nature portfolio

Peer Review File

ABHD6 loss-of-function in mesoaccumbens postsynaptic but not presynaptic neurons prevents diet-induced obesity in male mice

Corresponding Author: Professor Stephanie Fulton

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)
Overall, this was an excellent and potentially very useful manuscript that highlights the role of cannabinoid signaling in nucleus accumbens inputs and outputs in feeding in an obesity paradigm and locomotion. The studies were well conducted, and my specific comments, which are designed to help the authors sharpen the wording and thinking in specific passages, are listed below.

Major Comments:

The studies were technically elegant and yielded an interesting and important pattern of results. Although the literature review was comprehensive in terms of the number of references, some key areas of the literature were omitted. While the experiments are focused on modulation of cannabinoid signaling, DA mechanisms figure in conceptually, and are useful to discuss to provide context for the present results. For example, the idea that pharmacological or neurotoxic interference with mesoaccumbens DA transmission impairs locomotor activity but does not reduce food intake has been known for several decades (Koob et al. 1978; Salamone et al. 1993; Baldo and Kelley 2002). More recent studies show that antagonism of DA transmission switches choice behavior from running in a running wheel to sucrose drinking (Correa et al. 2016). Randall et al. (2012, 2014) reported that while DA antagonism shifts effort-based decision making, decreasing food-reinforced progressive ratio lever pressing but increasing chow intake, CB1 receptor antagonism or inverse agonism decreased both lever pressing and chow intake. There is much discussion of effortful behavior in motivation and the role of cannabinoid signaling in the present manuscript, but a discussion of DA is relevant for establishing the context for the present pattern of results.

Overall, the introduction and discussion were conceptually sophisticated in terms of behavioral processes, and the authors are to be congratulated. However, as is the case with many articles, the present manuscript has some passages use the term ‘reward’ as a neurobehavioral process, without actually defining it. For example, the statement that “VTA is a key substrate for neural processing of palatable food reward” is ambiguous. Does this refer to facilitating response-outcome associations that underlie reinforcement learning, or is it intended to refer more generally to various aspects of food motivation? ‘Reward’ as a synonym for reinforcement or reinforcement learning is distinct from the effort-related motivational processes that also are being discussed in relation to the present fundings. With this in mind, I urge the author to re-think the use of some of their terminology.

Minor Comments:

In the discussion, the phrase “including general inhibition of locomotion, immobility, and catalepsy” is ambiguously worded, and should read as “including general inhibition of locomotion, and induction of immobility and catalepsy”.

Reviewer #2

(Remarks to the Author)
This manuscript reports the impact of ABHD6 loss-of-function in mesoaccumbens postsynaptic and presynaptic neurons on multiple behavioral readouts of diet-induced obesity in mice. The manuscript is clearly written and the premise of the study

well-articulated. Behavioral analyses are thorough and most interpretation sound. Overall, this study will represent an important addition to the field.
Concerns that must be addressed:
Introduction: This sentence if incorrect “2-AG release from VTA DA neurons targets presynaptic CB1Rs localized on NAc GABAergic terminals13,14”. Rather, it should say “CB1Rs localized on VTA GABAergic and glutamatergic terminals”.
Results: The ABHD6-KO mediated downregulation of CB1R mRNA is unexpected and novel and should be explored further. Do the authors have an explanation of the mechanism by which a link exists between ABHD6 and CB1R expressions? Does it reduce CB1R expression in NAc interneurons, in output neurons or in both? How about other components involved in regulating 2-AG levels, including DAGLa, DAGLb and MAGL? A better understanding of these compensatory mechanisms will help interpret the electrophysiology results presented in Figure 4.
Figure 4: WIN was tested at 10 uM and produced small responses, including when measuring mIPSC amplitudes. The authors need to perform a WIN concentration-dependent dose response by adding 0.1% BSA in their perfusion media, to reduce WIN binding to plastics and as do most slice electrophysiology studies. Alternatively, or in addition, testing the MAGL inhibitor, JZL184, might help further unravel the difference in electrophysiological responses gathered between CTR and ABHD6-KD.
Discussion: While the discussion is already lengthy and could be shortened by 1/3 when combining most concepts discussed, it would be important to emphasize that ABHD6 is also involved in AMPA receptor trafficking. In fact, the authors could mention that the absence of changes in EPSCs in ABHD6-KO neurons argues against the involvement of ABHD6 trafficking function in the responses reported here.

Suggestions:
Introduction: “Endocannabinoids regulate… strong association to obesity”. It would be useful to also mention that THC users have a tendency to be leaner than non-users. A suggestion is to add a couple of sentences to address this notion and how if contrast with your study.
Reviewer: Dr. Nephi Stella

Reviewer #3

(Remarks to the Author)
The study by Lau et al., addresses an important question: what are the feeding, metabolic and motivational contributions of the 2-AG degrading enzyme ABHD6? This is addressed using viral-gene strategies in a manner that allows to elucidate pre- and post-synaptic contributions of this enzyme throughout the mesoaccumbal dopamine pathway. The results are interesting: while NAc ABHD6 -unexpectedly- facilitates food intake and obesity development, VTA ABHD6, specifically in TH+ cells, dampens motivation for food rewards without affecting body weight. Despite the marked phenotypes, several limitations undermine enthusiasm for the study at its current stage. Authors must address the lack of absolute controls (WT mice), low sample sizes, lack of validation of ABHD6 KD in cre+ and cre- (or TH+-) neurons, as well as rule out potential metabolic confounds in the motivational tasks performed on ABHD6 NAc KO mice before acceptance for publication. More details below:
Major concerns:
o The provided images satisfactorily demonstrate expression of ABHD6 in NAc MSN and VTA TH+ and – neurons. However, these images do not prove that such expression is halted when cre-recombinase is incorporated. Please, provide representative images illustrating the reduction of abdh6 expression in cre-expressing NAc cells. Similarly, provide IHC or RNAscope images to confirm effective and selective knockdown of ABHD6 in VTA TH+, but not TH-, neurons of the TH-cre cohort.

• The operant task is not properly explained in-text. Instead of having a figure of an operant chamber, please illustrate the training schedule and reinforcement schedules employed. Data related to all points in this training schedule is crucial to understanding whether there was a problem with the acquisition or expression of the motivated behavior.
• In addition, this is one of the most important findings of the paper, and there is only a n=4 in the cKO group. Sample sizes must be increased.
• Related to the above, it is plausible that the blunted reward seeking phenotype is caused by lower body weight of NAc cKO mice. Given that the caloric value of pellets is the same between groups, motivational effects might have been irrelevant in the expression of reduced reward seeking. One way to deconvolve these two conflating possibilities would be to match the calorific content of the reward to the weight and feeding efficiency of each group. This could be easily adjusted with sucrose solutions and lickometers. Alternatively, the authors could test a neuroeconomic demand task (e.g., Siciliano et al., 2018 JoN) and compare the rates at which reward demand is modified by motivational/price requirements (Pmax, alpha).

o I think it is necessary to include at least one WT control group in the most important metrics: body weight, food intake and operant reward seeking in NAc and VTA cKO experiments. As it is, the manuscript does not provide evidence that ABHD6-floxed mice are a normal, valid control.
Minor concerns:
• Please, in the introduction, compare and contextualize ABHD6 in relation to MAGL, the canonical 2AG synthesizing enzyme in the brain.

• What s the hypothesized reason for the decrease in cnr1 expression after abdh6 knockdown?

• Report exact p values, and d.f.

• The expression of conditioned place preference is not a measure of “conditioned motivational responses to amphetamine reward”. It is unclear exactly why animals approach drug-paired contexts and there are no clear correlates of motivation. Please, correct this imprecision.

• I am not sure about the authors interpretation of Figure 3 running wheel findings. They claim that “ABHD6 loss-of-function protects against inactivity in diet-induced obesity”. However, ABHD6 cKO mice clearly do not show evidence of obesity. Therefore, ABHD6 does not protects against inactivity in diet-induced obesity, it protects against diet-induced obesity. Likewise, ABDH6 cKO mice are not ‘safeguarded from the sedentary influence of diet-induced obesity’, they simply do not display an overweight phenotype.

• All the effects of figure 4 could be explained by the observed reduction in cnr1 expression (Figure 1) and would not necessarily reflect a consequence of increased 2AG signaling. This is satisfactorily addressed by figure 4J, but it is hard to inderstand why the Authors only tested eIPSCs in this condition (with WIN). eIPSCs and eEPSCs should be described in drug-free as well as WIN and AM251 bath conditions.

o Please, show representative traces and summary bar plots in all cases. This also applies to the mIPSCs plots. For some reason, WIN mIPSC plots are time-resolved and have no bar plots, but AM251 mIPSC are only showed in bar plots.

o How exactly ABHD6 expression was determined in Figure 5c?

o The number of cKO subjects in figures 6G-H and L-M, respectively, does not match. Please, correct.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors did an excellent job of addressing my questions.

Reviewer #2

(Remarks to the Author)
The authors have addressed most of my concerns, however their response to concerns #2 and #3 are unfortunately incorrect, unsatisfactory and will confuse our field.

Concern #2: “Cnr1 expression among striatal output neurons (Gokce et al., 2016, Cell Reports; Stanley et al., 2020, Neuron) with a lack of CB1R expression among MSNs of the NAc”. This statement is incorrect as their multiple studies show functional CB1R in MSN, both direct and indirect pathways, as they show themselves using electrophysiology. The topographic transcriptomic gradient data are not conclusive, most likely because of low sensitivity. Thus, my concern stands, and the authors need to address it: “The ABHD6-KO mediated downregulation of CB1R mRNA is unexpected and novel and should be explored further. Do the authors have an explanation of the mechanism by which a link exists between ABHD6 and CB1R expressions? Does it reduce CB1R expression in NAc interneurons, in output neurons or in both? A better understanding of these compensatory mechanisms will help interpret the electrophysiology results presented in Figure 4”.

Concern #3: “we did not add BSA to the WIN preparation”. The authors are correct that BSA will prevent loss of the agonists, which is crucial to prevent up to 98% loss of cannabinoid agonists as best demonstrated in this publication (https://pubmed.ncbi.nlm.nih.gov/15209515/) and well known by electrophysiologist studying cannabinoid agonists. Thus, my concern stands, and the authors need to address it: “WIN was tested at 10 uM and produced small responses, including when measuring mIPSC amplitudes. The authors need to perform a WIN concentration-dependent dose response by adding 0.1% BSA in their perfusion media, to reduce WIN binding to plastics and as do most slice electrophysiology studies”. Thus, experiment is justified by the fact that WIN activates multiple non-CB1R target when applied a high micromolar concentrations and should already fully activate CB1R at 30 nM.

Reviewer #3

(Remarks to the Author)
The authors have satisfactorily addressed this reviewer's concerns.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have answered my concerns and clarified the manuscript.

REVIEWER COMMENTS

Thanks very much to the reviewers for their detailed comments which guided revision and significantly improved the manuscript. We have carefully addressed the reviewer comments in our point-by-point responses below in blue. Changes to the manuscript text are also in blue.

Reviewer #1:

Overall, this was an excellent and potentially very useful manuscript that highlights the role of cannabinoid signaling in nucleus accumbens inputs and outputs in feeding in an obesity paradigm and locomotion. The studies were well conducted, and my specific comments, which are designed to help the authors sharpen the wording and thinking in specific passages, are listed below.

We thank the reviewer for the positive comments and interest in our work.

Major comments:

1. The studies were technically elegant and yielded an interesting and important pattern of results. Although the literature review was comprehensive in terms of the number of references, some key areas of the literature were omitted. While the experiments are focused on modulation of cannabinoid signaling, DA mechanisms figure in conceptually, and are useful to discuss to provide context for the present results. For example, the idea that pharmacological or neurotoxic interference with mesoaccumbens DA transmission impairs locomotor activity but does not reduce food intake has been known for several decades (Koob et al. 1978; Salamone et al. 1993; Baldo and Kelley 2002). More recent studies show that antagonism of DA transmission switches choice behavior from running in a running wheel to sucrose drinking (Correa et al. 2016). Randall et al. (2012, 2014) reported that while DA antagonism shifts effort-based decision making, decreasing food-reinforced progressive ratio lever pressing but increasing chow intake, CB1 receptor antagonism or inverse agonism decreased both lever pressing and chow intake. There is much discussion of effortful behavior in motivation and the role of cannabinoid signaling in the present manuscript, but a discussion of DA is relevant for establishing the context for the present pattern of results.

We fully agree that we should discuss our findings within the knowledgebase of mesoaccumbens DA function. We have modified the manuscript as follows:

Discussion, paragraph 8:
"NAc dopamine mediates a crucial role in the control of locomotion and motivated behaviour[131,132]. Emphasizing this, effort-related choice and behavioural economic paradigms have demonstrated that manipulations of NAc dopamine function powerfully alter cost-benefit computations underlying motivated behaviour[133,134,135], which can occur orthogonally to changes in intake[136,137,138]. "

2. Overall, the introduction and discussion were conceptually sophisticated in terms of behavioral processes, and the authors are to be congratulated. However, as is the case with many articles, the present manuscript has some passages use the term 'reward' as a neurobehavioral process, without actually defining it. For example, the statement that "VTA is a key substrate for neural processing of palatable food reward" is ambiguous. Does this refer to facilitating response-outcome associations that underlie reinforcement learning, or is it intended to refer more generally to various aspects of food motivation? 'Reward' as a synonym for reinforcement or reinforcement learning is distinct from the effort-related motivational processes that also are being discussed in relation to the present findings. With this in mind, I urge the author to re-think the use of some of their terminology.

We thank the reviewer for the critical reading of terminology. We very much appreciate that the reviewer raises this point as the term “reward” was indeed nebulously defined in manuscript. We revised the text throughout to refer to more specific processes of motivated behavior, while on occasion keeping the use of “reward” when it is clear we are talking about the goal object.

Minor comments:

In the discussion, the phrase “including general inhibition of locomotion, immobility, and catalepsy” is ambiguously worded, and should read as “including general inhibition of locomotion, and induction of immobility and catalepsy”.

Thank you for this correction. We have revised the manuscript text as suggested.

Reviewer #2:

This manuscript reports the impact of ABHD6 loss-of-function in mesoaccumbens postsynaptic and presynaptic neurons on multiple behavioral readouts of diet-induced obesity in mice. The manuscript is clearly written and the premise of the study well-articulated. Behavioral analyses are thorough and most interpretation sound. Overall, this study will represent an important addition to the field.

We thank the reviewer for their positive comments.

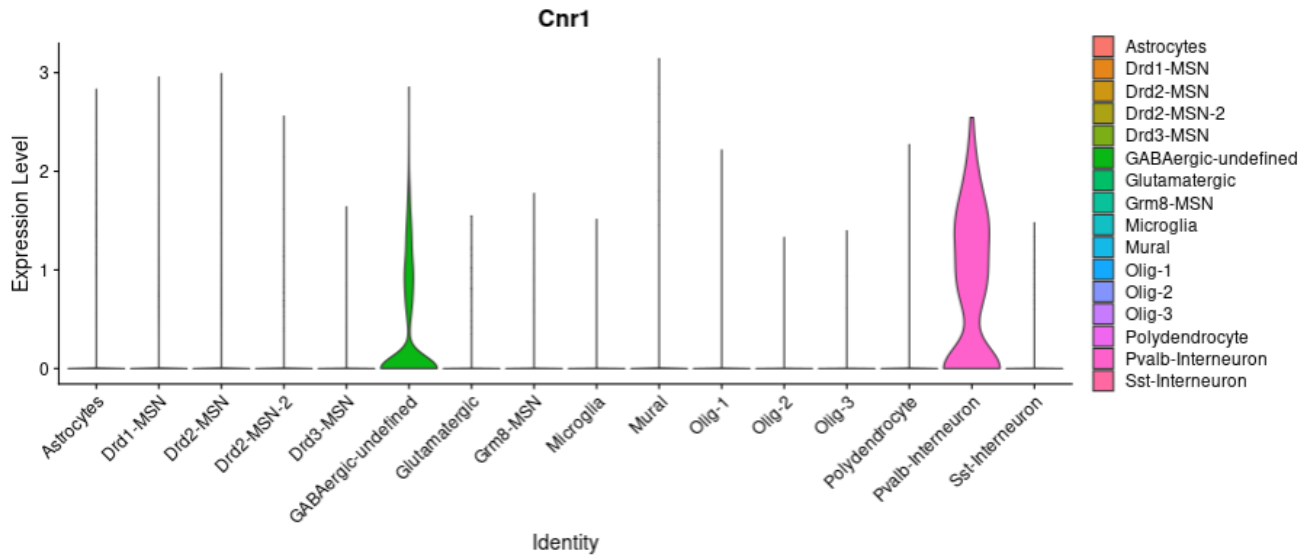
Concerns that must be addressed:

1. Introduction: This sentence is incorrect “2-AG release from VTA DA neurons targets presynaptic CB1Rs localized on NAc GABAergic terminals13,14”. Rather, it should say “CB1Rs localized on VTA GABAergic and glutamatergic terminals”.

Thank you for noting this error, we made the correction.

2. Results: The ABHD6-KO mediated downregulation of CB1R mRNA is unexpected and novel and should be explored further. Do the authors have an explanation of the mechanism by which a link exists between ABHD6 and CB1R expressions? Does it reduce CB1R expression in NAc interneurons, in output neurons or in both? How about other components involved in regulating 2-AG levels, including DAGLa, DAGLb and MAGL? A better understanding of these compensatory mechanisms will help interpret the electrophysiology results presented in Figure 4.

Based upon previous literature which has defined the topographic transcriptomic gradient of *Cnr1* expression among striatal output neurons (Gokce et al., 2016, *Cell Reports*; Stanley et al., 2020, *Neuron*) with a lack of CB1R expression among MSNs of the NAc (Winters et al., 2012, *PNAS;* Wright et al., 2017, *NPP*), we believe that changes in NAc CB1R expression are unlikely to be related to altered expression within NAc MSN output neurons themselves. Our electrophysiological results suggest CB1R signalling at inhibitory synapses to MSNs is altered (Fig. 4), implicating neuroplastic changes may be linked to NAc *Pvalb*-expressing fast-spiking interneurons, which are the exclusive known local inhibitory cell type within the murine NAc expressing CB1R (Winters et al., 2012, *PNAS*; Wright et al., 2017, *NPP*). We note that our study has not formally evaluated the possibility of whether distally localized (i.e. presynaptic axonal) *Cnr1* mRNA transcripts within the NAc may have contributed to our results (Holt et al., 2019, *Nat Struct Mol Biol*), nor, to our knowledge, has any other study to date. We additionally note that a recent single-nucleus RNA sequencing study suggests that some CB1R in the NAc may additionally be expressed in a currently uncharacterized GABAergic cell type, referred to as “GABAergic-undefined” within the dataset of Savell et al., (2020, *Sci Adv*). For reference, we provide a violin plot of NAc *Cnr1* expression from this dataset below.



Violin plot depicting distribution of *Cnr1* expression across cell clusters. Data from murine NAc single-nucleus RNA sequencing study (Savell et al., 2020, *Sci Adv*), available: https://day-lab.shinyapps.io/ratlas/.

Our RT-qPCR results revealed no changes in expression of NAc *Mgll* in ABHD6$^{NAc\ KO}$ mice (Fig. 1d), and new RT-qPCR experiments we carried out found no changes in *Dagla* or *Daglb* expression (Supplementary Figure 1a), suggesting no compensatory changes in NAc 2-AG biosynthetic and MAGL enzyme expression. Further RT-qPCR experiments unexpectedly revealed reduced expression of both *Faah* and *Napepld* (Supplementary Figure 1a), however our LCMS/MS results found no changes in anandamide levels in the NAc of ABHD6$^{NAc\ KO}$ mice (Fig. 1e). As FAAH and NAPEPLD enzymes regulate a number of bioactive lipid species, we cannot rule out that compensatory changes in other lipid species beyond 2-AG might have contributed to our findings. Moreover, our RT-qPCR analysis represents mRNA from bulk NAc tissue.

Future investigations using single-cell RNA sequencing would more comprehensively resolve the relative contributions of decreased CB1R expression within individual cell-types. We hope the reviewer agrees that such experiments are beyond the scope of the current manuscript but would be valuable for future studies to provide an entry point for elucidating potential translational and/or post-translational regulatory mechanisms controlling NAc CB1R expression.

3. Figure 4: WIN was tested at 10 uM and produced small responses, including when measuring mIPSC amplitudes. The authors need to perform a WIN concentration-dependent dose response by adding 0.1% BSA in their perfusion media, to reduce WIN binding to plastics and as do most slice electrophysiology studies. Alternatively, or in addition, testing the MAGL inhibitor, JZL184, might help further unravel the difference in electrophysiological responses gathered between CTR and ABHD6-KD.

As the reviewer points out, we did not add BSA to the WIN preparation. The product sheet (Enzo, BML-CR105-0010) does not mention adding BSA when reconstituting WIN. We have confirmed this is also the case for other WIN product sheets from other suppliers (e.g. Tocris). This is in contrast to product sheets for other compounds we have used previously, and we note that stickiness on tubing tends to be less of a concern for small molecules compared to proteins. We acknowledge that the magnitude of the impact of WIN on synaptic input to NAc MSNs is expected to be dose-dependent (Robbe et al., 2001, *J Neurosci;* Hoffman and Lupica, 2001, *J Neurophysiol*). However, if the reasoning for running a concentration dose-dependent response is to determine whether the impact of WIN was mitigated by binding to plastics we wish to note that the effect of WIN (10µM) on mIPSC frequency was reduced by ~40% 15 minutes after application in ABHD6$^{NAc\ KO}$ mice as compared to ABHD6$^{NAc\ GFP}$ controls (main effect $P<0.05$). We consider this an ample effect. There was a clear lack of genotype difference in the

effect of WIN on mIPSC amplitude (main effect P=0.66). It is not clear if the reviewer expects us to see a larger effect on frequencies. While a direct comparison is not possible due to differences in how data were quantified, our results appear similar to a previous publication measuring the effects of 10µM WIN on NAc MSN mIPSCs (Manzoni and Bockaert, 2001, *Eur J Pharmacol*).

We agree that testing a MAGL inhibitor would be interesting as an additional experiment. However, our RT-qPCR results found that NAc ABHD6 neuronal invalidation did not impact NAc *Mgll* expression (Fig. 1d), suggesting changes in MAGL expression do not contribute to our results. We feel that the cost, resources, and time required for an additional JZL184 experiment (re-deriving colony and breeding, injecting a new batch of mice with AAV, and repeat of electrophysiological recordings) are considerable for what it is likely to add to the manuscript. We hope the reviewer will agree.

4. Discussion: While the discussion is already lengthy and could be shortened by 1/3 when combining most concepts discussed, it would be important to emphasize that ABHD6 is also involved in AMPA receptor trafficking. In fact, the authors could mention that the absence of changes in EPSCs in ABHD6-KO neurons argues against the involvement of ABHD6 trafficking function in the responses reported here.

We completely agree with the reviewer's suggestion and thank them for correcting this oversight in the discussion. We have revised the manuscript text as indicated below.

Discussion, paragraph 3:
"ABHD6 has been demonstrated to modulate synaptic signaling and plasticity independently of enzymatic 2-AG degradation via negative regulation of postsynaptic AMPA receptor trafficking[85,86,87]. However, our electrophysiological results revealed no differences in EPSCs in NAc MSNs from ABHD6$^{NAc\ KO}$ mice, arguing against involvement of ABHD6-mediated control of AMPA receptor trafficking."

5. Suggestions:

Introduction: Endocannabinoids regulate… strong association to obesity". It would be useful to also mention that THC users have a tendency to be leaner than non-users. A suggestion is to add a couple of sentences to address this notion and how it contrasts with your study.

We thank the reviewer for this insight, we have revised the manuscript text as indicated below.

Discussion, paragraph 3:
"Chronic cannabis usage is also paradoxically associated with reduced BMI[90,91,92], an effect that is not driven by reduced energy intake[93,94,95]. A mechanistic basis for this phenomenon remains unclear. However, it has been hypothesized that compensatory downregulation of CB1R may be involved[96].. "

Reviewer #3:

The study by Lau et al., addresses an important question: what are the feeding, metabolic and motivational contributions of the 2-AG degrading enzyme ABHD6? This is addressed using viral-gene strategies in a manner that allows to elucidate pre- and post-synaptic contributions of this enzyme throughout the mesoaccumbal dopamine pathway. The results are interesting: while NAc ABHD6 -unexpectedly- facilitates food intake and obesity development, VTA ABHD6, specifically in TH+ cells, dampens motivation for food rewards without affecting body weight. Despite the marked phenotypes, several limitations undermine enthusiasm for the study at its current stage. Authors must address the lack of absolute controls (WT mice), low sample sizes, lack of validation of ABHD6 KD in cre+ and cre- (or TH+-) neurons, as well as rule out potential metabolic confounds in the motivational tasks performed on ABHD6 NAc KO mice before acceptance for publication. More details below:

We thank the reviewer for their interest in our work and detailed input that has improved the manuscript

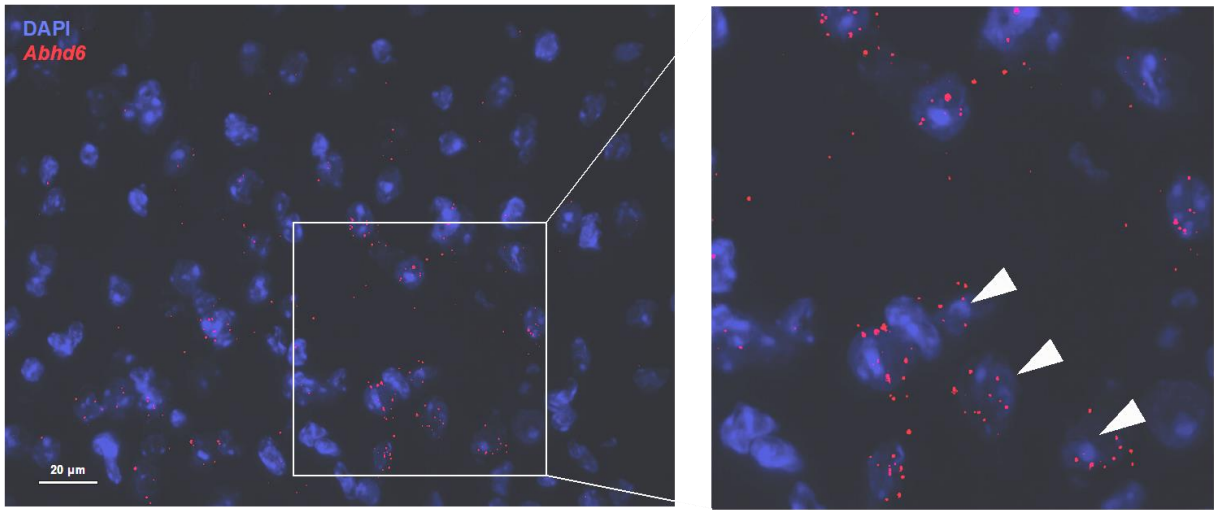
Major concerns:

1. The provided images satisfactorily demonstrate expression of ABHD6 in NAc MSN and VTA TH+ and – neurons. However, these images do not prove that such expression is halted when cre-recombinase is incorporated. Please, provide representative images illustrating the reduction of abdh6 expression in cre-expressing NAc cells. Similarly, provide IHC or RNAscope images to confirm effective and selective knockdown of ABHD6 in VTA TH+, but not TH-, neurons of the TH-cre cohort.

We provided qPCR evidence to demonstrate knockout of *Abhd6* mRNA in the NAc and VTA whereas revelation of ABHD6 knockout in specific cell types using IHC or RNAscope was not included for the reasons described below. We did however carry out IHC experiments to demonstrate the neuronal specificity of synapsin-driven AAV infection in nucleus accumbens neurons using the recommended control virus expressing GFP (Figure 1c). We now present new IHC data below revealing the infection specificity of the TH-AAV (AAV9.rTH.PI.Cre.SV40) in the midbrain VTA.

We have previously undertaken RNAscope experiments in attempts to provide cell-type specific illustration of ABHD6 invalidation, but found that the feasibility of this approach is significantly limited for the following reasons:

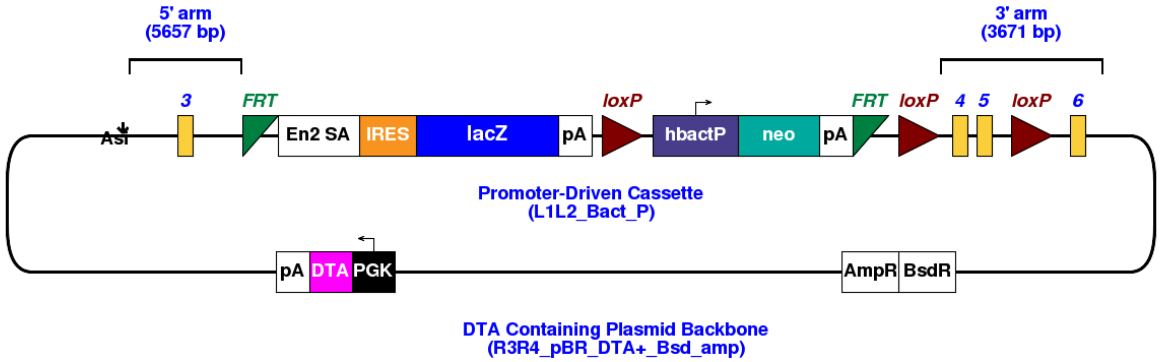
In our hands, antibodies for ABHD6, including a custom-made one from a collaborator, are not specific. This was our reason to apply RNAscope which has been demonstrated to minimize non-target signal by utilizing a novel proprietary probe design with high target specificity and sensitivity (Wang et al., 2012, *J Mol Diagn*). Our past experiment incorporated brain slices from ABHD6 full-body knockout mice as a negative control. Though the magnitude of the signal was attenuated compared to *Abhd6*$^{lox/lox}$ mice, we still observed *Abhd6* mRNA signal in ABHD6 full-body knockout mice, as illustrated below.



Representative photomicrograph of anterior hypothalamic area from full-body ABHD6 knockout mouse. Scale bar 20µm. Arrows indicate cells with *Abhd6* mRNA signal.

To provide context for these results, it is important to describe the specific design of the *Abhd6*$^{lox/lox}$ mice, ABHD6 whole-body knockout mice, and RNAscope *Abhd6* probes in detail. Full-body ABHD6 knockout mice were generated using a "knockout-first" strategy (Skarnes et al., 2011, *Nature*) of the *Abhd6* gene (NCBI RefSeq NM_001331064.1). Such a deletion includes a gene trap cassette upstream of exon 4/5 flanked by FRT sites; crossing this mouse with an FlpO driver line was used to excise the trap cassette and generate the floxed alleles used in this study, with LoxP sites flanking exon 4/5 (schematic of the

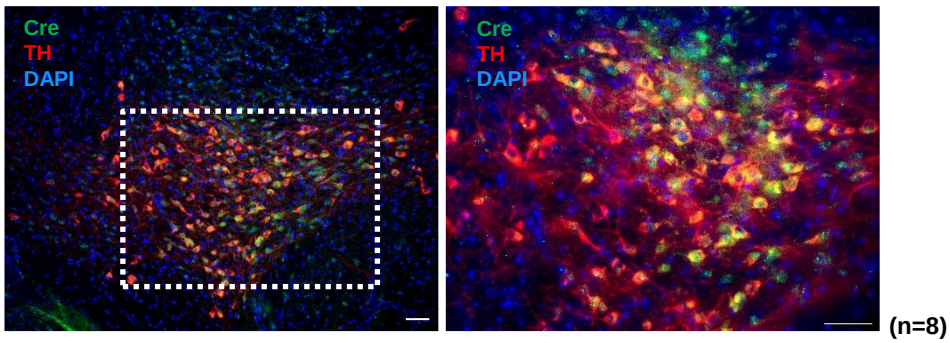
targeting vector is shown below). Additional details are available in a previously published manuscript (Zhao et al., 2014, *Cell Metabolism*).

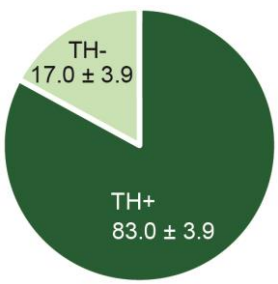


Schematic of targeting vector (IMPC), available:
https://www.mousephenotype.org/data/genes/MGI:1913332

By contrast, the design of *Abhd6* RNAscope probes (as is common for all RNAscope probes) features twenty pairs of complimentary sequences of 18-25 base-pair oligomers, which in the case of the *Abhd6* probes span exon 2 (beginning at bp 140) to exon 9 (up to bp 2301) of the mouse *Abhd6* transcript. Given the observed signal in ABHD6 full-body knockout mice, these results suggest RNAscope signal from oligomers targeting transcript outside the knockout region, even from transcript upstream of exon 4, is significant, and that using RNAscope to validate *Abhd6* knockout is compromised for this reason. We note that this RNAscope probe design broadly reflects the goal of maximizing the detection sensitivity for mRNA transcripts, rather than increasing the targeting specificity of mRNA transcript localized specifically between the LoxP sites in our particular study. We also highpoint that evidence from current manuscript (Fig. 1e) and previous studies using *Abhd6*$^{lox/lox}$ alleles with Cre recombination have established removal of exon 4/5 is effective in impairing the functionality of the ABHD6 protein (Zhao et al., 2014, *Cell Metabolism*; Zhao et al., 2015, *Molecular Metabolism*; Fisette et al., 2016, *Cell Reports*; Poursharifi et al., 2020, *JCI Insight*).

The TH-Cre virus has been used to target VTA dopamine neurons in previous publications (e.g., Stauffer et al., 2016, *Cell*; Parker et al., 2019, *Cell*; Robinson et al., 2019, *eLife*, Kim et al., 2021, *Sci Adv*; Kutlu et al., 2023, *Cell Reports*; Luján et al., 2024, *Nature Communications*). Moreover, our own validation experiments have shown this TH-Cre AAV specifically targets dopamine neurons of the VTA, as shown in a representative photomicrograph below. While we found that 17% of VTA cells expressing Cre lacked TH protein (TH-), it may well be the case that these cells are positive for TH mRNA with minimal to no TH protein (Lenartowski and Goc, 2011, *Int J Dev Neurosci*).



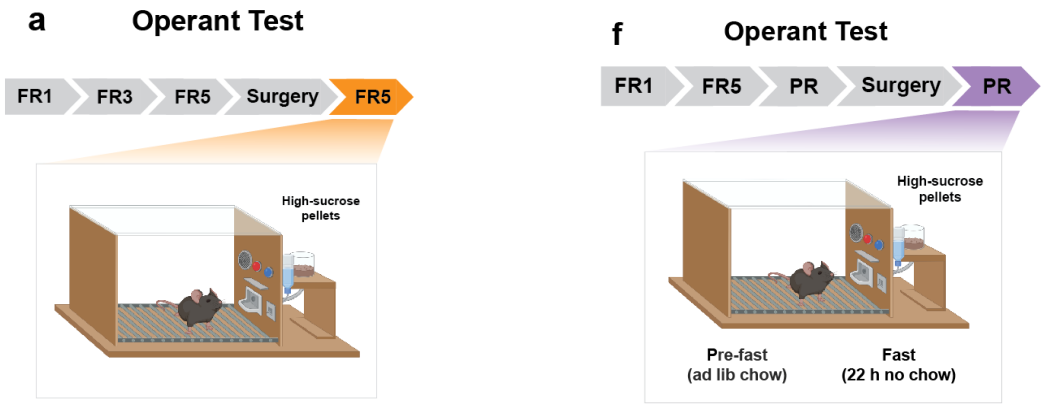


(n=8)

Representative IHC photomicrograph illustrating that the majority of Cre+ cells are TH+ in the VTA of *Abhd6*$^{lox/lox}$ mice injected with AAV9.rTH.PI.Cre.SV40 (left; scale bar 50µM). Proportion of Cre+ neurons that are TH+ and without TH- (right).

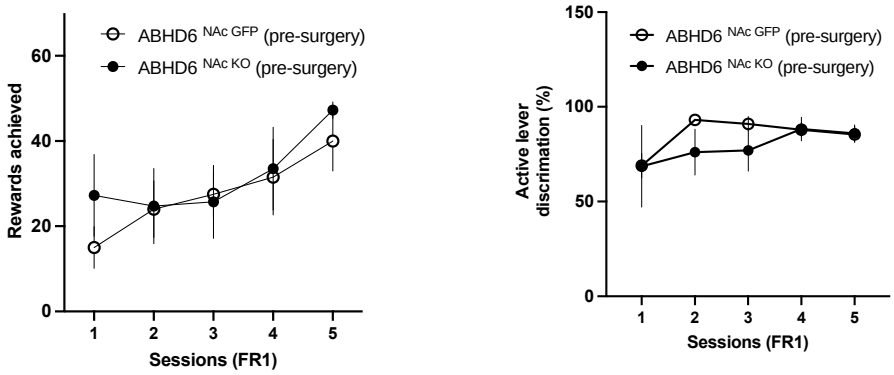
2. The operant task is not properly explained in-text. Instead of having a figure of an operant chamber, please illustrate the training schedule and reinforcement schedules employed. Data related to all points in this training schedule is crucial to understanding whether there was a problem with the acquisition or expression of the motivated behavior.

We have revised figures as suggested to include the training schedule and reinforcement schedules, as shown below.

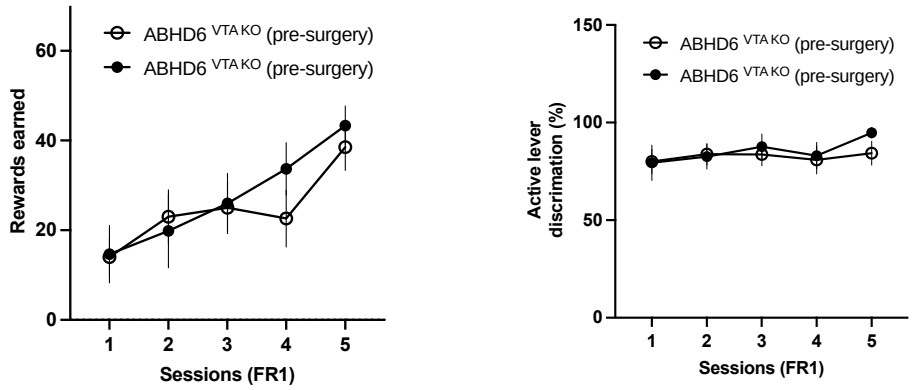


New Fig. 2a (left) and Fig. 6f (right).

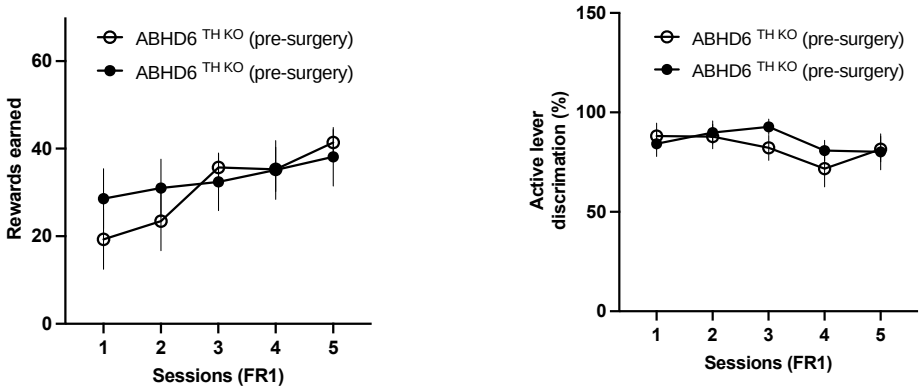
It is also important to note that all training occurred prior to AAV-injection surgeries for ABHD6 invalidation, thus differences between groups are unlikely to be due to changes in task acquisition. We include below FR1 data illustrating no significant differences between groups.



ABHD6 NAc pre-surgery FR1 training data. Operant training data from fixed ratio 1 (FR1) schedule of reinforcement for rewards achieved (left; n=4-6/group) and active lever discrimination (right; n=4-6/group). No significant differences between groups were observed in either the rewards achieved (two-way ANOVA: Group x time interaction $F_{(4,32)}$=0.6157, P=0.6545; Time $F_{(4,32)}$=6.110, P=0.0009; Group $F_{(1,8)}$=0.2331, P=0.6422) or active lever discrimination (two-way ANOVA: Group x time interaction $F_{(4,32)}$=1.281, P=0.2980; Time $F_{(4,32)}$=4.043, P=0.0092; Group $F_{(1,8)}$=0.4555, P=0.5188).



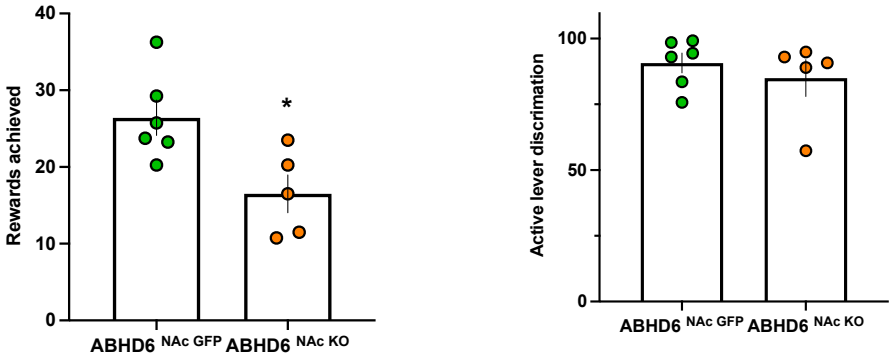
ABHD6 VTA pre-surgery FR1 training data. Operant training data from fixed ratio 1 (FR1) schedule of reinforcement for rewards achieved (left; n=6-10/group) and active lever discrimination (right; n=6-10/group). No significant differences between groups were observed in either the rewards achieved (two-way ANOVA: Group x time interaction $F_{(4,56)}$=1.051, P=0.3894, Time $F_{(4,56)}$=13.95, P<0.0001, Group $F_{(1,14)}$=0.1535, P=0.7011) or active lever discrimination (mixed effects model (REML): Group x time $F_{(4,53)}$=0.5462, P=0.7026, Time $F_{(4,53)}$=1.116, P=0.3590, Group $F_{(1,14)}$=0.1061, P=0.7495)



ABHD6 TH pre-surgery FR1 training data. Operant training data from fixed ratio 1 (FR1) schedule of reinforcement for rewards achieved (left; n=7/group) and active lever discrimination (right; n=7/group). No significant differences between groups were observed in either the rewards achieved (two-way ANOVA: Group x time interaction $F_{(4,48)}$=1.237, P=0.3077 ; Time $F_{(4,48)}$=5.589, P=0.0009; Group $F_{(1,12)}$=0.08513, P=0.7754) or active lever discrimination (two-way ANOVA: Group x time interaction $F_{(4,48)}$=0.6495, P=0.6300; Time $F_{(4,48)}$=1.756, P=0.1532 ; Group $F_{(1,12)}$=0.2849, P=0.6032).

3. In addition, this is one of the most important findings of the paper, and there is only a n=4 in the cKO group. Sample sizes must be increased.

We agree that the low sample size in the operant experiment of Figure 2 is a limitation. One mouse from the ABHD6$^{NAc\ KO}$ group was removed from the dataset due to operant discrimination below the threshold for inclusion (excluded mouse active lever discrimination 57.35%, threshold for inclusion 70%). We included for reference the graphs with the inclusion of this mouse (n=5 KO group) in the dataset below. The removal of this data did not influence the conclusions as ABHD6$^{NAc\ KO}$ exhibit significantly reduced rewards achieved (Unpaired t-test, $t_{(9)}$=2.928, p=0.0168).



Data from Fig. 2b (left) and 2c (right) with the inclusion of 1 additional mouse in ABHD6 $^{NAc\ KO}$ group.

4. Related to the above, it is plausible that the blunted reward seeking phenotype is caused by lower body weight of NAc cKO mice. Given that the caloric value of pellets is the same between groups, motivational effects might have been irrelevant in the expression of reduced reward seeking. One way to deconvolve these two conflating possibilities would be to match the calorific content of the reward to the weight and feeding efficiency of each group. This could be easily adjusted with sucrose solutions and lickometers. Alternatively, the authors could test a neuroeconomic demand task (e.g., Siciliano et al., 2018 JoN) and compare the rates at which reward demand is modified by motivational/price requirements (Pmax, alpha)

We completely agree with the reviewer that variation in body weight can exert powerful effects on food-seeking behaviour. Indeed, studies dating back to Hodos (1961, *Science*) have noted dramatic effects of decreased body weight (or increased reward size) to positively modulate operant behaviour, and hypoactive mesolimbic dopamine function (Stice et al., 2008, *Science*, Volkow et al., 2011, *Trends Cogn Sci*) and altered cost discounting (Bickel et al., 2021, *Neurosci Biobehav Rev*) continue to attract significant research interest as potential neural mechanisms linked to overweight and obesity. However, our observed findings of are not in the direction that would be anticipated by reduced body weight in ABHD6$^{NAc\ KO}$ mice, as we observed decreased, rather than increased operant responding. We also note that we did not observe any difference in free-feeding intake between chow-fed KO and controls (Fig. 1h).

5. I think it is necessary to include at least one WT control group in the most important metrics: body weight, food intake and operant reward seeking in NAc and VTA cKO experiments. As it is, the manuscript does not provide evidence that ABHD6-floxed mice are a normal, valid control.

We agree with the reviewer that comparison to WT mice is an important consideration. We have previously independently validated that *Abhd6*$^{lox/lox}$ mice have similar body weight gain, food intake, and fed/fasting glycemia to wild-type mice (Zhao et al., 2014, *Cell Metabolism*; Zhao et al., 2015, *Molecular Metabolism*). ABHD6-knockout mice and *Abhd6*$^{lox/lox}$ mice have previously proven to be a valuable tool in the study of energy metabolism, and *Abhd6*$^{lox/lox}$ mice have been utilized as a valid control from a number of studies on energy-homeostasis topics spanning insulin secretion/glucose tolerance, feeding and energy expenditure, and responses to dietary/cold challenges (Zhao et al., 2014, *Cell Metabolism*; Zhao et al., 2015, *Molecular Metabolism*; Fisette et al., 2016, *Cell Reports*; Poursharifi et al., 2020, *JCI Insight*). For this reason, *Abhd6*$^{lox/lox}$ mice injected with control viruses are the most appropriate control for our study. We have added the following text to the manuscript to make it clear that the floxed alleles do not modify key parameters:

Methods, paragraph 1:
"Previous work has established that *Abhd6*$^{lox/lox}$ mice have similar body weight gain and food intake to wild-type mice[50,165]."

Finally, as an additional means to support our findings and emphasize the potential clinical utility of targeting ABHD6 for protection against obesity, we have added an experiment that tested the impact of chronic central pharmacological inhibition of ABHD6 on wild-type mice on a high-fat diet. Our results demonstrate that chronic central ABHD6 inhibition also is protective against diet-induced obesity (Fig. 7). The following manuscript text has been added:

Results, paragraph 13:
Central ABHD6 pharmacological inhibition prevents diet-induced obesity
We next examined whether sustained central pharmacological ABHD6 inhibition may similarly prevent diet-induced obesity in WT mice. Adult male mice on a HFD were subcutaneously implanted with minipumps for chronic intracerebroventricular infusions of either vehicle or the ABHD6-specific inhibitor WWL7079 on HFD. WWL70-treated mice exhibited reduced body weight gain on HFD (Fig. 7a-b). WWL70 reduced food intake and increased energy expenditure on HFD (Fig. 7c-f). RER was reduced in WWL70-treated mice, suggesting increased fat utilization (Fig. 7g). Similar to ABHD6VTA KO mice, a trend towards reduced dark cycle RER and significantly reduced light cycle spontaneous locomotor activity were observed in WWL70-treated mice (Fig. 7g-j). Central ABHD6 inhibition did not alter anxiety-like behaviour (Fig. 7k-l).

Minor concerns:

1. Please, in the introduction, compare and contextualize ABHD6 in relation to MAGL, the canonical 2AG synthesizing enzyme in the brain.

We thank the reviewer for this important suggestion. We have revised the introduction text as indicated below.

Introduction, paragraph 4:
"In contrast to the canonical, presynaptically-localized 2-AG degrading enzyme MAGL, ABHD6 is localized postsynaptically at the site of 2-AG synthesis[5] and is uniquely positioned to control 2-AG spatiotemporal accumulation."

2. What is the hypothesized reason for the decrease in cnr1 expression after abdh6 knockdown?

We speculate based on previous research on chronic pharmacological MAGL inhibition and genetic invalidation of MAGL that it is possible that CB1R may be downregulated as a compensatory response to increased 2-AG tone following ABHD6 invalidation (Schlosburg et al., 2010, *Nature Neuroscience*). The reviewer is also referred to response to point # 2 of Reviewer 2 above for additional discussion.

3. Report exact p values, and d.f.

We have included exact p values and d.f. in Supplementary Tables 2-13.

4. The expression of conditioned place preference is not a measure of "conditioned motivational responses to amphetamine reward". It is unclear exactly why animals approach drug-paired contexts and there are no clear correlates of motivation. Please, correct this imprecision.

We revised the manuscript text as follows:

Results, paragraph 3:
"ABHD6$^{NAc\ KO}$ mice exhibited decreased conditioned approach behaviour to a context paired with drug reward (Fig. 2e)."

5. I am not sure about the authors interpretation of Figure 3 running wheel findings. They claim that

"ABHD6 loss-of-function protects against inactivity in diet-induced obesity". However, ABHD6 cKO mice clearly do not show evidence of obesity. Therefore, ABHD6 does not protect against inactivity in diet-induced obesity, it protects against diet-induced obesity. Likewise, ABDH6 cKO mice are not 'safeguarded from the sedentary influence of diet-induced obesity', they simply do not display an overweight phenotype.

Thank you for noting this. We have corrected the manuscript text as indicated below.

Results, paragraph 4:
"While ABHD6$^{NAc\ GFP}$ controls showed markedly reduced wheel running activity in response to diet-induced obesity, ABHD6$^{NAc\ KO}$ mice exhibited robust wheel-running (Fig. 3k-l) at levels approximating chow-fed controls (Supplementary Figure 3b-c), suggesting that NAc neuronal ABHD6 loss-of-function prevents inactivity resulting from diet-induced obesity."

And, we removed the following sentence from the manuscript:

Discussion, paragraph 1:
"Emphasizing the beneficial effects of NAc neuronal ABHD6 invalidation, we found knockout mice to be markedly safeguarded from the sedentary influence of diet-induced obesity."

6. All the effects of figure 4 could be explained by the observed reduction in cnr1 expression (Figure 1) and would not necessarily reflect a consequence of increased 2AG signaling. This is satisfactorily addressed by figure 4J, but it is hard to understand why the Authors only tested eIPSCs in this condition (with WIN). eIPSCs and eEPSCs should be described in drug-free as well as WIN and AM251 bath conditions.

The results of Figure 4, particularly of reduced m/sIPSC frequency and amplitude, would not readily be explained by reduced *Cnr1* expression. Reduced attenuation of presynaptic input resulting from reduced CB1R expression/signalling would be expected to increase rather than decrease these parameters. Additionally, we note mRNA levels alone are not always indicative of protein abundance and/or functionality (Monory et al., 2006, *Neuron*; Buccitelli and Selbach, 2020, *Nat Rev Genet*). As we saw a change in IPSCs and not EPSCs and for reasons related to time/resource limitations, we prioritized testing WIN and AM251 only on mIPSCs.

7. Please, show representative traces and summary bar plots in all cases. This also applies to the mIPSCs plots. For some reason, WIN mIPSC plots are time-resolved and have no bar plots, but AM251 mIPSC are only showed in bar plots.

We added representative traces on all the frequency graphs with exception of the WIN mIPSC frequency time plot data (Fig. 4h). We believe that all the data in the figure is represented in a manner that clearly communicates the results.

8. How exactly was ABHD6 expression was determined in Figure 5c?

ABHD6 expression was determined by RT-qPCR from VTA tissue punches.

9. The number of cKO subjects in figures 6G-H and L-M, respectively, does not match. Please, correct.

The number of subjects in these figures are correct and are matching. Some individual data points in Fig. 6g and 6l are overlapping (more likely with discrete data).

References

Bickel WK, Freitas-Lemos R, Tomlinson DC, Craft WH, Keith DR, Athamneh LN, Basso JC, Epstein LH (2021) Temporal discounting as a candidate behavioral marker of obesity. Neurosci Biobehav Rev 129:307–329.

Buccitelli C, Selbach M (2020) mRNAs, proteins and the emerging principles of gene expression control. Nat Rev Genet 21:630–644.

Fisette A, Tobin S, Décarie-Spain L, Bouyakdan K, Peyot M-L, Madiraju SRM, Prentki M, Fulton S, Alquier T (2016) α/β-Hydrolase Domain 6 in the Ventromedial Hypothalamus Controls Energy Metabolism Flexibility. Cell Rep 17:1217–1226.

Gokce O, Stanley GM, Treutlein B, Neff NF, Camp JG, Malenka RC, Rothwell PE, Fuccillo MV, Südhof TC, Quake SR (2016) Cellular Taxonomy of the Mouse Striatum as Revealed by Single-Cell RNA-Seq. Cell Rep 16:1126–1137.

Hodos W (1961) Progressive ratio as a measure of reward strength. Science 134:943–944.

Hoffman AF, Lupica CR (2001) Direct Actions of Cannabinoids on Synaptic Transmission in the Nucleus Accumbens: A Comparison With Opioids. Journal of Neurophysiology 85:72–83.

Holt CE, Martin KC, Schuman EM (2019) Local translation in neurons: visualization and function. Nat Struct Mol Biol 26:557–566.

Kim Y-J, Kong Q, Yamamoto S, Kuramoto K, Huang M, Wang N, Hong JH, Xiao T, Levine B, Qiu X, Zhao Y, Miller RJ, Dong H, Meltzer HY, Xu M, He C (2021) An autophagy-related protein Becn2 regulates cocaine reward behaviors in the dopaminergic system. Sci Adv 7:eabc8310.

Kutlu MG, Tat J, Christensen BA, Zachry JE, Calipari ES (2023) Dopamine release at the time of a predicted aversive outcome causally controls the trajectory and expression of conditioned behavior. Cell Rep 42:112948.

Lenartowski R, Goc A (2011) Epigenetic, transcriptional and posttranscriptional regulation of the tyrosine hydroxylase gene. Int J Dev Neurosci 29:873–883.

Luján MÁ, Covey DP, Young-Morrison R, Zhang L, Kim A, Morgado F, Patel S, Bass CE, Paladini C, Cheer JF (2023) Mobilization of endocannabinoids by midbrain dopamine neurons is required for the encoding of reward prediction. Nat Commun 14:7545.

Manzoni OJ, Bockaert J (2001) Cannabinoids inhibit GABAergic synaptic transmission in mice nucleus accumbens. Eur J Pharmacol 412:R3-5.

Monory K et al. (2006) The Endocannabinoid System Controls Key Epileptogenic Circuits in the Hippocampus. Neuron 51:455–466.

Parker KE, Pedersen CE, Gomez AM, Spangler SM, Walicki MC, Feng SY, Stewart SL, Otis JM, Al-Hasani R, McCall JG, Sakers K, Bhatti DL, Copits BA, Gereau RW, Jhou T, Kash TJ, Dougherty JD, Stuber GD, Bruchas MR (2019) A Paranigral VTA Nociceptin Circuit that Constrains Motivation for Reward. Cell 178:653-671.e19.

Poursharifi P, Attané C, Mugabo Y, Al-Mass A, Ghosh A, Schmitt C, Zhao S, Guida J, Lussier R, Erb H, Chenier I, Peyot M-L, Joly E, Noll C, Carpentier AC, Madiraju SRM, Prentki M (2020) Adipose ABHD6 regulates tolerance to cold and thermogenic programs. JCI Insight 5:e140294, 140294.

Robbe D, Alonso G, Duchamp F, Bockaert J, Manzoni OJ (2001) Localization and mechanisms of action of cannabinoid receptors at the glutamatergic synapses of the mouse nucleus accumbens. J Neurosci 21:109–116.

Robinson JE, Coughlin GM, Hori AM, Cho JR, Mackey ED, Turan Z, Patriarchi T, Tian L, Gradinaru V (2019) Optical dopamine monitoring with dLight1 reveals mesolimbic phenotypes in a mouse model of neurofibromatosis type 1. Elife 8:e48983.

Savell KE, Tuscher JJ, Zipperly ME, Duke CG, Phillips RA, Bauman AJ, Thukral S, Sultan FA, Goska NA, Ianov L, Day JJ (2020) A dopamine-induced gene expression signature regulates neuronal function and cocaine response. Sci Adv 6:eaba4221.

Schlosburg JE, Blankman JL, Long JZ, Nomura DK, Pan B, Kinsey SG, Nguyen PT, Ramesh D, Booker L, Burston JJ, Thomas EA, Selley DE, Sim-Selley LJ, Liu Q, Lichtman AH, Cravatt BF (2010) Chronic monoacylglycerol lipase blockade causes functional antagonism of the endocannabinoid system. Nat Neurosci 13:1113–1119.

Skarnes WC, Rosen B, West AP, Koutsourakis M, Bushell W, Iyer V, Mujica AO, Thomas M, Harrow J, Cox T, Jackson D, Severin J, Biggs P, Fu J, Nefedov M, de Jong PJ, Stewart AF, Bradley A (2011) A conditional knockout resource for the genome-wide study of mouse gene function. Nature 474:337–342.

Stanley G, Gokce O, Malenka RC, Südhof TC, Quake SR (2020) Continuous and Discrete Neuron Types of the Adult Murine Striatum. Neuron 105:688-699.e8.

Stauffer WR, Lak A, Yang A, Borel M, Paulsen O, Boyden ES, Schultz W (2016) Dopamine Neuron-Specific Optogenetic Stimulation in Rhesus Macaques. Cell 166:1564-1571.e6.

Stice E, Spoor S, Bohon C, Small DM (2008) Relation Between Obesity and Blunted Striatal Response to Food Is Moderated by *Taq* IA A1 Allele. Science 322:449–452.

Volkow ND, Wang G-J, Baler RD (2011) Reward, dopamine and the control of food intake: implications for obesity. Trends Cogn Sci 15:37–46.

Wang F, Flanagan J, Su N, Wang L-C, Bui S, Nielson A, Wu X, Vo H-T, Ma X-J, Luo Y (2012) RNAscope: a novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J Mol Diagn 14:22–29.

Winters BD, Krüger JM, Huang X, Gallaher ZR, Ishikawa M, Czaja K, Krueger JM, Huang YH, Schlüter OM, Dong Y (2012) Cannabinoid receptor 1-expressing neurons in the nucleus accumbens. Proc Natl Acad Sci U S A 109:E2717-2725.

Wright WJ, Schlüter OM, Dong Y (2017) A Feedforward Inhibitory Circuit Mediated by CB1-Expressing Fast-Spiking Interneurons in the Nucleus Accumbens. Neuropsychopharmacology 42:1146–1156.

Zhao S, Mugabo Y, Iglesias J, Xie L, Delghingaro-Augusto V, Lussier R, Peyot M-L, Joly E, Taïb B, Davis MA, Brown JM, Abousalham A, Gaisano H, Madiraju SRM, Prentki M (2014) α/β-Hydrolase domain-6-accessible monoacylglycerol controls glucose-stimulated insulin secretion. Cell Metab 19:993–1007.

Zhao S, Poursharifi P, Mugabo Y, Levens EJ, Vivot K, Attane C, Iglesias J, Peyot M-L, Joly E, Madiraju SRM, Prentki M (2015) α/β-Hydrolase domain-6 and saturated long chain monoacylglycerol regulate insulin secretion promoted by both fuel and non-fuel stimuli. Mol Metab 4:940–950.

RESPONSE LETTER

Reviewer #1 (Remarks to the Author):

The authors did an excellent job of addressing my questions.

We thank the reviewer for the re-evaluation and positive comment.

Reviewer #2 (Remarks to the Author):

The authors have addressed most of my concerns, however their response to concerns #2 and #3 are unfortunately incorrect, unsatisfactory and will confuse our field.

Concern #2: “Cnr1 expression among striatal output neurons (Gokce et al., 2016, Cell Reports; Stanley et al., 2020, Neuron) with a lack of CB1R expression among MSNs of the NAc”. This statement is incorrect as their multiple studies show functional CB1R in MSN, both direct and indirect pathways, as they show themselves using electrophysiology. The topographic transcriptomic gradient data are not conclusive, most likely because of low sensitivity. Thus, my concern stands, and the authors need to address it: “The ABHD6-KO mediated downregulation of CB1R mRNA is unexpected and novel and should be explored further. Do the authors have an explanation of the mechanism by which a link exists between ABHD6 and CB1R expressions? Does it reduce CB1R expression in NAc interneurons, in output neurons or in both? A better understanding of these compensatory mechanisms will help interpret the electrophysiology results presented in Figure 4”.

We thank the reviewer for their re-evaluation and for the opportunity to clear ambiguity on this matter. We wish to clarify that we did not intend to imply that CB1R is not expressed in striatal MSNs. We unreservedly agree with the reviewer that such a statement would be incorrect and that multiple studies, including studies we cite, have demonstrated the importance of functional CB1R expressed in striatal MSNs (Uchigashima et al., 2007, *J Neurosci*; Horne et al., 2013, *Eur J Neurosci*; Mathur et al., 2013, *Nat Neurosci*; Davis et al., 2018, *PLoS One*; Bonm et al., 2021, *Eur J Neurosci*; Mariani et al., 2023, *Current Biology*).

The distinction we discussed in our previous response relates to the expression of CB1R amongst MSNs of the dorsal striatum versus nucleus accumbens (ventral striatum), the latter being the exclusive striatal region studied in our investigation. Our manuscript referred to anatomical substructure when discussing a lack of CB1R expression among MSNs of the NAc. For clarity, we have revised the manuscript text as follows:

Discussion, paragraph 7:
“Within the dorsal striatum, CB1R is prominently expressed within MSNs to mediate important behavioural and synaptic functions, including modulation of MSN-to-MSN lateral inhibition[32,40,45,119,120,121]. In contrast, CB1R is not expressed within MSNs of the murine NAc (ventral striatum), with GABAergic fast-spiking interneurons (FSIs) representing the exclusive cellular source of local CB1R expression within the NAc[39,41,42,52]. While parvalbumin-expressing NAc FSIs comprise a small minority of NAc cells, they mediate powerful feed-forward inhibition onto NAc MSNs[122]. This FSI-to-MSN feed-forward inhibition is negatively modulated by CB1R signalling on FSI terminals to MSNs[38,39,40,41,42,43].”

Beyond the transcriptomic data we discussed in our previous response (Savell et al., 2020, *Sci Adv*), we describe below the results of Winters et al., 2012 (*PNAS*), Wright et al., 2017 (*NPP*) and Yu et al., 2017 (*PNAS*) (hyperlinked) demonstrating that FSIs are the sole cell type in the NAc expressing CB1R:

Winters et al., 2012 (*PNAS*) utilized a bicistronic *Cnr1*-tdTomato knock-in strategy to identify CB1R-expressing cells, in which fluorescent tdTomato was expressed under the control of the endogenous *Cnr1* promotor. They confirmed the pattern of fluorescent cells faithfully phenocopied reported endogenous mesoscale CB1R expression within the striatum. Applying whole-cell patch-clamp electrophysiological recordings from fluorescence-identified neurons within the NAc, they reported that all recorded tdTomato-positive cells within the NAc were identified as fast-spiking interneurons (FSIs), which have electrophysiological characteristics that are dissociable from MSNs. Highlighting the implausibility that such an observation could occur by chance when recording from hundreds of NAc tdTomato neurons, FSIs are estimated to comprise a minority of less than 1% of striatal neurons (Tepper and Bolam, 2004, *Curr Opin Neurobiol*).

The study of Wright et al., 2017 (*NPP*) performed paired patch-clamp electrophysiological recordings from pairs of NAc MSNs or pairs of one MSN and one CB1R-expressing FSI in the NAc. This approach allowed for a comparison of MSN-to-MSN lateral inhibition and FSI-to-MSN feed-forward inhibition in the NAc. They reported that FSI-mediated feed-forward inhibition onto MSNs was significantly modulated by CB1R signalling, while MSN-to-MSN lateral inhibition was not affected by pharmacological manipulations of CB1R signalling. These results demonstrate the selectivity for CB1R pharmacological manipulations to modulate FSI-, but not MSN-, inhibitory synapses onto NAc MSNs.

Yu et al., 2017 (*PNAS*) corroborated the observation that CB1R-expresing neurons in the NAc are exclusively FSIs and confirmed that FSI-to-MSN plasticity is modulated by CB1R signalling using electrophysiology.

The data from these three studies are in agreement with the transcriptomic data that FSIs represent the exclusive known source of local CB1R expression the NAc, in marked contrast to the dorsal striatum.

Concern #3: “we did not add BSA to the WIN preparation”. The authors are correct that BSA will prevent loss of the agonists, which is crucial to prevent up to 98% loss of cannabinoid agonists as best demonstrated in this publication (https://pubmed.ncbi.nlm.nih.gov/15209515/) and well known by electrophysiologist studying cannabinoid agonists. Thus, my concern stands, and the authors need to address it: “WIN was tested at 10 uM and produced small responses, including when measuring mIPSC amplitudes. The authors need to perform a WIN concentration-dependent dose response by adding 0.1% BSA in their perfusion media, to reduce WIN binding to plastics and as do most slice electrophysiology studies”. Thus, experiment is justified by the fact that WIN activates multiple non-CB1R target when applied a high micromolar concentrations and should already fully activate CB1R at 30 nM.

We fully acknowledge that many scientists in the cannabinoid field add BSA to WIN preparations and other CB1R agonists to prevent loss of agonists. We appreciate the reviewer referring us to an article demonstrating the importance of BSA addition to anandamide to prevent sticking to plastics and loss of agonists. We were not aware of this at the time of the experiments. The laboratory of Dr. David Stellwagen that completed these experiments is accustomed to preparing proteins with BSA for bath slice perfusion, but prepared WIN without BSA according to

manufacture instructions and in consideration that it is a small molecule. We elected to perform our WIN electrophysiological experiments at 10uM as this is a commonly used dose (e.g., Manzoni and Bockaert, 2001, *Eur J Pharmacol*; Robbe et al., 2001, *J Neurosci*; Patwardhan et al., 2006, *PNAS*; Flores-Otero et al., 2014,*Nature Communications*; Geddes et al., 2016, *PNAS*; Bridi et al., 2019, *Neuron*; Patzke et al., 2021, *Molecular Psychiatry*; Bohmbach et al., 2022, *Nature Communications*, Nufer et al., 2023, *NPP*; Shang et al., 2023, *NPP*).

We have added the following text to the Discussion to assert the limitations in our study:

Discussion, paragraph 9:
"Our electrophysiological experiments with WIN55,212-2 were performed in the absence of BSA, which may well underestimate the magnitude of CB1R stimulation[163,164]. Additionally, it is unclear if differences we observed in response to WIN are due to CB1R activation as the 10uM dose of WIN applied may activate non-CB1R targets [165,166]."

Performing extra experiments to characterize the influence of WIN across a range of doses would provide additional information. However, such experiments would require us to revive the mouse colony, perform AAV injection surgeries and ship mice to the lab of Dr. Stellwagen. Recordings would not be possible before October. With this in mind, we sincerely hope that the reviewer understands why we wish to avoid carrying out these costly experiments which are not central the main results and message of the manuscript.

Our key findings demonstrate that ABHD6 loss-of-function in NAc neurons produces behavioral and metabolic effects (that are seemingly paradoxical and opposing to our VTA pan-neuronal and DA neuron ABHD6 deletions) that inhibit food-seeking and increase energy expenditure to prevent weight gain. These effects can be explained by the reduced inhibitory synaptic inputs onto MSNs that we observed. The aim of our electrophysiological experiments with WIN was to evaluate the impact of exogenous cannabinoid receptor stimulation on inhibitory synaptic input to MSNs of the NAc from Abhd6$^{NAc\ KO}$ and Abhd6$^{NAc\ GFP}$ mice. To this end, our results demonstrate that WIN application at 10uM in the absence of BSA: 1) significantly attenuated eIPSC amplitude and mIPSC frequency and amplitude (Two-way ANOVA main effect Time $P<0.0001$ and paired t-tests (Pre-WIN baseline vs. post-WIN) $P<0.05$ for all comparisons), and 2) reduce mIPSC frequency onto NAc MSNs from ABHD6$^{NAc\ KO}$ mice to a significantly lower extent relative to ABHD6$^{NAc\ GFP}$ controls (Figure 4h).

Reviewer #3 (Remarks to the Author):

The authors have satisfactorily addressed this reviewer's concerns.

We thank the reviewer for their re-evaluation and approval.

References

Bohmbach K, Masala N, Schönhense EM, Hill K, Haubrich AN, Zimmer A, Opitz T, Beck H, Henneberger C (2022) An astrocytic signaling loop for frequency-dependent control of dendritic integration and spatial learning. Nat Commun 13:7932.

Bonm AV, Elezgarai I, Gremel CM, Viray K, Bamford NS, Palmiter RD, Grandes P, Lovinger DM, Stella N (2021) Control of exploration, motor coordination and amphetamine sensitization by cannabinoid CB_1 receptors expressed in medium spiny neurons. Eur J of Neuroscience 54:4934–4952.

Bridi MCD, Zong F-J, Min X, Luo N, Tran T, Qiu J, Severin D, Zhang X-T, Wang G, Zhu Z-J, He K-W, Kirkwood A (2020) Daily Oscillation of the Excitation-Inhibition Balance in Visual Cortical Circuits. Neuron 105:621-629.e4.

Davis MI, Crittenden JR, Feng AY, Kupferschmidt DA, Naydenov A, Stella N, Graybiel AM, Lovinger DM (2018) The cannabinoid-1 receptor is abundantly expressed in striatal striosomes and striosome-dendron bouquets of the substantia nigra Lee J, ed. PLoS ONE 13:e0191436.

Flores-Otero J, Ahn KH, Delgado-Peraza F, Mackie K, Kendall DA, Yudowski GA (2014) Ligand-specific endocytic dwell times control functional selectivity of the cannabinoid receptor 1. Nat Commun 5:4589.

Geddes SD, Assadzada S, Lemelin D, Sokolovski A, Bergeron R, Haj-Dahmane S, Béïque J-C (2016) Target-specific modulation of the descending prefrontal cortex inputs to the dorsal raphe nucleus by cannabinoids. Proc Natl Acad Sci USA 113:5429–5434.

Gokce O, Stanley GM, Treutlein B, Neff NF, Camp JG, Malenka RC, Rothwell PE, Fuccillo MV, Südhof TC, Quake SR (2016) Cellular Taxonomy of the Mouse Striatum as Revealed by Single-Cell RNA-Seq. Cell Reports 16:1126–1137.

Horne EA, Coy J, Swinney K, Fung S, Cherry AET, Marrs WR, Naydenov AV, Lin YH, Sun X, Dirk Keene C, Grouzmann E, Muchowski P, Bates GP, Mackie K, Stella N (2013) Downregulation of cannabinoid receptor 1 from neuropeptide Y interneurons in the basal ganglia of patients with Huntington's disease and mouse models. Eur J of Neuroscience 37:429–440.

Manzoni OJ, Bockaert J (2001) Cannabinoids inhibit GABAergic synaptic transmission in mice nucleus accumbens. Eur J Pharmacol 412:R3-5.

Mariani Y, Covelo A, Rodrigues RS, Julio-Kalajzić F, Pagano Zottola AC, Lavanco G, Fabrizio M, Gisquet D, Drago F, Cannich A, Baufreton J, Marsicano G, Bellocchio L (2023) Striatopallidal cannabinoid type-1 receptors mediate amphetamine-induced sensitization. Current Biology 33:5011-5022.e6.

Maroto IB et al. (2023) Control of a hippocampal recurrent excitatory circuit by cannabinoid receptor-interacting protein Gap43. Nat Commun 14:2303.

Mathur BN, Tanahira C, Tamamaki N, Lovinger DM (2013) Voltage drives diverse endocannabinoid signals to mediate striatal microcircuit-specific plasticity. Nat Neurosci 16:1275–1283.

Nufer TM, Wu BJ, Boyce Z, Steffensen SC, Edwards JG (2023) Ethanol blocks a novel form of iLTD, but not iLTP of inhibitory inputs to VTA GABA neurons. Neuropsychopharmacology 48:1396–1408.

Patwardhan AM, Jeske NA, Price TJ, Gamper N, Akopian AN, Hargreaves KM (2006) The cannabinoid WIN 55,212-2 inhibits transient receptor potential vanilloid 1 (TRPV1) and evokes peripheral antihyperalgesia via calcineurin. Proc Natl Acad Sci U S A 103:11393–11398.

Patzke C, Dai J, Brockmann MM, Sun Z, Fenske P, Rosenmund C, Südhof TC (2021) Cannabinoid receptor activation acutely increases synaptic vesicle numbers by activating synapsins in human synapses. Mol Psychiatry 26:6253–6268.

Robbe D, Alonso G, Duchamp F, Bockaert J, Manzoni OJ (2001) Localization and mechanisms of action of cannabinoid receptors at the glutamatergic synapses of the mouse nucleus accumbens. J Neurosci 21:109–116.

Savell KE, Tuscher JJ, Zipperly ME, Duke CG, Phillips RA, Bauman AJ, Thukral S, Sultan FA, Goska NA, Ianov L, Day JJ (2020) A dopamine-induced gene expression signature regulates neuronal function and cocaine response. Sci Adv 6:eaba4221.

Shang H, Li P, Lin X, Cai Q, Li Z, Deng L, Song Y, Chen J-F, Zhou J (2023) Neuronal and astrocytic CB1R signaling differentially modulates goal-directed behavior and working memory by distinct temporal mechanisms. Neuropsychopharmacology 48:1520–1531.

Stanley G, Gokce O, Malenka RC, Südhof TC, Quake SR (2020) Continuous and Discrete Neuron Types of the Adult Murine Striatum. Neuron 105:688-699.e8.

Tepper JM, Bolam JP (2004) Functional diversity and specificity of neostriatal interneurons. Current Opinion in Neurobiology 14:685–692.

Uchigashima M, Narushima M, Fukaya M, Katona I, Kano M, Watanabe M (2007) Subcellular Arrangement of Molecules for 2-Arachidonoyl-Glycerol-Mediated Retrograde Signaling and Its Physiological Contribution to Synaptic Modulation in the Striatum. J Neurosci 27:3663–3676.

Winters BD, Krüger JM, Huang X, Gallaher ZR, Ishikawa M, Czaja K, Krueger JM, Huang YH, Schlüter OM, Dong Y (2012) Cannabinoid receptor 1-expressing neurons in the nucleus accumbens. Proc Natl Acad Sci USA 109 Available at: https://pnas.org/doi/full/10.1073/pnas.1206303109 [Accessed June 20, 2024].

Wright WJ, Schlüter OM, Dong Y (2017) A Feedforward Inhibitory Circuit Mediated by CB1-Expressing Fast-Spiking Interneurons in the Nucleus Accumbens. Neuropsychopharmacol 42:1146–1156.

Yang B, Sanches-Padilla J, Kondapalli J, Morison SL, Delpire E, Awatramani R, Surmeier DJ (2021) Locus coeruleus anchors a trisynaptic circuit controlling fear-induced suppression of feeding. Neuron 109:823-838.e6.

Yu J, Yan Y, Li K-L, Wang Y, Huang YH, Urban NN, Nestler EJ, Schlüter OM, Dong Y (2017) Nucleus accumbens feedforward inhibition circuit promotes cocaine self-administration. Proc Natl Acad Sci USA 114 Available at: https://pnas.org/doi/full/10.1073/pnas.1707822114 [Accessed June 20, 2024].