questions on presentation and interpretation of the data. As described point-by-point below, we have addressed these questions with the provision of new data analyses and presentation, and revisions to the text.

Referee 2 raised four questions or suggestions. We addressed all of these points below and made appropriate changes in the manuscript, including the addition of new data and analyses.

Referee 3 requested additional experimental details, alternative presentation of the data, additional discussion and additional data analysis. We have addressed all of these points below and provided the additional details in the manuscript as requested.

Referee 4's main concern was that we did not cite an existing paper that has precedence for one of the components of our findings. We acknowledge that this omission was a veritable oversight on our part and have remedied this by citing the paper in multiple key places in the revised manuscript. We also followed the reviewer's advice to qualify the anatomical specificity of our recordings and manipulations throughout the manuscript. In addition, we addressed the reviewer's other points by revisions to the manuscript and by including new figures to depict behavior in the subset of animals that had successful voltammetry recordings.

We have provided detailed responses to all of the reviewers' comments below. The bolded parts are the original reviews, and the italicized parts are our responses. Revisions to the manuscript in response to these comments are underlined.

Referees' comments:

Referee #1 (Remarks to the Author):

Burgeno, Farero and colleagues investigate the relationship between dopamine signaling in the nucleus accumbens core and the progression of addiction-related drug seeking behaviors in rats. Consistent with previous studies, they demonstrate individual differences in drug cue reactivity and escalation of cocaine seeking behavior, finding a subset of rats of more vulnerable rats. Using fast scan voltammetry recording they track dopamine signaling across extended access cocaine self admin, finding that non-contingent drug cue presentations evoke ever larger dopamine responses in the accumbens of susceptible rats, which has a functional role in promoting seeking. At the same time, the dopamine responses to drug seeking actions and drug itself diminish in susceptible rats, which is thought to separately promote drug seeking motivation to maintain drug highs, consistent with their own previous work. Overall, these results support a multifaceted role for accumbens core dopamine in addiction and motivation in general. They also underscore the importance of considering factors like individual differences, and the various ways that sensory stimuli can intersect with behavior/decision making states, when defining functional roles for brain systems in disease. These data help reconcile some long-standing ideas about the role of dopamine in drug-related motivation, by more or less concluding that multiple theoretical perspectives are true.

Overall the manuscript is clearly written and the results are intuitively presented. I have a few comments about limitations in the study design, analysis, and interpretations based on the current data.

My primary comment is that unfortunately, the use of only male subjects, in a report with what would otherwise be broad implications, is a major shortcoming and limitation. At this stage in biomedical research and given the large number of animals used in these studies, including for the difficult voltammetry approaches, it is not clear why female rats were excluded, and no justification is provided in the paper. Without confirmation that at least some of these effects hold/don't hold in female rats, the generalizability of these findings and therefore broad impact is heavily weakened.

It is entirely possible that the general take home message would not differ in male and female rats, but there a number of ways these effects could be sex biased that could be critical to understand in the context of the real-world problem of addiction. There are known basic differences in drug-seeking motivation and relapse and, especially pertinent to these

studies – dopamine responses to drugs and drug-related cues and incubation of craving. Those sex biases could intersect here in ways that would be really important to know.

I also note that the lack of some attempt at assessing data in males and females does not comply with the standards set for expectations for NIH-funded grants, which have been in place for several years now.

We acknowledge that the inclusion of only male subjects is a limitation of the study, and we agree that the possibility exists that the current results in males are not predictive of the neurochemistry in females. Throughout the manuscript we have now qualified that the results were collected in males, and we have discussed the limitations of extrapolating them to females.

This work began before the field adopted best-practice procedures of including both male and female subjects in this type of research. All of our ongoing studies include both males and females. We hope that we will, in the future, be able to get a better understanding of sexual dimorphisms relating to the current findings. However, the work took many years to get to this point and, as noted by the reviewer, used a large number of animals. Therefore, waiting until we have comparable data in females would delay dissemination of the findings very significantly.

Despite this limitation, we still believe the work has broad implications to the scientific community and beyond, consistent with the reviewer's acknowledgement that the "data help reconcile some long-standing ideas about the role of dopamine in drug-related motivation." Given the value of our findings to behavioral neuroscientists, clinical neuroscientists, psychologists and physicians, we believe that expeditious publication of the work would be greatly advantageous to the scientific community.

Over the past few years in rodent addiction neuroscience there has been somewhat of a pivot or broadening of access patterns studied, with intermittent/binge models becoming popular, versus the long access approaches here. These tend to involve a lot less drug exposure overall, but there is growing evidence this still engenders a sensitized behavioral state and striatal dopamine system – so a major question I'm left with is if the intake amount vs dopamine signal relationship seen here is dependent on a certain amount of cocaine exposure, or something else.

While we did not use intermittent access, we did address the relationship between intake and dopamine. We found that dopamine release increases, mirroring incubation of craving, following withdrawal (Figure 2). These concomitant changes in psychological state and neurochemistry take place without additional drug intake. We have clarified this point in the manuscript.

There is a kind of chicken and egg situation at play – do the escalating rats somehow have a dopamine system that primes them to escalate, or do rats who happen to escalate develop unique dopamine responses based on the fact they take a lot more cocaine. The lack of yoked controls limit insight somewhat in this regard. Presumably unpaired cue and cocaine exposure matched to the intake of the self administering escalators would be insufficient to drive some of the dopamine responses seen here?

This point is related to the previous point, but in the context of individual differences: whether the dopamine dynamics are directly related to the history of drug intake or whether these factors can be divorced. Differences in cue-evoked dopamine release between escalators and non-escalators emerge before escalators have taken more cocaine than non-escalators. This interesting observation comes about because, as it turns out, escalators actually take less cocaine per session initially, catch up with non-escalators, and then surpass them. Specifically, the cumulative intake in escalators crosses that in non-escalators during W3 (all escalators: $1,577 \pm 51$ total infusions, $n=24$, all non-escalators: $1,595 \pm 59$ total infusions, $n=35$, $p=0.8313$, unpaired t-test; voltammetry escalators: $1,609 \pm 63$ total infusions, $n=8$, voltammetry non-escalators: $1,505 \pm 62$ total infusions, $n=11$, $p=0.2677$, unpaired t-test). At this time point, dopamine release to (non-contingent) drug cues is much greater (~4 times) in escalators compared to non-escalators. Therefore, this difference is not due to the total drug intake history (or the number of drug-cue pairings) since these metrics are not different at this time point. We have now provided more information on the absolute drug intake over time (Supplementary Figures 2a, 2b, 4a and 4b) and the cumulative history of escalators and non-escalators, and we have elaborated on how dopamine signaling changes relative to these intake statistics.

Are cocaine-experienced rats extra responsive to cues in general, or is the dopamine effect specific to the drug-paired cue? There is no control cue or neutral stimulus for comparison. This kind of relates to the notion set up in the paper of the impact of Pavlovian cues on instrumental actions in PIT tasks, as there can be specific or general PIT. It also seems like these components could dissociate – a neutral cue may still evoke a large DA novelty response in the context of the sensitized state the drug experienced animals are in, but maybe it would not evoke the same approach behavior.

Ito et al (2000) used a CS+/- design to demonstrate that dopamine responses in cocaine-experienced rats were selective for drug-related cues. We have now cited this paper with respect to this selectivity. Furthermore, the main thesis of the current manuscript is that changes in dopamine signaling are context dependent based upon how the conditioned stimulus is presented (contingent versus non-contingent). We would argue that this result indicates that there is not a generalized sensitization of the dopamine system underlying the changes we observed.

Related to this – it would be useful to get a sense of how much drug intake it happening. There are only % baseline values shown – it's unclear if this is 50 infusions, 20, etc. Overall there is not much detail about the behavior which makes comparisons to other papers challenging.

We used baseline normalized intake plots since the individual differences we report are based upon the change in intake over time (i.e., escalation) rather than the absolute intake. However, the reviewer makes a good point that it is valuable to also see the absolute intake. The value of this point is further reinforced by our response above, pertaining to the cumulative intake of escalators versus non-escalators. We have now added supplementary figures showing the absolute daily intake, and inactive nose pokes (Supplementary Figures 2a, 2b, 4a and 4b).

It would be helpful to have more histological examination of the viral approach for the optogenetic experiments. The current pictures are very cropped and small – hard to evaluate. While the viral approach appears to be relatively specific, it remains a bit unconventional – versus using something like a DAT or TH-cre rat, or a TH-promotor virus to directly target dopamine neurons. Given the optically evoked dopamine signals, it's clear that dopamine neurons are being targeted, but the lack of specificity in the viral approach means other things might be also, and more so than for other techniques. They are hard to see but just eyeballing the images in figure 2, I see lots of red but not green cells (the whole IPN is lit up, also). I don't think these are necessarily major concerns given the effects are based on terminal stimulation, but it would be helpful to include a rationale for the use of this viral approach (and why off targeting isn't an issue) instead of a more common and validated targeting method. It's unclear to me why this CamKII virus would be specific for dopamine neurons per se.

DAT or TH-cre rats were not used because these animals are a different strain from those used in the voltammetry studies. We felt that if we changed the strain, we would need to carry out significant characterization to be confident that we are working within the same domain of relative numbers of escalator versus non-escalator rats. We were particularly concerned about this point given that the rate of escalation differs between rat strains (e.g., Picetti et al, 2010). Therefore, we opted to gain cell-type specificity using a promoter.

In terms of the selection of a promoter, we avoided the TH promoter because it can be 'leaky' in the experience of some of the authors and their collaborators. Rather, we followed the extensive characterization of cell-type selective genetic markers in the VTA, carried out by authors MES and LSZ, which identified CaMKII α as having good selectivity for TH-containing neurons in the VTA. As we showed in the manuscript using the promoter for this gene confers high selectivity for cells that positively label for TH (89.6% of all mCherry+ cells were TH+) and also is highly efficient (90.7% of all TH+ cells were mCherry+). These statistics are a conservative representation of the selectivity and efficiency because of the likelihood of false negatives. Importantly, these statistics relate to cells within the boundaries of the VTA. The selectivity is

not sustained when the virus has infected surrounding nuclei, most notably the interpeduncular nucleus (IPN). However, efferents from those surrounding nuclei do not project to the nucleus accumbens, and because the optical stimulation is targeted to the nucleus accumbens core, off-target stimulation of those neurons is not an issue.

We have added a concise version of the above justification to the manuscript and also updated the histology figure.

Roberto Picetti R, Ho A, Butelman ER, Kreek MJ (2010) Dose preference and dose escalation in extended-access cocaine self-administration in Fischer and Lewis rats. Psychopharmacology 211:313-23. doi: 10.1007/s00213-010-1899-3. PMID: 20559822, PMCID: PMC2926930.

Referee #2 (Remarks to the Author):

This is a conceptually innovative study, in which the authors provided convincing evidence demonstrating different dopamine dynamics in the NAcc in response to contingent versus noncontingent CS associated with cocaine taking.

These findings are novel and push our understanding about in vivo dopamine dynamics to the next level. The results and conclusions indeed make a lot of sense.

The logic flow is very clear, results are robust, conclusions are reasonable and informative, previous work is appropriately cited and discussed, and manuscript is extremely well written

Some suggestions/questions:

1) The actual meanings of noncontingent versus contingent SC were not immediately clear in the text, figures or legends. I did some digging to figure out the details of these two experimental conditions.

We have now better defined contingent and non-contingent stimulus presentations in the abstract and introduction.

Abstract: "Dopamine evoked by non-contingent cue presentation (independent of the animal's actions) increased over drug use, producing greater cue reactivity; whereas dopamine evoked by contingent cue presentation (dependent on the animal's actions) decreased over drug use, producing escalation of drug consumption."

Introduction: "When a drug-paired stimulus is presented in response to a drug-taking action (contingent), it acts as a feedback signal indicating that drug delivery is imminent and no further action is required, whereas the same stimulus, presented independently of the subject's actions

(non-contingently) outside the active drug-taking context, acts as a cue (eliciting stimulus) to promote drug seeking.”

2) Figure 1 (and other related figures): It appears that there were 5 days of training in each week for ShA as well as LgA procedures. Were there any particular reasons that there had to be a two-day break (withdrawal) for this procedure?

As noted by the reviewer, we did not carry out self-administration sessions every consecutive day. This was not designed as a manipulation specific to the current avenue of investigation, but is our standard practice (e.g., Willuhn et al, 2014) as well as that from other labs (e.g., Briand et al, 2008; Durand et al, 2022). Importantly, the intake in a session relative to the previous session is not different following a break compared to consecutive sessions. We have now added this information to the manuscript (Supplementary Figures 2d and 4d). In addressing this point, we noticed that we were not explicit in stating that there were days off and so have clarified in the methods.

Briand LA, Flagel SB, Garcia-Fuster MJ, Watson SJ, Akil H, Sarter M, Robinson TE (2008) Persistent alterations in cognitive function and prefrontal dopamine D2 receptors following extended, but not limited, access to self-administered cocaine. Neuropsychopharmacology 33:2969-80. doi: 10.1038/npp.2008.18. PMID: 18305460, PMCID: PMC3092154.

Durand A, Girardeau P, Freese L, Ahmed SH (2022) Increased responsiveness to punishment of cocaine self-administration after experience with high punishment. Neuropsychopharmacology 47:444-453. doi: 10.1038/s41386-021-01159-3. PMID: 34429520, PMCID: PMC8674259.

3) Fig. 1g: In the DA results for Week 3, there was a “bump” right after the 20-sec measurement window in both ShA and LgA troupes. what are they? do they carry any meaningful information?

We agree that this aspect of the signal is curious. However, none of our analyses has gleaned any relevant information encoded by the bump. Specifically, it does not correlate with CS-onset signal, approach score, drug consumption or escalation slope. In addition, changes in this signal over time also do not correlate with any of those variables. We have now commented on this signal in the manuscript: “A secondary peak was apparent at the CS offset, especially in week 3. However, this signal did not correlate with the primary peak ($r^2=0.002$, $p=0.785$), approach score ($r^2=0.0007$, $p=0.933$), drug intake ($r^2=0.02$, $p=0.463$) or escalation ($r^2=0.05$, $p=0.446$)”.

4) In exploring the cellular-behavioral causal relationship, the authors used several manipulations, all focusing on stimulation/activation of dopamine release. Have the authors considered the inhibition angle (e.g., manipulations that inhibit dopamine release, contrasting to Figure 2 and 4)?

We agree that the use of inhibition would be useful in further characterizing these phenomena. The reason we elected not to include inhibition experiments at this time is because the onset of the inhibitory stimulation would need to precede the cue presentation. To do this for behavior-contingent cue presentations, we would need to anticipate the time at which the animal would respond. One approach we considered was to impose a delay between the nose poke and the cue presentation so that we could turn on the inhibitory stimulation at the time of the nose poke (preceding the cue presentation). However, we were concerned that this, in and of itself, would change the drug taking behavior and complicate interpretation of the data. In addition, there have been reports of behavior driven by rebound excitation following optogenetic inhibition of the VTA (e.g., Hughes et al, 2020). This potential confound was another factor that we considered might complicate interpretation of the results.

Hughes RN, Bakhurin KI, Petter EA, Watson GDR, Kim N, Friedman AD, Yin HH (2020) Ventral Tegmental Dopamine Neurons Control the Impulse Vector during Motivated Behavior. Curr Biol. 30:2681-2694.e5. doi: 10.1016/j.cub.2020.05.003. PMID: 32470362, PMCID: PMC7590264.

Referee #3 (Remarks to the Author):

Here, the authors identify an opposing change in dopamine dependent upon cue stimuli being response-contingent or non-contingent. A major finding of this work is new knowledge that may inform us about why certain animals may escalate and why others may not and how this relates to environmental stimuli. This work is very interesting; but is likely of interest mostly to behavioral neuroscientists. For some time, new investigations have informed the field that dopamine is more than just a signal for valence; but is much more complicated in regards to motivated behavior. While the authors have focused on the differences between the dopamine response to non-contingent stimuli in two subsets of rats, there is still some agreement with prior reports (Mohebi et al., 2019; Kutlu et al., 2021) in regards to the dopamine signal being related to other aspects of motivated behavior. However, as written there is a lack of necessary details in a few areas that would need to be addressed.

Major Comments

1. Self-administration data should be presented as number of infusions instead of percent baseline (Figure 1) or percent baseline of ShA rats (Figure 3). Additionally, the authors should

present the active and inactive lever data (even if this is simply put in the supplemental section).

We chose to present relative (percent baseline), rather than absolute responding since we felt that this metric was more relevant for escalation (i.e., the dynamic change in drug consumption over time from baseline, rather than the absolute amount). That being said, the absolute responding provides useful additional information. Therefore, we have included supplementary figure depicting the absolute responding as well as inactive nose pokes (Supplementary Figures 2a, 2b, 4a and 4b).

2. There needs to be a few more details provided for the probe sessions. First, the authors state these sessions were conducted with the CS presented twice; however, the authors don't clearly state the duration of the entire probe session. Additionally, since the probe session was immediately prior to the last self-administration session in some contexts, did the self-administration session begin immediately prior to the probe session. To be clear, there needs to be additional details regarding the specific timing of these sessions. Also, since the CS was presented twice, is it true that an FSCV recording was only recorded twice during the probe sessions? This should all be clearly stated.

Conditioned approach behavior was assessed in drug-free, probe sessions in the self-administration chamber where cues associated with self-administration sessions were presented non-contingently (experimenter delivered) at three-minute intervals. Self-administration sessions began three minutes after the last cue presentation. We were intentionally conservative on the number of presentations for short-access animals, to minimize extinction effects. As noted by the reviewer, this meant that there were recordings of only two cue presentations averaged per week. For animals that underwent long-access self-administration, we increased the number of cue presentations to five (still spaced out by three minutes) to increase the statistical power as we had less concern about extinction in these animals.

We have now provided more information on the sessions in the methods, the relevant results sections, and in the timeline schematics in the figures.

3. In regards to Figure 3, the authors state they re-analyzed the data. Thus, is all of the data in Figure 3 a re-analysis of the previously presented data. With the current way this section of the results is written, it is not clear if this is re-analyzed data or if it is new data. I assume the latter. Additionally, Figure 1 and 2 and the associated results text were very clear that the FSCV was completed during probe session only; however the section associated with Figure 3 does not do this (and there is no timeline figure to clarify this). Thus, it is not clear when the FSCV was completed for this presented data and if it only represent a single session for the associated weeks displayed in Figure 3. This will need to be clarified. The legend of figure 3

does refer to probe session for panel 3D; but this should be made clear in the main text if this is the case.

The animals from Figure 3 were the same ones as the LgA cohort in Figure 1. However, we compared the responses to five cue presentations in the probe sessions (since all animals were long-access cohorts). We also included data from an additional week of LgA in this analysis. We have clarified these points, and have added timelines to Figures 3 and 4, and more details to the timelines in Figures 1 and 2.

4. Figure 4 presents the FSCV recordings that were collected during the self-administration sessions and not the probe sessions. This highlights the fact that the methods should be clear about which sessions were used as FSCV recording sessions. Were all the self-administration sessions simultaneously recording dopamine release? If so, is the representative data for the waveforms in Figure 4 a mean of the entire week for each rat? Why is there no data presented for weak 2 (in regards to week 2, this same question applies to Figure 3)?

For self-administration, voltammetric recordings were conducted in the session at the end of weeks 1, 3 and 4. These time points were chosen a priori to represent the last session of ShA (end of week 1), the last session of the regular LgA block (end of week 3) and an additional time to match our previous data (end of week 4). We did not record in every self-administration session to preserve the integrity of the headcaps and electrodes. Therefore, each trace represents the average responses from each subject from a single recording session. Likewise, non-contingent probe sessions took place at the end of week 1 and the end of week 3 (see Figure 1e). A subset of animals continued into a third week of LgA training (i.e., week 4) and were also tested at the end of that week. We have added timelines to Figures 3 and 4 to clarify when recordings were carried out.

5. Is it possible that the non-escalators and escalators have a different landscape of dopamine machinery in the NAc (DA transporter, D1 and/or D2 receptor density)? The methods state the rats were house individually; but was that just from the start of the surgery and self-administration? When receiving the rats were they group housed originally and then separated?

The reviewer raises an interesting question on trait differences between escalators and non-escalators. As far as we know, transcriptional analyses (single cell or otherwise) have never been carried out to compare animals exhibiting these behavioral differences (escalators versus non-escalators). This analysis would be complicated by the fact that, at this time, we do not have means to predict which animals will become escalators and non-escalators, and so the analysis would need to be performed after drug-self administration, conflating whether any transcriptional differences were predisposing or a result of the behavior. Animals were pair

housed prior to surgery, and then single housed. This information has been added to the Methods section.

6. Something else may be occurring in this paradigm and it may be centered upon novelty. Kutlu et al., (2021) used photometry to show that dopamine release may be enhanced with novel stimuli. The probe sessions are spaced far enough apart and are given in contexts that are novel and this may be something that factors into the observations of this report.

We probably were not clear that the probe sessions took place in the self-administration chamber and, therefore, were not in a novel environment. This lack of clarity may have come about due to our reference to dopamine dynamics in different contexts. To this end, we were referring to different situational contexts (the means by which the stimulus is presented) rather than environmental contexts (the place in which the stimulus is perceived). We have now clarified this point in the manuscript.

7. Since key findings of sex differences continue to be revealed, the authors should provide a justification for completing this study only in male rats.

We have now discussed our rationale for including only males and highlighted the limitations thereof.

8. This is more of a question rather than a comment: Given that the LgA rats exhibit greater self-administration and CS evoked dopamine release, if you were to run a linear regression for individual rats would drug intake and dopamine amplitude correlate?

These variables do significantly correlate. We have included a graph of this relationship in Supplementary Figure 4e.

Minor Comments

1. Abstract (page 1), line 6. The authors should specify that this was completed in male rats, instead of rats in general.

We made this change.

2. The authors should include individual data points in the bar charts.

We have added individual data points to all the bar charts, including those in extended data figures.

3. Page 19, line 295-296. Here, the authors should provide the exact n for the escalators and non-escalators.

We have provided this information.

4. In the methods, there are some details that are missing that would facilitate reproducing the authors' methods. The authors should provide the details for the camera and software that was used to record the rats' drug seeking behavior. In the methods, the authors should provide the specific software used for the FSCV acquisition and analysis (Tarheel, Demon, other?). Some of the specifics for resources used in surgical procedures should be provided (type of dental cement/acrylic, titer/volume of AAVs).

We have added the requested details.

Referee #4 (Remarks to the Author):

This manuscript, from Burgeno and colleagues, examines phasic changes in dopamine in the nucleus accumbens core in response to cocaine self-administration cues. The authors report that different changes in cue-elicited dopamine depending on whether the cue is presented contingent upon the cocaine instrumental response or if the cue is presented non-contingently, and that these differences correlate with patterns of drug-taking over days. The authors show that optogenetically enhancing the cue-elicited dopamine signal increases the behavioral impact of non-contingent cues, yet decreases responding for cocaine when paired with response-contingent cues. Overall, they show that the rats show opposite changes in cue-elicited dopamine depending on how that cue is presented (non-contingent vs contingent) and that the behavioral function of these two types of dopamine elicitation are distinct. These results are quite interesting and will have an impact on preclinical research on substance abuse as well as dopamine and reward learning research in general. The findings from the optogenetic manipulation are especially notable in their demonstration of

functional roles for the observed dopamine signals. I also note that the work is presented in a clear manner – indeed, the paper was a pleasure to read.

The main drawback of the work in its present form is the lack of appropriate citation and discussion of prior work that discovered the difference in accumbens core dopamine elicited by response contingent and non-contingent cocaine-paired cues. Ito et al (J Neuroscience, 20 (19) 7489-7495, 2000) found that a cocaine cue elicited increases in dopamine when presented non-contingently, but no increase when presented in a response-contingent manner. The authors surely know this work well. While this prior work used microdialysis, and thus lacks the temporal specificity shown here, the prior experiments were well-designed (the use of a CS- was a strength absent from the current studies) and resulted in interpretable data. The current results confirm the prior findings, and then extend them by relating the dopamine signals to whether or not subjects “escalate” responding over weeks, and by using optogenetics for a causal manipulation.

We appreciate that the reviewer brought this notable oversight to our attention. We agree that the current work dovetails with the previous findings of Ito and colleagues and we believe, those results strengthen our conclusions by providing precedence for differential dopamine signaling in the NAcc to CS presentation based upon the contingency of their presentation. Accordingly, we have corrected our omission by citing the work in key places of the manuscript. In the introduction, we have noted that differences in dopamine release for contingent versus non-contingent cues were observed by Ito and colleagues. We also revisit this point in the results and discussion.

A second shortcoming of the present work is the treatment in the text of accumbens core dopamine measures as a stand-in for all dopamine. Indeed, the abstract does not mention where the dopamine recordings are made. Prior work, again from the Robbins-Everitt group, found unique patterns in accumbens core vs shell and in the dorsal striatum. Ideally the authors would have a comparison region. At the least, the authors must modify their conclusions to reflect the anatomical limitations of their studies.

We have added qualifiers throughout the manuscript to note that recordings and manipulations of dopamine transmission were specifically in the nucleus accumbens core.

Other points:

The authors use only male rats but this does not figure in the discussion at all. It is critical that the conclusions are qualified until the authors know whether or not the same effects would be observed in female rats.

We have added qualifiers throughout the manuscript labeling the subjects as males. We also included a discussion on the caveat of only using male subjects.

It is surprising that the cocaine self-administration group is of such a very large sample size (n=66). I assume this was not the plan setting out. How was this group assembled? Why is the sample size so large? This is for the behavioral data presented in Figure 1 F. Then in Figure 1 G we see average dopamine traces for much smaller sample sizes. Why? How were subjects whose data are shown chosen from the larger group? Sample sizes again are different in the data in Figure 2 when reporting the behavior (Fig 2b,c) vs dopamine signals to non-contingent cues (Fig 2 d). This pattern is found throughout. I understand the difficulties in obtaining good signals from this cyclic voltammetry technique; the behavior shown should be from the rats from whom the dopamine data were obtained.

The larger numbers represent all of the animals on which we attempted voltammetry. However, there was a high level of attrition of successful recordings (see criteria at the end of the Fast-Scan Cyclic Voltammetry section of the Methods). This was especially true when we needed to obtain within-animal repeated recordings on specific timepoints. Nonetheless, the behavior in the animals from which successful recordings were obtained was representative of the whole dataset. On each of the behavioral figures, we have now indicated the animals included in the voltammetric recordings with unique symbols. We have also provided extended data figures of the behavior of the voltammetry animals alone including statistical analyses (Supplementary Figures 2b and 4b).

The finding that the non-contingent cue-evoked dopamine response is greater in rats trained in the self-administration procedure than in naïve rats does not as the authors say confirm that the change is learning-related. Although this certainly is the most likely conclusion, the authors do not have a control group that received prior cocaine to control for non-associative

sensitized responses to cue presentation in the cocaine self-administration group. The wording should be altered.

The reviewer makes a good point. We changed "...confirming that these are learned, rather than innate responses." to "...the capacity for the CS to elicit dopamine is dependent on experience, rather than being an innate response."

We appreciate the reviewers' thoughtful feedback. We have carefully addressed all of their points and made changes to the manuscript where warranted. Our point-by-point responses are listed below (reviewers' comments bolded, our responses italicized). Changes to the manuscript are underlined in the main text.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The manuscript by Burgeno et al investigates changes in dopamine (DA) levels in the nucleus accumbens core (Nacc) in response to contingent or non-contingent presentation to drug-related cues as function of chronic voluntary exposure to cocaine. They report that while, as previously reported by the same group, DA release induced by contingent cues decreases over time (i.e.undergoes tolerance), DA release induced by non-contingent cues increases over time (i.e. undergoes sensitization). The authors also show that this effect is mostly driven a subpopulation of rats that initially do not take much cocaine but over time, develops strong escalation of cocaine taking, leading to high levels of cocaine self-administration. Finally, using optogenetics the authors show that the reported changes in DA neurotransmission have causal effects of behavior because stimulation or inhibition of DA release mimics the effects observed. I think the data are compelling and they are of interest for a wide audience. However, I think there are several points that the authors should consider to improve their manuscript.

Main points.

1) One of my main concerns is with the approach measure. First of all, it is not clear how it was obtained and there is not enough information in the methods to understand it. At page 3 (results), the authors states that the nose-poke was inaccessible but do not explain how. Moreover, according to the methods, the highest score (=5) included interaction with the nose-poke. How was this possible if the nose-poke was inaccessible?

Approach scores were assessed by video review, by investigators that were blinded to dopamine response and escalator status at the time of the review. The individual approach scores are the average response to all five cue presentations in the session. Nose-pose ports were covered with clear self-adhesive tape to prevent entry, without obstructing observation of the cues. Therefore, animals were able to approach and make contact with the outside of the port and the covering tape but, to mitigate

extinction, were prevented from enacting a full operant response. Interaction with the nose-poke port (as required to obtain a score of 5) was assessed as contact with the tape covering the port. This information has been added to the methods.

Also, in figure 2, both extinction responses (I guess nose-pokes) and approach scores are reported in this order. However, it appears (page 7) that approach responses were measured the day before extinction. Why inverting the order of presentation in the manuscript? Were these measures correlated? If they are correlated, are the authors sure that they measure different processes (in their words, CS-reinforced and CS-elicited responses) or simply rats were prevented from nose-poking because the nose-poke was inaccessible? If they are not correlated, how the authors interpret this?

The extinction responses were reported first because they are the standardized method for assessing incubation of craving. We carried out that test and reported the results to validate that the withdrawal paradigm had produced a behavior state that replicated previous studies using this procedure. However, since the primary interest of this study was to test the effects of non-contingent cue-elicited dopamine release, we felt it was necessary to test the behavior that was directly elicited by non-contingent cue presentation, rather than extrapolate from a related behavior. Therefore, we first report the validation of the paradigm to produce incubation of craving and then test how our primary experimental behavior is affected by that behavioral manipulation. We have clarified this rationale in the text.

Finally, in this figure, the mean value in the highest group was 3, which consists in “animal orients body towards nose-poke port” which makes me wonder about the interpretation of this measure as indicator of strong drug seeking.

The number represents the average of all five cue presentations in the session. In general, the early responses are stronger than the later ones (i.e., there is extinction of the response). The response to the early cue presentations was often 4 or 5. We have now added the breakdown of each cue presentation as a supplementary figure that demonstrates this point.

2) Studies aiming to dissect seeking and taking behavior normally use a “seek” operandum and a “take” operandum. Given my previous comment, I wonder whether the authors should revise the text (and the title) to better frame their

methods and results. For the title, I suggest focusing on the difference in contingent vs non-contingent cue-induced responses.

We agree that using “drug seeking” and “drug taking” presented in opposition in the title and elsewhere may be indicative that we used a seeking–taking chained schedule of reinforcement. To avoid that perception, we changed “taking” to “consumption” in the title and in the results when we are describing our metric of drug intake. We feel that consumption is a good operational definition of this behavior. Likewise (and as reinforced by the reviewer in minor point 1 below), “drug seeking” is a veritable definition for the behavioral cue reactivity we measured throughout the manuscript.

3) Introduction. One interesting aspect of this manuscript is that reconciles two sets of results in the literature and two theoretical frameworks about the role of DA in addiction. The first is the incentive salience framework that suggest that DA release increases over time in the course of addiction. As the authors clearly explains this hypothesis is mostly related to Pavlovian learning and therefore it is logical that this should be related to non-contingent presentation. The second framework, based on results by the group of Roy Wise in the context of contingent presentation of cues during active self-administration, proposes that levels of dopamine provide a sort of satiation signal and therefore, when levels are low animals should work more to obtain cocaine injections. This is explained in the discussion, but I find it surprising that this second framework is not presented in the introduction. I think that both hypotheses should be presented in the introduction to allow the reader to fully appreciate the interest of the paper.

As requested, we have modified the introduction to include both frameworks.

4) A third theoretical framework about the role of DA in addiction that appears relevant to this work is the reward prediction error. Can the authors discuss how these results fit with this framework?

We have now added extensive discussion on reward prediction errors in the context of the current findings.

Minor points:

1) Abstract. The abstract states “Drug-cue reactivity, such as craving and automatized drug use” suggesting that these terms are synonyms. In contrast, drug-cue reactivity, craving and automatized drug use are conceptually very

different processes and concepts and should not be confused. Elsewhere in the manuscript, the authors use the term “craving” for drug seeking. I suggest using the term drug seeking in all the descriptions in the results and limit use of the term “craving” to the instances in which they speculated about underlying behavioral processes.

Our intention was for craving and automatized drug use to be examples of different types of cue reactivity. We introduced automatized drug seeking as an alternative to craving based upon different conceptual frameworks. We have now moved the reference to automatized behavior to later in the introduction and presented it in a way that should be clearer that it is an alternative framework: “For example, following chronic drug use in some individuals, a drug cue that was previously received as a result of drug-seeking actions (contingent presentation) can in and of itself initiate drug seeking when experienced in other situations (non-contingent presentation). This drug seeking may be driven by cue-induced craving, or may be an automatized behavior elicited directly by the cue.”

As requested, we have removed the use of “craving” in the results and adhered to “cue reactivity”, “CS-elicited approach”, “conditioned approach” or “cue-induced drug seeking”.

2) Introduction. The sentence “We observed increasing dopamine release, a trajectory that is diametrically opposed to changes in dopamine evoked by the same stimulus when presented in response to a drug-taking action, measured in the same subjects” is not clear. Can the authors rephrase?

We replaced the sentence with the following: “Dopamine release elicited by non-contingent cue presentation increased over the course of chronic cocaine use. This trajectory is diametrically opposed to changes in dopamine evoked by the same stimulus when presented in response to a drug-taking action (contingent presentation). These opposing dopamine trajectories were observed concurrently in individual subjects, particularly those that escalated their cocaine consumption over this period.”

3) Introduction. The sentence “decreased NAcc dopamine to behavior-contingent stimuli producing increased drug consumption, and increased NAcc dopamine to non-contingent cues producing craving” does not explain what the measures are to separate consumption and craving. A better description of the actual measure would be more informative for the reader. Moreover, as in my main point 2 above, I wonder whether the authors can distinguish these processes in their procedure.

We have provided operational definitions of the metrics we recorded in the sentences preceding this one. We have also changed “craving” to “drug seeking” in this sentence as advised in the reviewer’s minor point 1 above.

4) A more detailed description of the methods including detailed description of the behavioral procedures and how data were analysed is needed (eventually as supplementary material). For examples, data in the bottom panel in supplementary figures are % of % and it is not clear how they were obtained and what they represent.

The double percentage in the axis label was a typographic error. The data represent the proportion of total nose pokes that were in the active nose-poke port, as a means to demonstrate the status of learning the discrimination between the active and inactive ports. For clarity we have changed the label to “Discrimination (% active pokes)”.

Similarly, for escalation, linear regression was used but it is not clear what parameters were used for this analysis and what was the criterion for escalation.

The linear regression used the individual’s intake from each session over time. If the regression produced a significant upward slope, they were classified as escalators. Otherwise, they were classified as non-escalators. We have clarified the description in the methods section.

Also, how was the delta conditioned approach value (fig. 3d and fig.3g) calculated? What does it represent?

The delta conditioned approach was the difference between an individual’s approach score after extended drug use (W2-3) and that after early drug use (W1). It represents the change in cue reactivity over chronic drug use. We have now clarified this point in the description of these results.

5) Results. Although the authors states that increase in DA release from week 1 to week 3 only occurs in LgA rats, it appears that an increase may also have occurred also in ShA and did not reach statistical difference mostly because of the low n in the 3 week group. Can the authors indicate the n at each time point? Why was a lower n used in this group? I understand that running additional rats may be very complicated, the authors should acknowledge this problem and present the results in a more nuanced way.

Our intention was not to indicate that the increase in dopamine was only in the LgA group. We agree with the reviewer that there is an apparent trend that did not achieve statistical significance in the ShA group. Accordingly we stated that “we observed an increase in CS-evoked dopamine between week 1 and week 3, which was significant in the LgA cohort”. There was a main effect of access (LgA vs ShA) but not a significant interaction with time. We do not think that whether the ShA signal is different to the LgA signal or not is consequential to the manuscript. The main point we were trying to make is that the dopamine response to non-contingent cues increases during drug use, which can be observed robustly in LgA animals.

It is true that the ShA W3 group was smaller ($n = 6$). This was due to attrition in this group. Note that the group sizes are in this figure next to the traces on the left. As requested, we have updated the results to include this nuance: “we observed an increase in CS-evoked dopamine between week 1 and week 3 (main effect of week: $F(1,55)=11.43$, $p=0.0013$), which was significant in the LgA cohort (Sidak post-hoc test: $p=0.0002$; Fig. 1g), but not in the ShA cohort which had fewer subjects ($n = 6$ versus $n = 15$).”

6) Escalation of cocaine intake implies increased number of injections. Why only nose-pokes were shown in supplementary figures?

Since the drug was available on FR1 schedule (with time outs) the number of active nose pokes is exactly synonymous with the number of infusions. Note that we do not classify responses in the cocaine nose-poke port during time outs (when it is inactive) as active responses. The active responses are the number of reinforced responses. We labeled the axis as active nose pokes in the supplementary figures because they are being contrasted with responses in the inactive port. We have clarified in the supplementary figure legends that the number of active responses is the same as the number of infusions.

Reviewer #2 (Remarks to the Author):

The manuscript by Burgeno and colleagues is an interesting and timely study on how long-term cocaine intake alters rapid dopamine signals in the nucleus accumbens, as well as these signals play in cocaine reinforcement (operant behavior) and incentive sensitization (Pavlovian conditioning). The authors discovered that dopamine signals increase following non-contingent exposure to cues that were previously associated with chronic cocaine, but decreased when these cues followed contingent administration of cocaine. Additionally,

optogenetic experiments showed that the heightened signals were causal to conditioned approach in the Pavlovian / non-contingent condition and the decreased signals were causal to escalation of cocaine intake in the long-access cocaine rats. This is a difficult experiment to perform, but the experimental design is strong. The within subject nature of the design for both the non-contingent and contingent exposures made this study particularly compelling for the multifaceted role that dopamine plasticity plays in cocaine / drug reinforcement. The writing, figures, and discussion were exceptionally clear, perhaps due in part, to this being a revised manuscript in response to a prior review. Given the timeliness and clarity of the experiments / design / writing, most of my remaining questions could be classified as minor. I feel the authors did a good job revising the manuscript and responding according to the prior reviewers' comments.

Minor Comments:

1. prior reviewer commented on differences in nucleus accumbens core vs shell dopamine, and so brain region differences have been addressed to some degree. However, this group previously reported that in addition to the decreasing dopamine signals in the nucleus accumbens following chronic cocaine already discussed in the contingent condition, there is a corresponding dopamine signal increase in the dorsolateral striatum following contingent chronic cocaine (Willhuhn et al., 2012; PNAS). Some reference to this study, and possibly a sentence or two in the discussion, might help reader place this work in the context of broader understanding of concomitant dopamine signals in other regions that might be a neural substrate for habit formation, yet still play a role in this behavior.

We have now cited this work in the suggested context.

Reviewer #3 (Remarks to the Author):

Burgeno et al. investigate how mesolimbic dopamine signaling to drug-associated cues changes over the course of cocaine self-administration in rats, and how these changes relate to addictive behaviors. Using fast-scan cyclic voltammetry (FSCV) in the nucleus accumbens (NAcc) core during cocaine self-administration, they show that dopamine responses to a cocaine-paired cue evolve in opposite directions depending on the context of the cue presentation. Specifically, when the cue was presented non-contingently, cue-evoked dopamine release increased with prolonged drug use. In contrast, when the same cue was

presented contingent on an operant response during drug self-administration, the dopamine signal decreased over time in those animals that escalated their cocaine intake. Behaviorally, these neurochemical changes were linked to core addiction-like behaviors: the growing dopamine response to non-contingent cues was associated with enhanced cue-induced “craving” (operationalized as elevated approach behavior and lever pressing for the cue), whereas the diminishing dopamine response to the contingent cue correlated with escalating drug consumption. The authors causally tested these links using optogenetics. Together, the results support a model in which “diametrically opposite” dopamine adaptations both promote addiction: increased phasic dopamine to passive drug cues promotes craving and relapse risk, whereas decreased phasic dopamine during drug-taking promotes greater consumption.

Surprisingly few studies have examined dopamine dynamics during protracted abstinence and during cue-evoked seeking / conditioned reinforcement. These are highly challenging experiments to perform, and the authors should be commended for completing them in a highly rigorous fashion (e.g. well-powered, within-subject design, appropriate statistics, etc.). There are a few issues, which are readily addressable, detailed below.

Major:

1. Was contingent cue-evoked dopamine measured during abstinence? The diagram and behavioral data in figure 2 suggest that it was measured, but not reported. These data would be useful in determining whether abstinence-induced changes reflect an interaction with the cue presentation type, as the authors demonstrate for the changes occurring during ongoing self-administration, or if abstinence-induced changes reflect non-selective plasticity similar to PMID: 29175958.

As noted to reviewer 1, the sole utility of the “extinction” sessions was to provide a standard test to validate the incubation effect. Therefore, we did not measure dopamine during these sessions. In the timeline diagrams, we indicate the sessions in which voltammetric recordings were made. Accordingly, in Figure 2 we label that recordings were made in the non-contingent-cue-presentation sessions, but not the “extinction” sessions. We have modified the text to clarify that recordings were not made during these sessions.

2. The framing of the findings within the human literature take an overly narrow / somewhat cherry-picked view. For example, it is stated in the introduction that evidence corroborating the incentive sensitization theory has accumulated over

the years, and this concept is revisited in the discussion. While a few studies can be found to support this, it ignores a much larger literature that has found null or opposite effects in humans (for review see PMID: 18720516). While it may not be necessary to fully flesh out the human literature, as currently written there are several broad statements made that are not well supported; the authors should either provide a more nuanced, well-cited argument or remove the statements entirely.

When we framed our findings within the human literature, we make the following points:

- *Humans substance use is subject to individual differences. We cited one review paper (George & Koob, 2017). While this is only one reference, the consensus of the literature, as we understand it, suggests that human substance use is heterogeneous.*
- *In humans, cue reactivity predicts clinical outcomes. For this point we reference one primary research paper (Regier et al, 2021), and one meta-analysis (Vafaie & Kober, 2022). The meta-analysis follows PRISMA guidelines and includes 51,788 participants from 237 studies. A failsafe analysis revealed that 180,092 null studies would need to be published to nullify the conclusion that cue reactivity predicts clinical outcomes.*
- *Drug-paired cues can elicit dopamine release in humans. For this point we cited one primary research paper (Boileau et al, 2007) which was the original observation.*
- *Drug-paired cues can produce craving in humans. For this point we cited three papers, which are all widely cited, and we know of no controversy over this point.*
- *Incubation of craving was first observed in humans. We cite the single paper which, to our knowledge, is the original report of the incubation phenomenon (Gawin & Kleber, 1986).*
- *Physiological correlates of incubation of craving have been measured in humans. We cite one study (Parvaz et al, 2016) which is the first one that we are aware of. We did not make any conclusion based upon the specific physiological correlates reported, just that they can be measured.*
- *Cocaine resulted in 2 % of deaths in males between 15 and 49 in the USA in 2019. We cite the Institute for Health Metrics and Evaluation's report on Global Burden of Disease.*

We do not think that any of these points are controversial and, therefore, are having difficulty in understanding why the reviewer characterizes our citations as selective in the discussion.

For the introduction, the reviewer uses, as an example, our citation of the 30-year update on the Incentive Sensitization Theory (which itself cites 231 other papers but is not primarily focused on human literature). By noting that “corroborative evidence for the theory has accumulated over the thirty years since its inception”, we did not mean to indicate that all of the previous work corroborated the Incentive Sensitization Theory, or even that the majority did. Moreover, we were making the statement that despite the existence of corroborating (indirect) evidence, the central tenet has never been tested directly. To avoid the perception that we are postulating that all previous empirical work consistently corroborates the theory, we have removed the sentence. In addition, we have acknowledged that there is controversy over whether dopamine transmission increases or decreases following substance use: “Many contemporary theories of substance use disorders propose that enduring changes in mesolimbic dopamine transmission underlie aberrant behaviors. However, there is no agreement on the manner and even the direction of these changes.”

3. Along these same lines, statements throughout the introduction and discussion regarding clinical findings are supported by references studying amphetamine, alcohol, and opioids, though the clinical data for these drugs often differs from cocaine. The references should be replaced by cocaine studies, and/or should be qualified in the text.

The references on amphetamine, alcohol and opioids were intentionally selected and presented alongside references on cocaine. The references are all used to support statements about addictive drugs and substance use disorders broadly, not specifically cocaine use. Again though, every one of these non-cocaine citations has a cocaine co-citation.

Minor:

4. The sessions in which animals respond for presentation of the cue should be labeled as conditioned reinforcement (the contingent occurrence of a conditioned stimulus) rather than extinction (the complete discontinuation of an operant contingency). See APA dictionary and Ferster & Skinner, 1957.

The reviewer is correct that the sessions we labeled “extinction sessions” incorporate conditioned reinforcement to the cue alongside the extinguishing effect of the removal of the drug. We prefer not to label it as conditioned reinforcement because of the conflation of these effects (a standard test of conditioned reinforcement pairs a conditioned stimulus with an unconditioned stimulus and then offers presentation of the conditioned stimulus for responses on an operandum that has never been paired with

the unconditioned stimulus). As noted to Reviewer 1, the sole utility of this session was to provide a standard test to validate the incubation effect and we want this point to be clear to the reader. Therefore, we have elected to relabel these sessions as “incubation test”, consistent with other publications (e.g., Venniro & Shaham, 2020, PMID: 32203485).

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Burgeno et al did a good job at addressing most of my concerns. The only doubt I still have is the relationship between CS-reinforced and CS-elicited responses. The authors now clearly explain that CS-elicited responses were obtained by blocking physical access to the nose-pokes but they do not address the question of whether CS-reinforced and CS-elicited are related phenomena (as it is expected) or not. As suggested in my previous review, they could perform correlation analysis between individual subjects' CS-reinforced and CS-elicited response measures. If the two measures are correlated, this would indicate that they represent related phenomena; if they don't, this may indicate that they are somewhat different processes. In my opinion, this analysis and clarifying the relationship between these two measures will further improve the manuscript.

We conducted the correlation analysis as requested and did not observe significant correlation between the incubation of CS-reinforced and CS-elicited measures. We also did not observe significant correlation between the raw measures at either time point (day 1 or day 30). Therefore, on the surface, it appears that these metrics are not directly related. However, we are reticent to make a strong statement about their independence since the two measures form different types of distributions (the approach scores are non-parametric) and, therefore, linear regression does not provide a definitive test of their association. That being said, we agree that this information may be useful to the reader and so have included the following sentence "However, the degree of this incubation did not significantly correlate with the incubation of CS-reinforced responding across individuals ($r^2=0.24$, $p=0.147$, $n=11$)".

Also, I still find somewhat confusing the sentence "We then assayed whether behavioral cue reactivity also underwent incubation" given that this was tested BEFORE CS-reinforced responses according to the experimental sequence described in the methods.

The sentence was intended to indicate that we analyzed the data from the probe session once we established the animals exhibited incubation. We recognize now that "assayed" could imply that we performed the behavioral test subsequent to completing the incubation test. We have attempted to clarify this point by modifying the sentence as follows: "We then analyzed behavior from the probe sessions that followed withdrawal to test whether behavioral cue reactivity also underwent incubation."

These points are minor and should not delay the publication of this excellent study.