Dual action of ketamine confines addiction liability

Simmler, Linda; Li, Yue; Hadjas, Lotfi; Hiver, Agnès; Van Zessen, Ruud; Luescher, Christian

Peer Review File

Manuscript Title: Dual-action of ketamine confines addiction liability

Editorial Note- Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The study by Simmler, Li et al examines a dynamic role of ketamine on the dopaminergic reward circuit. Using a series of well-designed and rigorous experiments with state of art techniques the authors show that ketamine increases VTA DA neuron activity and NAc DA transients similar to used substances with high addiction liability. They show that this occurs via NMDAR inhibition in VTA GABA neurons. The authors then demonstrate that ketamine does not induce locomotor sensitization or synaptic plasticity in VTA and NAc compared to drugs like cocaine. They argue that NMDAR antagonism and D2R mediated rapid off-kinetics of DA transients preclude drug-evoked plasticity in VTA and NAc to limit ketamine addiction liability. This study provides novel and important insight into ketamine-mediated mechanisms in the dopaminergic reward circuitry that differ from other drugs that are associated with increased addiction vulnerability. Given the use of ketamine to treat neuropsychiatric disorders like major depressive disorder, this new insight into ketamine specific mechanisms in reward circuitry is important.

Major comments:
1. The authors conclude that ketamine evokes reinforcing responses similar to cocaine. However, Figure 1e suggests that a high number of mice display reduced reinforcing behavior to ketamine. This appears to be an average of all 10 sessions and the data in Figure 1d suggests that this variation in behavior may be occurring from day 3 onward. In the supplemental data they include individual data on the active vs. inactive lever for day 1 or averaged during all days or day 1-3. Please show this data for each day. This will provide a more accurate assessment into reinforcing responses from each subject on each day. Additionally, identifying the sex in these graphs would inform about whether the variation in responses occurs in both sexes. If there are a subset of mice that do not show reinforcing responses to ketamine then this is very interesting and should be discussed.

2. In Figure 1 it is unclear which conditions the d-Light studies occur. From the methods it seems like this was with an acute dose of ketamine. The authors should provide more clarity in the main text about this as well as the other experiments that use only acute ketamine, which are the majority of the experiments in the manuscript.

3. The authors nicely validate reduced AMPA/NMDA ratios with the NR1 CRISPR KO. However, validation that NR1 expression is reduced in GABA neurons is needed. Additionally, while the authors cite the Hunker study in the main text there is little information about the CRISPR vector in the methods. They should clarify that the vector and guide RNA was generated and validated in the Hunker study.

4. The authors nicely demonstrate distinct mechanisms of ketamine on the VTA that they argue preclude drug-evoked plasticity in VTA and NAc to limit ketamine addiction liability. However, the current study does directly link these mechanisms to long term behavioral responses relevant to addiction (e.g. operant self administration and relapse like behaviors). Thus, the strong arguments that these mechanisms limit ketamine addiction liability should be toned down.

5. The field is shifting to the use of terms that are sensitive to individuals with substance use disorders. Please modify statements like “drug abuse” to substance use disorders or drug use.

Referee #2 (Remarks to the Author):

A Summary of the key results
In this manuscript the authors carry out in the mouse a detailed and precise investigation of ketamine effects on VTA neurons and their targets in the nucleus accumbens. They use behavioral approaches and sophisticated biosensor imaging of dopamine (DA) release and neuronal activity, as well as ex vivo electrophysiological recording. The main conclusion is that although ketamine shares some of the actions of cocaine, such as generating self-administration and increasing DA release in the nucleus accumbens, there are major differences including a shorter action, due at least in part to stimulation of D2 receptors, and the ability to prevent synaptic plasticity through blockade of NMDA receptors. The authors also show the absence of ketamine-induced locomotor sensitization in support of the lack of effect on synaptic plasticity. The authors conclude that these differences between cocaine and ketamine and the lack of effect of ketamine on plasticity “strongly suggest that ketamine’s addiction liability is low”.

B Originality and significance: if not novel, please include reference
Because of the development of ketamine as an antidepressant drug and its existing use as a "recreative" drug, the issue of its addictive properties is of high importance and debated. Therefore this work is important. There are already a large number of published papers on ketamine in relation to synaptic plasticity in various systems and to addiction, from the existence of stress-induced ketamine addictive behavior in female rats (see Wright et al Chronic Stress 2019) to potential use of ketamine to improve abstinence in human addicts (see Jones et al. Front. Psychiatry, 2018)... However there is no thorough study of the cellular and molecular actions of ketamine on the reward system and the current manuscript is an important strong contribution. Yet the existing literature and its contradictions underline the need to include both cellular mechanisms and a demonstrative behavioral study in the current manuscript to have broad and strong impact (see G below).

C Data & methodology: validity of approach, quality of data, quality of presentation

The results that are presented in the manuscript are of high quality and very convincing, supporting the authors' conclusions concerning the cellular mechanisms for which this manuscript brings very interesting new information.

D Appropriate use of statistics and treatment of uncertainties
The data analysis is satisfactory. Summary of statistics is reported in figure legends. A detailed report of all statistical results including post hoc as supplementary material would be useful. The number of male and female mice in each group should be indicated in this table.

E Conclusions: robustness, validity, reliability
The presented data are convincing and the conclusions at the circuit level robust. The main claim (low ketamine's addiction liability), which is relevant for therapeutics in patients, is, however, largely speculative, even if it is supported by strong logical presumptive elements. Following the initial work of Deroche-Gamonet et al in 2004, several groups (including the Lüscher lab with insightful approaches) have shown that the main criteria of human addiction can be evaluated in rodents. These criteria are reached only by a subset of self-administering animals and include the persistence of self-administration in the absence of drug, the resistance to punishment, and the strong motivation when necessary effort to obtain the drug is increased. If the prediction of the authors concerning ketamine is right, all mice self-administering ketamine should fail these behavioral "addiction tests". It is very important that such experiments are carried out and the results included before conclusions about low addictive potential of ketamine in mice are made.

F Suggested improvements: experiments, data for possible revision
In relation to the comments in E, to fully support the authors' conclusion that ketamine's addiction liability is low in mice, it is needed to include a new self-administration experiment comparing cocaine and ketamine in different groups with a sufficient number of mice in each group, and showing that the expected proportion of mice that self-administer cocaine reach addiction criteria (see comments in E), whereas those which self-administer ketamine don't.

G References: appropriate credit to previous work?
Yes

H Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions
The manuscript is clear and well written. The following points should be improved:
- Although the shorter duration of action of ketamine as compared to cocaine and also fentanyl is a key point in the manuscript, the differences in pharmacokinetics between these drugs are not clearly presented and discussed. The authors should be more precise about the differences in action they think are related to pharmacokinetic differences (e.g. half-life) versus those that are due to specific actions of ketamine such as stimulation of D2 receptors.
- The primary classic use of ketamine in anesthesia should be mentioned in the abstract or introduction to inform readers outside of the field.
- "Rectification index" should be abbreviated at the first occurrence.

Referee #3 (Remarks to the Author):

The addiction liability of ketamine is hotly debated. In this study, Simmler et al. focused on this fundamental question by first focusing on the effect of ketamine on dopamine release. The authors showed that, similar to cocaine, administration of ketamine increased dopamine levels in the nucleus accumbens and VTA. Further results suggest that this elevation of dopamine levels was initiated by ketamine-mediated NMDAR antagonism in VTA GABAergic neurons, and these effects were relatively transient because elevated dopamine quickly activated D2Rs, preventing further dopamine release. The authors then demonstrated that, unlike cocaine, administration of ketamine failed to change the ratio of AMPAR- / NMDAR-mediated EPSCs in both VTA and nucleus accumbens neurons, failed to change the rectification index of EPSCs in VTA and accumbens neurons, and failed to induce persistent increases in locomotor responses. These “negative results” led the authors to conclude that ketamine has low addiction liability.

The study targets the addiction liability of ketamine, which is an important and influential question in the research field of addiction neuroscience. The results about the in vivo action of ketamine are important. There are, however, several layers of concerns.

1) The addiction liability of ketamine is the central focus of this study. However, no experiments were designed to directly test it. The only experiments that used drug taking paradigms (that potentially induce addiction) showed ketamine was sufficient to maintain self-administration, similar to cocaine. Most of the currently included results just show different cellular effects between ketamine and cocaine after i.p. injections. They cannot be used to draw conclusions about addiction liability.

2) It has also been well established that an increased or decreased locomotor sensitization does not predict a high or low addiction liability. The related data set does not really support the argument that ketamine has low addiction liability.

3) The ketamine locomotor results are at odds with the well-established literature. There are a good number of studies examining ketamine-induced locomotor sensitization using a more systematic and thorough approach (e.g., different doses, durations, influence of other drugs), all showing that ketamine induces robust and reliable locomotor sensitization. Indeed, it is well known that not only ketamine, but also other NMDAR antagonists, induce locomotor sensitization. A lack of such behavioral effects (Fig. 4d-f) raises some concerns related to the experimental approaches employed by this study (see #4).

4) Related to #3, ketamine is usually used at 10 mg/kg for antidepressant studies in mice and 2.5-10 mg/kg for locomotor sensitization. At 5 mg/kg, ketamine starts to induce aversive conditioning in female mice. It is not clear why such a high dose (30 mg/kg) of ketamine, a dose that potentially induces aversive response, was chosen by this study to study the addiction liability of ketamine.

5) The data about accumbal drug-evoked synaptic plasticity are at odds with published results,

including the results from the authors' prior studies. Specifically, figure 4g-i shows a decreased AMPAR/NMDAR ratio after withdrawal from repeated cocaine administration. Several labs, including the authors' lab, have shown an increased A/N and increased strength of excitatory synapses on accumbens neurons after withdrawal from cocaine.

6) Male versus female mice exhibit differential ketamine sensitivity, but there is no mention of sex-based differences.

Author Rebuttals to Initial Comments:

Dual-action of ketamine confines addiction liability

Simmler, Li et al.

Response to reviewers

We thank the reviewers for their very thoughtful comments, which we address below (in blue). All changes made in the manuscript are highlighted in yellow.

Best

Christian Lüscher

Comments to authors.

The study by Simmler, Li et al examines a dynamic role of ketamine on the dopaminergic reward circuit. Using a series of well-designed and rigorous experiments with state of art techniques the authors show that ketamine increases VTA DA neuron activity and NAc DA transients similar to used substances with high addiction liability. They show that this occurs via NMDAR inhibition in VTA GABA neurons. The authors then demonstrate that ketamine does not induce locomotor sensitization or synaptic plasticity in VTA and NAc compared to drugs like cocaine. They argue that NMDAR antagonism and D2R mediated rapid off-kinetics of DA transients preclude drug-evoked plasticity in VTA and NAc to limit ketamine addiction liability. This study provides novel and important insight into ketamine-mediated mechanisms in the dopaminergic reward circuitry that differ from other drugs that are associated with increased addiction vulnerability. Given the use of ketamine to treat neuropsychiatric disorders like major depressive disorder, this new insight into ketamine specific mechanisms in reward circuitry is important.

Major comments:
1. The authors conclude that ketamine evokes reinforcing responses similar to cocaine. However, Figure 1e suggests that a high number of mice display reduced reinforcing behavior to ketamine. This appears to be an average of all 10 sessions and the data in Figure 1d suggests that this variation in behavior may be occurring from day 3 onward. In the extended data they include individual data on the active vs. inactive lever for day 1 or averaged during all days or day 1-3. Please show this data for each day. This will provide a more accurate assessment into reinforcing responses from each subject on each day. Additionally, identifying the sex in these graphs would inform about whether the variation in responses occurs in both sexes. If there are a subset of mice that do not show reinforcing responses to ketamine then this is very interesting and should be discussed.

We agree with the referee and now provide additional observations for ketamine self-administration using FR1 to FR3 in a 4h paradigm. We found that the mice typically abandoned after FR2, speaking to their low motivation. We also changed Extended data figure 1 as suggested.

As requested, we have broken down the data for the sex of the animal for each experiment in Extended data table 1. We indeed used mice of both sexes and found similar values for both sexes. For example, self-administration as shown in figure 4 was not statistically different for the number of infusions (Two-way RM ANOVA; $F(1,198) = 0.2$, $P = 0.6633$; N = 8 males and 12 females) or the breakpoint (Two-tailed t test; $t(18) =$

1.009, P = 0.3265; N = 8 males and 12 females). We would also like to stress that the study was not designed to reveal sex differences. The raw data deposited on Zenodo now also has sex identification.

2. In Figure 1 it is unclear which conditions the d-Light studies occur. From the methods it seems like this was with an acute dose of ketamine. The authors should provide more clarity in the main text about this as well as the other experiments that use only acute ketamine, which are the majority of the experiments in the manuscript.
Yes, the experiments with the d-Light1 were monitoring dopamine in response to an acute single injection of ketamine i.p. **The text has been edited for clarity.**

3. The authors nicely validate reduced AMPA/NMDA ratios with the NR1 CRISPR KO. However, validation that NR1 expression is reduced in GABA neurons is needed. Additionally, while the authors cite the Hunker study in the main text there is little information about the CRISPR vector in the methods. They should clarify that the vector and guide RNA was generated and validated in the Hunker study.
We agree with the referee that the validation of the NR1 KO is essential. Much literature confirms that there is an absence of NMDAR currents without the obligatory GluN1 subunit (e.g. Engblom 2008 and Zweifel 2008). By carrying out patch-clamp recordings, we can confirm that NMDA-EPSCs are strongly reduced in most cells (Extended data figure 3). For **the validation of the vector, we give more details in the methods section.**

4. The authors nicely demonstrate distinct mechanisms of ketamine on the VTA that they argue preclude drug-evoked plasticity in VTA and NAc to limit ketamine addiction liability. However, the current study does directly link these mechanisms to long-term behavioral responses relevant to addiction (e.g. operant self administration and relapse like behaviors). Thus, the strong arguments that these mechanisms limit ketamine addiction liability should be toned down.
We have carried out additional experiments with a 4h long access to ketamine and gradually increased the fixed ratio. This shows that self-administration fades already at FR2, leaving the mice with very few infusions (updated Figure 4j, k). We also determined the progressive ratio and found it to be very low (updated Figure 4l). Taken together this shows that motivation for ketamine self-administration is low, leading to insufficient injections to formally test for resistance to punishment. We nevertheless challenged the mice with an aversive air puff (electric shock was not an option because of the analgesic effect of ketamine), and the mice ceased self-administration (Extended data figure 6a, b). **These data are now shown in Extended data and updated in Figure 4.**

5. The field is shifting to the use of terms that are sensitive to individuals with substance use disorders. Please modify statements like “drug abuse” to substance use disorders or drug use.
Point well taken. We now use the more neutral term of “**drug use**”.

Referee #2 (Remarks to the Author):

A Summary of the key results
In this manuscript the authors carry out in the mouse a detailed and precise investigation of ketamine effects on VTA neurons and their targets in the nucleus accumbens. They use behavioral approaches and sophisticated biosensor imaging of dopamine (DA) release and neuronal activity, as well as ex vivo electrophysiological recording. The main conclusion is that although ketamine shares some of the actions of cocaine, such as generating self-administration and increasing DA release in the nucleus accumbens, there are major differences including a shorter action, due at least in part to stimulation of D2 receptors, and the ability to prevent synaptic plasticity through blockade of NMDA receptors. The authors also show the absence of ketamine-induced locomotor sensitization in support of the lack of effect on synaptic plasticity. The authors conclude that these differences between cocaine and ketamine and the lack of effect of ketamine on plasticity "strongly suggest that ketamine's addiction liability is low".

We thank the referee for this accurate summary of our study.

B Originality and significance: if not novel, please include reference
Because of the development of ketamine as an antidepressant drug and its existing use as a "recreative" drug, the issue of its addictive properties is of high importance and debated. Therefore this work is important. There are already a large number of published papers on ketamine in relation to synaptic plasticity in various systems and to addiction, from the existence of stress-induced ketamine addictive behavior in female rats (see Wright et al Chronic Stress 2019) to potential use of ketamine to improve abstinence in human addicts (see Jones et al. Front. Psychiatry, 2018)... However, there is no thorough study of the cellular and molecular actions of ketamine on the reward system and the current manuscript is an important strong contribution. Yet the existing literature and its contradictions underline the need to include both cellular mechanisms and a demonstrative behavioral study in the current manuscript to have broad and strong impact (see G below).
We agree with the referee that a combination of "cellular mechanisms and a demonstrative behavioral" is essential. We have indeed expanded our study with extended self-administration (see section E).

C Data & methodology: validity of approach, quality of data, quality of presentation
The results that are presented in the manuscript are of high quality and very convincing, supporting the authors' conclusions concerning the cellular mechanisms for which this manuscript brings very interesting new information.
We thank the referee for this assessment.

D Appropriate use of statistics and treatment of uncertainties
The data analysis is satisfactory. A summary of statistics is reported in figure legends. A detailed report of all statistical results including post hoc as supplementary material would be useful. The number of male and female mice in each group should be indicated in this table.
We have now included **Extended data Table 1** with statistics and the breakdown of the sex of the mice used. For the balanced groups, no statistical differences were observed. For example the number of infusions (Two-way RM ANOVA; $F(1,198) = 0.2$, $P = 0.6633$; N = 8 males and 12 females) or the breakpoint (Two tailed t test; $t(18) = 1.009$, $P = 0.3265$; N = 8 males and 12 females) were similar in both sexes. We would also like to stress that the study was not designed to reveal sex differences. The raw data deposited on Zenodo now also has sex identification.

E Conclusions: robustness, validity, reliability
The presented data are convincing and the conclusions at the circuit level robust. The main claim (low ketamine's addiction liability), which is relevant for therapeutics in patients, is, however, largely speculative, even if it is supported by strong logical presumptive elements. Following the initial work of Deroche-Gamonet et al in 2004, several groups (including the Lüscher lab with insightful approaches) have shown that the main criteria of human addiction can be evaluated in rodents. These criteria are reached only by a subset of self-administering animals and include the persistence of self-administration in the absence of drug, the resistance to punishment, and the strong motivation when necessary effort to obtain the drug is increased. If the prediction of the authors concerning ketamine is right, all mice self-administering ketamine should fail these behavioral "addiction tests". It

is very important that such experiments are carried out and the results included before conclusions about low addictive potential of ketamine in mice are made.

We have carried out additional experiments with a 4h long access to ketamine and gradually increased the fixed ratio. This shows that self-administration fades already at FR2, leaving the mice with very few infusions (updated Figure 4j, k). We also determined the progressive ratio and found it to be very low (updated Figure 4l). Taken together this shows that motivation for ketamine self-administration is low, leading to insufficient injections to formally test for resistance to punishment. We nevertheless challenged the mice with an aversive air puff (electric shock was not an option because of the analgesic effect of ketamine), and the mice ceased self-administration (Extended data Figure 6a, b). **These data are now shown in Extended data and updated in Figure 4.**

F Suggested improvements: experiments, data for possible revision
In relation to the comments in E, to fully support the authors' conclusion that ketamine's addiction liability is low in mice, it is needed to include a new self-administration experiment comparing cocaine and ketamine in different groups with a sufficient number of mice in each group, and showing that the expected proportion of mice that self-administer cocaine reach addiction criteria (see comments in E), whereas those which self-administer ketamine don't.
Since most animals stopped self-administering ketamine with an extended access FR2 schedule, we could not test for compulsion (see above). When compared to cocaine self-administration, the number of infusions and active lever presses are significantly lower (updated Fig 4j, k). The breakpoint in a progressive ratio task was also much lower for ketamine than cocaine, indicating a low motivation for the former (updated Fig 4l).

G References: appropriate credit to previous work?
Yes

H Clarity and context: lucidity of abstract/summary, appropriateness of abstract, introduction and conclusions
The manuscript is clear and well written. The following points should be improved:
- Although the shorter duration of action of ketamine as compared to cocaine and also fentanyl is a key point in the manuscript, the differences in pharmacokinetics between these drugs are not clearly presented and discussed. The authors should be more precise about the differences in action they think are related to pharmacokinetic differences (e.g. half-life) versus those that are due to specific actions of ketamine such as stimulation of D2 receptors.

We agree with the referee that the term "pharmacokinetics" has many underlying factors (absorption, distribution, and elimination of the drug). Here we focussed on the "kinetics of the pharmacodynamic effect" (i.e., the time course of DA elevation in the NAc induced by ketamine). **We have therefore edited the wording for clarity.**

- The primary classic use of ketamine in anesthesia should be mentioned in the abstract or introduction to inform readers outside of the field.

We have **edited the abstract as suggested**.

- "Rectification index" should be abbreviated at the first occurrence.

The text has been edited as suggested.

Referee #3 (Remarks to the Author):

The addiction liability of ketamine is hotly debated. In this study, Simmler et al. focused on this fundamental question by first focusing on the effect of ketamine on dopamine release. The authors showed that, similar to cocaine, administration of ketamine increased dopamine levels in the nucleus accumbens and VTA. Further results suggest that this elevation of dopamine levels was initiated by ketamine-mediated NMDAR antagonism in VTA GABAergic neurons, and these effects were relatively transient because elevated dopamine quickly activated D2Rs, preventing further dopamine release. The authors then demonstrated that, unlike cocaine, administration of ketamine failed to change the ratio of AMPAR- / NMDAR-mediated EPSCs in both VTA and nucleus accumbens neurons, failed to change the rectification index of EPSCs in VTA and accumbens neurons, and failed to induce persistent increases in locomotor responses. These “negative results” led the authors to conclude that ketamine has low addiction liability.

The study targets the addiction liability of ketamine, which is an important and influential question in the research field of addiction neuroscience. The results about the in vivo action of ketamine are important. There are, however, several layers of concerns.

1) The addiction liability of ketamine is the central focus of this study. However, no experiments were designed to directly test it. The only experiments that used drug taking paradigms (that potentially induce addiction) showed ketamine was sufficient to maintain self-administration, similar to cocaine. Most of the currently included results just show different cellular effects between ketamine and cocaine after i.p. injections. They cannot be used to draw conclusions about addiction liability.
We have carried out additional experiments with a 4h long access to ketamine and gradually increased the fixed ratio. This shows that self-administration fades already at FR2, leaving the mice with very few infusions (updated Figure 4j, k). We also determined the progressive ratio and found it to be very low (updated Figure 4l). Taken together this shows that motivation for ketamine self-administration is low, leading to insufficient injections to formally test for resistance to punishment. We nevertheless challenged the mice with an aversive air puff (electric shock was not an option because of the analgesic effect of ketamine), and the mice ceased self-administration (Extended data Figure 6a, b). **These data are now shown in Extended and updated Figure 4.**

2) It has also been well established that an increased or decreased locomotor sensitization does not predict a high or low addiction liability. The related data set does not really support the argument that ketamine has low addiction liability.
We agree with the referee that sensitization cannot predict compulsion (see also Steketee and Kalivas 2011). Moreover the experiments with the extended self-administration now directly examine the possibility of a transition to compulsion (see Extended data).

3) The ketamine locomotor results are at odds with the well-established literature. There are a good number of studies examining ketamine-induced locomotor sensitization using a more systematic and thorough approach (e.g., different doses, durations, influence of other drugs), all showing that ketamine induces robust and reliable locomotor sensitization. Indeed, it is well known that not only ketamine, but also other NMDAR antagonists, induce locomotor sensitization. A lack of such behavioral effects (Fig. 4d-f) raises some concerns related to the experimental approaches employed by this study (see #4).
We are not sure which papers that referee is referring to. We indeed found a small number of papers that have looked at locomotor effects with ketamine with diverging results.

- Stronge et al, 2017 looked at sensitization after withdrawal (rats, 2.5 and 5 mg/kg), and finds a rather small effect, particularly in males: https://www.sciencedirect.com/science/article/pii/S0028390817301995?via%3Dihub
- Trujillio et al. 2008 (rats, 20 and 50 mg/kg) found an “escalated locomotor response” with one weekly injection, which is a protocol quite different from the classical sensitization experiment: https://www.sciencedirect.com/science/article/pii/S0006322307001503
- Popik et al. 2008 (rats; 50 mg/kg) on the other hand does not observe any long lasting effects on locomotion: https://link.springer.com/article/10.1007/s00213-008-1158-z
- Uchihashi et al. 1993 (mice; 12.5-50 mg/kg) saw sensitization at 3-4days intervals, which is again different from the protocol used here. https://pubmed.ncbi.nlm.nih.gov/8096258/

- Schoepfer et al. 2019 (rats, 5 and 10mg/kg), used daily injections that led to a build-up of the locomotor responses (just as we see it in our study), but the delayed test is done already after a single day of withdrawal. https://www.sciencedirect.com/science/article/pii/S0031938417303608?via%3Dihub
- Wiley at al, (rats, 3 and 10 mg/kg) found sensitization in adult, but not adolescent rats with a protocol that allowed for 48h of withdrawal. https://onlinelibrary.wiley.com/doi/epdf/10.1111/j.1369-1600.2007.00077.x

In summary, our reading of the literature, in line with our observations here, is that daily injections may lead to short-term sensitization. On the other hand, after a delay of withdrawal of a week, the effects are either very small or absent. We have **edited the text to reflect the above literature**.

4) Related to #3, ketamine is usually used at 10 mg/kg for antidepressant studies in mice and 2.5-10 mg/kg for locomotor sensitization. At 5 mg/kg, ketamine starts to induce aversive conditioning in female mice. It is not clear why such a high dose (30 mg/kg) of ketamine, a dose that potentially induces aversive response, was chosen by this study to study the addiction liability of ketamine.
We chose 30 mg/kg because it elicited an acute locomotion response and dopamine transient comparable to cocaine. The dose-response (Extended data figure 2) for dLight1 shows that the DA elevation for an injection of 15 mg/kg is much lower than 30 mg/kg. We have CPP data in male mice (shown below) to confirm that 30 mg/kg is not aversive (in male mice).

[REDACTED]

5) The data about accumbal drug-evoked synaptic plasticity are at odds with published results, including the results from the authors' prior studies. Specifically, figure 4g-i shows a decreased AMPAR/NMDAR ratio after withdrawal from repeated cocaine administration. Several labs, including the authors' lab, have shown an increased A/N and increased strength of excitatory synapses on accumbens neurons after withdrawal from cocaine.
We respectfully disagree with the referee's interpretation. Since we assessed synaptic transmission of the mPFC-to-NAc projection, where cocaine drives the insertion of GluA2-lacking AMPAR we see a reduced A/N ratio as previously reported (Pascoli et al. Nature 2014).

6) Male versus female mice exhibit differential ketamine sensitivity, but there is no mention of sex-based differences.

We have broken down the data for the sex of the animal for each experiment in Extended data table 1. We indeed used mice of both sexes and found similar values for both sexes. For example, self-administration as shown in figure 4 was not statistically different for the number of infusions (Two-way RM ANOVA; $F(1,198) = 0.2$, $P = 0.6633$; N = 8 males and 12 females) or the breakpoint (Two tailed t test; $t(18) = 1.009$, $P = 0.3265$; N = 8 males and 12 females). We would also like to stress that the study was not designed to reveal sex differences. The raw data deposited on Zenodo now also has sex identification.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have addressed all concerns. This is an important study that provides new information into ketamine regulated mechanisms in reward circuitry.

Referee #2 (Remarks to the Author):

The authors addressed my remarks and the data now fully support the authors' conclusions.
Minor points: a few sentences in the Discussion are difficult to understand and should be improved:
Line 170: "As interneurons elsewhere, NMDARs on VTA GABA neurons contribute to excitatory transmission at resting potential, maybe through …"
Should be "As in interneurons…"

Line 178: "However, fentanyl induced DA release, which occurs through disinhibition similar to ketamine, shows a much slower off-kinetics than ketamine."
Does it mean that the authors rule out that the inhibitory effects of dendritic release of DA is a relevant mechanism in the rapid off-kinetic of ketamine effects on acccumbens DA release and therefore propose that the D2 agonist effect of ketamine is the main mechanism? Please confirm/clarify.

Line 193: "First, with single doses, the fast off-kinetics of the DA elevation was insufficient to cause VTA plasticity…" is unclear. Perhaps "First, with single doses, because of its fast off-kinetics, the DA elevation was insufficient to cause VTA plasticity…" would be non-ambiguous.

Line 199: "While the nasal spray causes a rapid ketamine exposure similar to i.p. injections in our animal model, the i.v. infusion regimen may lead to enhanced DA levels during the 40-min of infusion where NMDAR blockade may predominantly confine drug adaptive plasticity. » Do the authors mean that i.v. infusion could lead to plasticity in contrast to i.p. injections? And thus be more prone to trigger addiction? I don't understand this sentence, please clarify.

Referee #3 (Remarks to the Author):

In the revised manuscript, the authors included additional results showing the relatively weak reinforcing capacity of ketamine compared to cocaine. The authors have also addressed several points raised from the first round of review.

Author Rebuttals to First Revision:

Dual-action of ketamine confines addiction liability

Simmler, Li et al.

Response to referee 2

Line 170: “As interneurons elsewhere, NMDARs on VTA GABA neurons contribute to excitatory transmission at resting potential, maybe through …”
Should be “As in interneurons…

Edited as suggested.

Line 178: “However, fentanyl induced DA release, which occurs through disinhibition similar to ketamine, shows a much slower off-kinetics than ketamine.”
Does it mean that the authors rule out that the inhibitory effects of dendritic release of DA is a relevant mechanism in the rapid off-kinetic of ketamine effects on acccumbens DA release and therefore propose that the D2 agonist effect of ketamine is the main mechanism? Please confirm/clarify.

The difference between fentanyl and ketamine would indeed suggest a direct mechanism of the drug on D2Rs. However, this mechanism remains controversial. More research will be required to solve this question. We have edited the text to highlight this unresolved question.

Line 193: “First, with single doses, the fast off-kinetics of the DA elevation was insufficient to cause VTA plasticity…” is unclear. Perhaps “First, with single doses, because of its fast off-kinetics, the DA elevation was insufficient to cause VTA plasticity…” would be non-ambiguous.

Thank you for the suggestion. We have edited the sentence accordingly.

Line 199: “While the nasal spray causes a rapid ketamine exposure similar to i.p. injections in our animal model, the i.v. infusion regimen may lead to enhanced DA levels during the 40-min of infusion where NMDAR blockade may predominantly confine drug adaptive plasticity. » Do the authors mean that i.v. infusion could lead to plasticity in contrast to i.p. injections? And thus be more prone to trigger addiction? I don’t understand this sentence, please clarify.

No, we are referring to the two regimes that we used in this study, i.p. vs repetitive i.v. Injections. We have edited the sentence to clarify.