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Npas4 regulates medium spiny neuron physiology and gates cocaine-induced hyperlocomotion

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Thank you for the transfer of your manuscript to EMBO reports. We have now received the enclosed referee reports.

As you will see, the referees acknowledge that the findings are potentially interesting. However, they also point out that significant revisions will be required for consideration of the manuscript for publication here. Given that you think that you can address all concerns, I am happy to invite you to revise your study along the lines suggested by the referees.

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Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further.

Regarding data quantification, please specify the number "n" for how many independent experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

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- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

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If your study has not produced novel datasets, please mention this fact in the Data Availability Section.

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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Kind regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

In this study, the authors investigate the immediate early gene Npas4 in regards to its role in response to cocaine as well as the effect in structural synaptic plasticity and behavior. They also show some molecular and cellular evidence in vitro about the pathways and molecular targets that participate in the upstream and downstream signaling of this transcription factor.

General comment:

The order of the experiments and the story behind the manuscript is not clear. It needs to be reorganized in order to tell a good story that is clear for the reader and make sense with the general hypothesis, as well as be careful with the conclusions from in vitro studies that lead the authors to design the follow up in vivo experiments. It seems from the introduction that the authors are interested in the D1R-MSNs, Npsa4 and the effect on Cocaine rewarding. The title is not taking the 'full picture' of what is showed in the manuscript. It looks to me that there are experiments that doesn't help to probe what they want to probe.
For instance, MSNs in vitro experiments are based on enriched D1-MSNs cultures and D1-agonists following the evidence that D1R-expressing MSNs are involved in the effect of Cocaine in locomotion activity. However, the effect of Npas4 knock-down in vivo after chronic Cocaine administration was studied specifically in D1R-MSNs for structural synaptic plasticity (Figure 5) but no specific knock-down in D1R-MSNs was used to study the behavioral effect in locomotion (Figure 7). Therefore, it is not possible

to corelate behavioral data (D1+D2) with spine density data (only D1). In the same page, electrophysiological data from naïve animals were collected from both D1-positive and D1-negative cells (Figure 6 and Supplementary Table 1). Apparently, there are no differences between the 2 populations and ephys data showed are pooled from both D1 and D2-MSNs. In this scenario the effect of Npas4 knock-down in decreasing spine density could be observed in D2-MSNs also. Furthermore, the authors should have included data from D2-MSNs relative to spine density to answer the question if Npas4 regulate synaptic structural plasticity of only D1 or both D1 and D2?

Specific comments:

1) IEGs: The administration of cocaine in home cage can induce lower levels of IEGs as c-fos in both dorsal Striatum and Nucleus Accumbens, compared with novel environment (see for instance Uslaner et al., Brain Res 2001). In addition, the effect observed might be more probably driven for the dopaminergic inputs than glutamatergic inputs to the NAc MSNs. The lack of Npas4-positive cells in dorsal Striatum might be due to home cage administration. A lower activation of the glutamatergic inputs to the Striatum when cocaine is administered in home cage could explain that differential regional activation. In that sense, additional experiment to show Fos or FosB expression would help to clarify if Striatum-differential Npas4 expression after chronic cocaine injection is specific of this transcription factor or is influenced by the context (Home Cage).
2) There is evidence of membrane depolarization effect over Npas4 induction but no D1R-mediated effect (Figure 2). It doesn't make sense the sentence "...with Fosb falling in the middle of these to group". Fosb mRNA induction is more than 1 order of magnitude higher than Npas4 in Figure 2d (1000 vs 60, see scale). Based on Figure 2d, the HiK/SKF in Figure 2e don't make sense either as well as the statement "...Npas4 has significantly higher value than all other IEGs analysed (Figure 2e)". Please correct and explain.

3) cFos mRNA induction is measured at 2 h (Figure 2c) but not at 1 h what is the maximum induction for the other IEGs analyzed. It should be a similar time point for cFos added to Figure 2d to be able to compare with Npas4 induction. Similarly, Fosb but not cFos, one of the most classical and used IEGs markers of strong activation, is missing in Figure 3 for comparison with other IEGs. Please explain why is excluded from the analysis.

4) It is not clear and is confusing the fact that transcriptome microarray analysis is performed using hippocampal instead of MSNs neurons as the other in vitro experiments. What is the relevance of those finding and how can be extrapolated to the main brain region/neurons that are in the objective of the manuscript (NAc/MSNs)? For instance, the rational for the experiment of spine density in vitro in Figure 4 it seems is based in the results of the transcriptome analysis in hippocampal neuron as can be read in the Result section.

5) Although spine density changes induced by Npas4 knock-down are interesting, the strength of the synapses and therefore the effect on synaptic activity can be more related with parameters such as spine head morphology (diameter or volume of the head or percentage of mushroom-like spines). That data can be analyzed from the images they have acquired, and it would add more interest to the field.

Referee #2:

This manuscript from Lissek and colleagues asserts that cocaine induces Npas4 expression in the NAc, which, in turn, regulates dendritic spine density, alters MSN excitability, and drives cocaine locomotor sensitization. With a thorough set of pharmacologic experiments in striatal culture and human IPSCs, the authors also show nuclear calcium, but not traditional kinases, controls Npas4 expression. By combining Npas4 overexpression with transcriptomics, they find Npas4 influences expression of a wide variety of genes involved in synaptic structure and function. They then confirm Npas4 contributes to structural changes to MSNs with dendritic spine analysis, and functional changes to electrophysiological properties through whole cell electrophysiology. Finally, they demonstrate that npas4 knockdown blunts locomotor sensitization to cocaine, presumably by acting on the established structural and functional mechanisms they have established. Overall, the manuscript is well-written and is quite thorough. While I think this work will be of great interest to the field, there are some major issues that must be addressed prior to publication.
Major Concerns
1. The title of the manuscript is too general for the tested hypotheses. The authors show Npas4 contributes to cocaine locomotor sensitization, not responsiveness to multiple rewarding stimuli. "striatal network function" is also too strong, given that the focus of the manuscript is on the nucleus accumbens, the transcriptomics are in hippocampal cultures, and there is no direct assessment of network function outside of the ex vivo slice electrophysiology.
2. The sex of the mice is not reported anywhere in the manuscript text. There are known differences in drug-abuse liability between males and females (mice, rats, humans) and this should be specified, and justified if only in a single sex.
3. There are no F stats reported anywhere in the text or figure legends. Please address.
4. In the in vitro experiments, the authors nicely demonstrate that depolarization and nuclear calcium drive Npas4 induction, not dopamine. But in mice, Npas4 expression is increased only in the NAc (not DStr) upon administration of cocaine. The cultured MSNs are generated from both dorsal and ventral striatal neurons. It is possible that DStr MSNs require different stimuli to induce Npas4 than NAc MSNs. To link the results of the cultured MSN experiments to the conditions in vivo and rule out any

"dilution effects" of DStr vs NAc originating MSNs, the authors need to stimulate the cultured MSNs with cocaine and demonstrate induction of Npas4 in the presence of SCH, and/or attenuated Npas4 induction with cocaine + Cyclosporin/FK506 or KN93.
5. The in silico analysis of Npas4 binding motifs and increased expression of gene targets after Npas overexpression (Nptk2, etc) does suggest that Npas4 binds to the promotors to control transcription. However, Npas4 overexpression also alters expression of other genes with no apparent binding motif (e.g. grin2a). To say definitively that Npas4 controls transcription of these genes (Nptk2, etc) the authors need to show Npas4 enrichment on these specific regions with chromatin immunoprecipitation or similar.
6. Several of the electrophysiological properties appear to be at odds with a wealth of literature reporting intrinsic properties of medium spiny neurons (e.g. the rheobase is much lower, the resting membrane potential is much more depolarized than many other reports---65 vs -80 -for examples see PMIDs 18945889, 18279319, 30899778) It is also surprising that there were no significant differences in rheobase between D1+ and D1- (putative D2-MSNs) (see PMIDs 18945889, 18279319, 23469183, 29975170). Please reconcile these differences with the literature.
7. Physiological effects of Npas4 knockdown appear to be driven by the D1(-) neurons in Suppl table 1 (rheobase, firing rate). Why are the neuron subtypes pooled in the main figure? If the effects are driven in D1(-) neurons, why were dendritic spines quantified only in D1(+) neurons? Put another way, if the neuron subtypes are to be pooled for the ephys experiments, why are D1(-) neurons excluded from dendritic spine analysis?
Other Concerns
1. Fig 1c Y axis- Is this also normalized to the number of NeuN positive cells? How many slices were counted from each mouse?
2. What is the p value for Fig 3b, Arc, +HiK, +TTX + verapamil. It is difficult to believe it is not statistically significant.
3. Fig 4e. Please specify if Npas4 overexpression alone (without HiK) causes the resulting upregulation in gene expression.
4. Fig 4f y axis- is 'mRNA level' fold change vs control?
5. Fig 5d- Were all GFP+ cells counted, or does this represent a subset? How many cells (or images) were counted per mouse?
6. By eye, Npas4 knockdown seems to reduce the number of dendritic spines with larger heads in Fig 5f. How does Npas4 knockdown impact different MSN spine types (I.e. thin, stubby, mushroom). This information should be easy to obtain since the authors indicate they used neuronstudio to count dendritic spines, which automatically classifies spine type.
7. For dendritic spine density--How many dendrites from how many cells per mouse? Were these secondary dendrites?

8. Fig 7b. Please provide units for the Y axis
9. Supplemental Table 1 Nasp instead of Npas. Please correct.
10. Please specify if independent cultures are from independent mice, and specify % of neurons that are positive for DARPP32 in addition to representative images.

11. In the methods, please specify which samples are analyzed via Taqman vs traditional SybrGreen
12. The locomotor testing appears to be called "cyclotron" in several figures, but is not referenced in the methods. Please either specifiy the locomotor sensitization device is called a "cyclotron" or change the figure timelines to read "locomotion"

13. References to "in vivo" MSN spine density is confusing, since spine density was determined in fixed brain slices, and not in an intact animal (say with 2p imaging, e.g.). Please remove "in vivo" to improve clarity.
14. Locomotor sensitization does not equal drug-abuse liability. References to "how responsive an animal is to cocaine" or "behavioral sensitivity to drugs of abuse" is too general, as the only behavioral test employed is cocaine locomotor sensitization (and not conditioned place preference or self-administration).
15. Please specify when MSNs are from striatal culture as opposed to in brain slices throughout the text.
16. The phrase "synapse to nucleus cascade" is inappropriate, since striatal cultured MSNs don't have the same glutamatergic inputs as in an intact brain, or even brain slice, (unless they are corticostriatal cultures, in which case, please clarify).
17. iPSC's are not human neurons. Please change the language on pg 10 "whether differential inducibility of Npas4 is conserved in human neurons."

18. Pg 20. "Npas4 induction is hence tightly coupled to membrane depolarization, which enables the cell to discriminate different circuit inputs." This was not tested. Please remove.

19. Pg 22. "What is especially intriguing about Npas4 as a treatment target is that changing Npas4 levels leaves basal locomotor behaviour unchanged, as shown in or study, implying the possibility of targeted interventions with minimal side-effects on other behaviour." This is entirely too strong and an ill-supported statement. Please remove.

20. Verapamil has also been shown to inhibit voltage-gated K+ channels. Please comment on this possibility.

Referee #3:

In this manuscript, Lissek et al. provide evidence that Npas4 uniquely controls baseline striatal physiology and transcriptional networks essential for responsiveness to cocaine. Overall, this is a wide-ranging study including transcriptional analysis, slice physiology, ca imaging and behavioral approaches. These data are generally well-executed, the data analysis is largely

appropriate, and the manuscript is well written with clear figures. In particular, Fig3 is an excellent investigation of the signaling pathways the induce Npas4 activity. Nevertheless, there are some remaining issues that should be done to strengthen these data prior to publication:

Major Points:

1. the use of organoids to complement the striatal cultures is viewed as a positive, however, what exactly are the identity of these "neurons"? Forebrain FoxG1+ neurons encompass all telencephalic neurons - does this imply that the authors see such clear Npas4 regulation because the same upregulation is happening in cortical neurons as well as striatal? Are there striatal neurons in this preparation?

2. regarding the transcriptional analyses, it would be good if the same candidates were shown in both hippocampal and striatal O/E experiments - these do not need to be identical but would help give a picture of the core transcriptional components regulated in O/E. In addition, it may be more meaningful to focus on the clear, consistent specific results (GRIN1; GRIN2b; Cacna1d) rather than focusing on GO groups that are vague.

3. furthermore, given the suggestion that Npas4 controls transcription in adults, the transcriptional data would be strengthened by validating some of these results in mice by qPCR from NAc tissue following NPas4 KD.

4. was the data for Fig4d done in a systematic way? or are these promoter elements anectodal in their description?

5. in general, in addition to the posthoc tests, the results of the ANOVAs should be included as supplemental materials. For example, in Fig.5F, is the interaction term of the 2w ANOVA significant or is there just a main effect of NPas4-kd on spine density - these are two very distinct outcomes. one supports a role in cocaine-induced plasticity, the other in general synaptogenesis.

6. given prior publications of SPN subtype-specific differences in both NAc (Greuter, NN 2010) and DStr (Kreitzer, Nature 2007), it seems problematic to lump together all recorded neuronal subtypes, particularly given that the authors used a viral tool to distinguish cell types. Do these physiological phenotypes hold up when comparing neurons of the same subtype?

7. what is the explanation for the increased firing rate given the other changes indicate a reduction in excitability?

8. For the behavioral figure, the potential effects on basal locomotion seem important for determining the specificity of NPas4 effects for cocaine or just locomotion in general. Along those lines: a) is basal locomotion calculated the same way as the movement in 7C? why is the variability so large? b) is there an effect of the shRNA on the day prior to cocaine administration in the group that will receive cocaine (7C, last two bars in 0d time group)?

Minor Points:

1. given the effects of NMDAR blockade on Npas4 induction, it seems likely that the striatal cultures have a reasonable number of glutamatergic neurons - this should be quantified in some way and mentioned b/c otherwise these effects are confusing.

2. "This decreased facilitation indicates an increase in c-str transmission" should be softened as this single assay suggests an increase in release probability (although in isolation, it could also reflect changes to AMPAR desensitization).

3. In Figure 1, details of quantification such as animal number, images collected per animal seem to be missing.

4. In Figure 3h co-application of HiK and SKF had no additional effect on Npas4 induction, leading the authors to conclude that there is no crosstalk between Ca2+ and D1 related signaling. This seems at odds with Figure 3e in which they show that inhibition of PKA by H-89 (a downstream effector of D1R signaling) increases Npas4 induction. The authors conclude that this result indicates negative regulation of Ca2+ Npas4 induction by PKA. To me this seems like crosstalk between the two pathways.

5. In Figure 5d, I am unclear on how the exact measure being displayed. It looks to be normalized so that the control condition is set to 100. If so this should be indicated for clarity. If the data is a raw percentage of Npas4+/GFP+ cells, this seems at odds with the images shown.

6. In Figure 5d, the figure legend says that 3 mice per group were used, but does not detail how many images were taken per mouse, or whether data is displayed with each image as a data point or with data from each mouse averaged together.

7. In Figure 5e, information about the number of animals, and number of cells imaged seems to be missing. Additionally, the figure legend says that normalization was done "to 10 um of dendrite for saline control and after cocaine injection". If this means that only 10 um of control dendrite was used for normalization (as opposed to the data simply being displayed as spines/10 um), this is not a large enough segment of dendrite to be taken as representative. Usually at least 50 um is counted.

8. In the Discussion section, suggestions of Npas4 as a potential drug target should be toned down. The data presented indicating that knockdown of Npas4 does not have non-specific effects on behavior is quite limited and manipulating the expression and/or function of a master transcriptional regulator is not likely to be a viable therapeutic strategy.

9. The authors mention that their results "imply that it (Npas4) controls overall stimulus-independent and -dependent synaptic input onto MSNs". This seems to be an overgeneralization. The data-presented show that Npas4 regulates spine density independently of experimentally induced stimuli (ex. Cocaine treatment) only. This does not rule out naturally occurring stimuli which are not being controlled for in this study.

Author's response – Lissek et al, 2021, EMBOR-2020-51882-T

We thank the reviewers for their valuable comments and suggestions. We have performed a substantial amount of additional experiments and analyses, including spine density assessments in D2-MSNs (Figure 5h, i), slice stainings for Fos after cocaine treatment (Expanded View Figure 1), as well as analysis of spine morphology through spine head diameter assessment (Expanded View Figure 2). We have also performed revisions to the manuscript and figures in line with the referees' suggestions. We have addressed all points and hope that the reviewers share our enthusiasm about this revised version of our study, with an even stronger impact.

Referee #1:

In this study, the authors investigate the immediate early gene Npas4 in regards to its role in response to cocaine as well as the effect in structural synaptic plasticity and behavior. They also show some molecular and cellular evidence in vitro about the pathways and molecular targets that participate in the upstream and downstream signaling of this transcription factor.

General comment:

The order of the experiments and the story behind the manuscript is not clear. It needs to be reorganized in order to tell a good story that is clear for the reader and make sense with the general hypothesis, as well as be careful with the conclusions from in vitro studies that lead the authors to design the follow up in vivo experiments. It seems from the introduction that the authors are interested in the D1R-MSNs, Npsa4 and the effect on Cocaine rewarding. The title is not taking the 'full picture' of what is showed in the manuscript.

Response:
We have reformulated the title to "Npas4 regulates medium spiny neuron physiology and gates cocaine-induced hyperlocomotion", which makes it a more specific description of the study's findings and also addresses Referee 2's Major Point 1. We have also made the abstract more specific by rephrasing the first sentence to: "This study identifies the transcription factor Npas4 as a strong regulator of medium spiny neuron spine density and electrophysiological parameters that determine the magnitude of cocaine-induced hyperlocomotion in mice." We have enhanced the clarity and preciseness of the phrasings throughout the text in line with the reviewers' suggestions (see also answers to other reviewers below).

It looks to me that there are experiments that doesn't help to probe what they want to probe.
For instance, MSNs in vitro experiments are based on enriched D1-MSNs cultures and D1-agonists following the evidence that D1R-expressing MSNs are involved in the effect of Cocaine in locomotion activity. However, the effect of Npas4 knock-down in vivo after chronic Cocaine administration was studied specifically in D1R-MSNs for structural synaptic plasticity (Figure 5) but no specific knock-down in D1R-MSNs was used to study the behavioral effect in locomotion (Figure 7). Therefore, it is not possible to corelate behavioral data (D1+D2) with spine density data (only D1). In the same page, electrophysiological data from naïve animals were collected from both D1-positive and D1-negative cells (Figure 6 and Supplementary Table 1). Apparently, there are no differences between the 2 populations and ephys data showed are pooled from both D1 and D2-MSNs. In this scenario the effect of Npas4 knock-down in decreasing spine density could be observed in D2-MSNs also. Furthermore, the authors should have included data from D2-MSNs relative to spine density to answer the question if Npas4 regulate synaptic structural plasticity of only D1 or both D1 and D2?

Response:

We agree with the points regarding the differentiation of D1- and D2-MSNs and have addressed these with additional experiments. We performed shRNA-mediated knockdown of Npas4 in the striatum and stained D2-MSNs via ppENK-selective tdTom expression (Fig. 5h, i). In line with the literature, D2-MSNs did not show a cocaine-dependent increase in spine density, and Npas4-knockdown has no effect on spine density in this neuronal population. Regarding the non-specific knockdown, it is true that the U6-promoter driven shRNA expression does not differentiate between D1- and D2-MSNs. Because spine adaptations happen in D1-MSNs and D1-MSNs have previously been shown to be the driver of hyperlocomotion after cocaine exposure, we deem it most likely that the effects are driven by Npas4 reduction in these neurons.

Specific comments:

1) IEGs: The administration of cocaine in home cage can induce lower levels of IEGs as c-fos in both dorsal Striatum and Nucleus Accumbens, compared with novel environment (see for instance Uslaner et al., Brain Res 2001). In addition, the effect observed might be more probably driven for the dopaminergic inputs than glutamatergic inputs to the NAc MSNs. The lack of Npas4-positive cells in dorsal Striatum might be due to home cage administration. A lower activation of the glutamatergic inputs to the Striatum when cocaine is administered in home cage could explain that differential regional activation. In that sense, additional experiment to show Fos or FosB expression would help to clarify if Striatum-differential Npas4 expression after chronic cocaine injection is specific of this transcription factor or is influenced by the context (Home Cage).

Response
This is an excellent suggestion and we have performed an additional staining for Fos (Expanded View Figure 1). We find that Fos is induced in both the NAc and dStr which stands in marked contrast to Npas4's induction in only the NAc. We thus rule out that the lack of Npas4 induction in the dStr is due to a broadly weaker IEG induction, since we observe robust Fos induction in the dStr. Npas4 induction is hence more restricted, something we subsequently also find with regard to different stimulation protocols in *in vitro* MSNs and iPSC-derived organoids (Fig. 2).

2) There is evidence of membrane depolarization effect over Npas4 induction but no D1R-mediated effect (Figure 2). It doesn't make sense the sentence "...with Fosb falling in the middle of these to group". Fosb mRNA induction is more than 1 order of magnitude higher than Npas4 in Figure 2d (1000 vs 60, see scale). Based on Figure 2d, the HiK/SKF in Figure 2e don't make sense either as well as the statement "...Npas4 has significantly higher value than all other IEGs analysed (Figure 2e)". Please correct and explain.

Response:
The statement "...with Fosb falling in the middle of these two groups" refers to the induction ratios between the two stimuli HiK and SKF (Ind_{HiK}/Ind_{SKF} in Fig 2e). It measures a relative difference between HiK and SKF induction values for the same gene, hence the absolute fold induction values compared to baseline do not matter. Regarding the HiK/SKF ratio, Fosb ($Ind_{HiK}/Ind_{SKF} = 4$) does fall in the middle of Npas4 ($Ind_{HiK}/Ind_{SKF} = 8$) and the other genes ($Ind_{HiK}/Ind_{SKF} = 2$) as also seen in the bar graph in Fig. 2e.

3) cFos mRNA induction is measured at 2 h (Figure 2c) but not at 1 h what is the maximum induction for the other IEGs analyzed. It should be a similar time point for cFos added to Figure 2d to be able to compare with Npas4 induction. Similarly, Fosb but not cFos, one of the most classical and used IEGs markers of strong activation, is missing in Figure 3 for comparison with other IEGs. Please explain why is excluded from the analysis.

Response:
The cFos mRNA measurement at 2h in Fig. 2c was done at a very early stage of the study to confirm D1-selectivity of the agonist. We needed a robust marker for activity-dependent transcription. As the reviewer mentions, cFos is such a ubiquitous marker for activity-induced transcription and hence a safe choice for an initial assessment. From previous experiments in hippocampal cells with different stimuli we knew that 2h is a timepoint where cFos is upregulated under most conditions. Going forward, we decided to use FosB rather than cFos as these two IEGs usually have extremely similar induction patterns and mechanisms but Fosb is a lot more relevant to the addiction field. The 1h time-point was chosen for these experiments because the observations in Fig. 2 revealed that this is the time-point of maximal IEG induction in MSNs after HiK stimulation.

4) It is not clear and is confusing the fact that transcriptome microarray analysis is performed using hippocampal instead of MSNs neurons as the other in vitro experiments. What is the relevance of those finding and how can be extrapolated to the main brain region/neurons that are in the objective of the manuscript (NAc/MSNs)? For instance, the rational for the experiment of spine density in vitro in Figure 4 it seems is based in the results of the transcriptome analysis in hippocampal neuron as can be read in the Result section.

Response:
The reason for using hippocampal neurons was that these neurons allowed us to perform microarray analysis. Striatal neuron cultures generally yield much lower RNA amounts that sufficed for qPCR based quantifications but not for microarray analysis. According to (Spiegel et al., 2014), Npas4 has a core target gene set that is conserved in various neuron types. Since we subsequently confirmed and investigated interesting gene groups, such as NMDAR subunit genes (Grin1, Grin2a, Grin2b) in striatal neurons via qPCR, we see this approach as justified. The approach of screening in experimentally more amenable cell types to subsequently confirm in a more specific manner is used often in the field.

5) Although spine density changes induced by Npas4 knock-down are interesting, the strength of the synapses and therefore the effect on synaptic activity can be more related with parameters such as spine head morphology (diameter or volume of the head or percentage of mushroom-like spines). That data can be analyzed from the images they have acquired, and it would add more interest to the field.

Response:
This is an excellent suggestion and we have performed this additional analysis (Extended View Fig. 2). We anaylzed spine head diameter for D1- and D2-MSNs from the dataset from Fig. 5 and plotted the results as a cumulative distribution. We find no significant impact of Npas4 knockdown on spine morphology.

Referee #2:
This manuscript from Lissek and colleagues asserts that cocaine induces Npas4 expression in the NAc, which, in turn, regulates dendritic spine density, alters MSN excitability, and drives cocaine locomotor sensitization. With a thorough set of pharmacologic experiments in striatal culture and human IPSCs, the authors also show nuclear calcium, but not traditional kinases, controls Npas4 expression. By combining Npas4 overexpression with transcriptomics, they find Npas4 influences expression of a wide variety of genes involved in synaptic structure and function. They then confirm Npas4 contributes to structural changes to MSNs with dendritic spine analysis, and functional changes to electrophysiological properties through whole cell electrophysiology. Finally, they demonstrate that npas4 knockdown blunts locomotor sensitization to cocaine, presumably by acting on the established structural and functional mechanisms they have established. Overall, the manuscript is well-written and is quite thorough. While I think this work

will be of great interest to the field, there are some major issues that must be addressed prior to publication.

Major Concerns

1. The title of the manuscript is too general for the tested hypotheses. The authors show Npas4 contributes to cocaine locomotor sensitization, not responsiveness to multiple rewarding stimuli. "striatal network function" is also too strong, given that the focus of the manuscript is on the nucleus accumbens, the transcriptomics are in hippocampal cultures, and there is no direct assessment of network function outside of the ex vivo slice electrophysiology.

Response:

We have amended the title to “Npas4 regulates medium spiny neuron physiology and gates cocaine-induced hyperlocomotion”. We believe that this is now more precise as the in vitro, ex vivo and in vivo experiments investigate MSN physiological parameters (i.e. transcriptome changes, spine parameters and electrophysiological properties), while the behavioural analysis is focused on cocaine induced hyperlocomotion, as suggested by the reviewer.

2. The sex of the mice is not reported anywhere in the manuscript text. There are known differences in drug-abuse liability between males and females (mice, rats, humans) and this should be specified, and justified if only in a single sex.

Response*:*

We agree that this information is important and have included it in the manuscript. We now specify in the methods section that we used male mice in the present work. Responses observed in some preclinical models of addiction or other psychiatric diseases revealed sex differences in behavioral adaptations. In the present study, we chose male mice for two reasons. Firstly, most of the papers studying non-operant addiction paradigms used males, including in our group (Pascoli et al., 2014) (Besnard et al., 2011) (Cahill et al., 2014) and in others (Salery et al., 2020). In order to compare our results with the majority of the studies in the field, we thus used male mice. Secondly, in morphological studies performed in our group, we observed that naïve male and female mice displayed differences in dendritic spine densities. This implies that we would not been have able to pool data from both sexes without introducing significant variability. This would have led us to either increase the number of mice in each experiment to reach statistical significance or we could have replicated all experiments done with males using females. In both cases this would not have agreed with the “3R” (Replacement, Reduction and Refinement) ethical rules.

3. There are no F stats reported anywhere in the text or figure legends. Please address.

Response:

We have included an additional Supplementary Table (Supplementary Table 5) with all ANOVA F-stats and P-values for all experiments.

4. In the in vitro experiments, the authors nicely demonstrate that depolarization and nuclear calcium drive Npas4 induction, not dopamine. But in mice, Npas4 expression is increased only in the NAc (not DStr) upon administration of cocaine. The cultured MSNs are generated from both dorsal and ventral striatal neurons. It is possible that DStr MSNs require different stimuli to induce Npas4 than NAc MSNs. To link the results of the cultured MSN experiments to the conditions in vivo and rule out any "dilution effects" of DStr vs NAc originating MSNs, the authors need to stimulate the cultured MSNs with cocaine and demonstrate induction of Npas4 in the presence of SCH, and/or attenuated Npas4 induction with cocaine + Cyclosporin/FK506 or KN93.

Response:
We agree with the observation that Npas4 being only induced in mice in the NAc and not the dStr is a highly interesting one. We believe that this point has significant overlap with the specific point 1 by Reviewer 1 and is addressed in with the additional analyses of Fos in Expanded View Figure 1. The differential induction *in vivo* (Npas4 only in NAc, Fos in both NAc and dStr) is most likely caused by different neurotransmitter inputs, as suggested by our additional analyses in Fig. 2 and Fig. 3. We do not believe that the suggested *in vitro* experiments of MSN stimulation with cocaine would be conclusive in this case. Cocaine primarily acts via dopamine transporter (DAT) blockade as a dopamine reuptake inhibitor and thus depends on the presence of dopaminergic presynapses. These however are not present in *in vitro* striatal cultures as all dopaminergic input regions (i.e. VTA) are dissected away during preparation. Cocaine would thus have no adequate target in *in vitro* experiments, which is why we and others use direct dopamine receptor agonists (Pascoli et al., 2014) (Cahill et al., 2014).

5. The in silico analysis of Npas4 binding motifs and increased expression of gene targets after Npas overexpression (Nptk2, etc) does suggest that Npas4 binds to the promotors to control transcription. However, Npas4 overexpression also alters expression of other genes with no apparent binding motif (e.g. grin2a). To say definitively that Npas4 controls transcription of these genes (Nptk2, etc) the authors need to show Npas4 enrichment on these specific regions with chromatin immunoprecipitation or similar.

Response:
We share the referee's view that the *in silico* analysis by itself is limited in its explanatory power. Due to suggestions made by referee 1 regarding the clear focus of the manuscript, we decided to exclude the in silico analysis and not further investigate with additional ChIP experiments. In this case, a detailed dissection of transcriptional mechanisms would be diverging too much from the main points of the study (differential Npas4 induction, Npas4 as a regulator of MSN physiology and cocaine hyperlocomotion).

6. Several of the electrophysiological properties appear to be at odds with a wealth of literature reporting intrinsic properties of medium spiny neurons (e.g. the rheobase is much lower, the resting membrane potential is much more depolarized than many other reports---65 vs -80 -for examples see PMIDs 18945889, 18279319, 30899778) It is also surprising that there were no significant differences in rheobase between D1+ and D1- (putative D2-MSNs) (see PMIDs 18945889, 18279319, 23469183, 29975170). Please reconcile these differences with the literature.

Response:
In the dorsal striatum, and more particularly in the dorsolateral striatum, MSNs exhibit a relatively hyperpolarized RMP and a relatively large rheobase. We reported this phenomenon in various papers including Fino et al., 2008 (PMID: 17499375) in which we found a RMP = -75.4±0.7 mV (n=64 MSNs) with rheobase = 155±6 pA and Ri=251±11MO, in full accordance with the papers (Planert et al., 2013; Cepeda et al., 2008; Gertler et al., 2008) cited by the reviewer. Therefore, when we used the same experimental conditions (used in this study) in the dorsolateral striatum we found passive and active membrane properties in full accordance with the literature. In the present study, we recorded MSNs in the ventral striatum. When compared to Meitzen's lab (Cao et al., 2018; Willett et al., 2019) as quoted by the reviewer, there are indeed differences in the RMP and Ri. There are several differences in the experimental conditions between the (Cao et al., 2018; Willett et al., 2019) studies and ours, such as the orientation of the brain slices, i.e. coronal versus oblique parasagittal (10°) or the chloride concentration in the intracellular solution. Nevertheless, we measured similar rheobase values as those in (Cao et al., 2018; Willett et al., 2019). Regarding excitability differences between D1 and D2 MSNs: these have been reported mainly in the dorsolateral striatum but do not seem to be so prominent in the dorsomedial striatum and ventral striatum. As an example, Grueter et al (2010, Nat Neurosci) reported differences between D1 and D2 MSNs concerning paired-pulse ratio with a higher facilitation in D2 then in D1, but no

significant difference in terms of membrane properties. Moreover, in the ventral striatum it seems that difference between D1 and D2 MSNs relies strongly on the mice gender (Cao et al., 2018; Willett et al., 2019). For example, in the Willet et al. (2019) study, differences in D1 vs D2 MSNs were observed for AP thresholds between female but not male animals, for RMP between male but not female animals, for rheobase mainly between male animals. In our present experiments, we used adult mice (male and female) whereas prepubertal mice were used in the (Cao et al., 2018; Willett et al., 2019) studies, possibly accounting for the observed differences.

7. Physiological effects of Npas4 knockdown appear to be driven by the D1(-) neurons in Suppl table 1 (rheobase, firing rate). Why are the neuron subtypes pooled in the main figure? If the effects are driven in D1(-) neurons, why were dendritic spines quantified only in D1(+) neurons? Put another way, if the neuron subtypes are to be pooled for the ephys experiments, why are D1(-) neurons excluded from dendritic spine analysis?

Response:
The reasons for performing the main analysis with a pooled neuron population were two-fold: (i) we initially operated in a subtype agnostic manner, and (ii) to correlate the data better with the non-specific knockdown for the locomotor behavior experiments. The Npas4 target gene analysis was performed in striatal cultures (which are heterogeneous with regard to D1- and D2 expression). To thus correlate pan-MSN in vitro experiments and pan-MSN locomotor experiments with the electrophysiological data, we decided to look at all the changes in all MSNs first, with subtype characterization as secondary endpoints. We agree however that the D1 specific labeling in Figure 5 conflicts somewhat with this approach. To resolve this discrepancy, we have performed additional experiments to analyze the effects of Npas4 knockdown on spine density in D2-MSNs (Figure 5h, i). We find no differences in spine density between shscr control and shNpas4 in D2-MSNs.

Other Concerns
1. Fig 1c Y axis- Is this also normalized to the number of NeuN positive cells? How many slices were counted from each mouse?

Response:
The NeuN staining has been included in order to visualize the relative density of neurons. It has not been used for normalization. The Y axis represents the ratio (fold increase) between the number of Npas4-positive cells in the cocaine-treated cells over the number of Npas4-positive cells in the saline group. Quantifications were performed from n = 4 mice per group. We have used one slice per mouse corresponding to 0.98 mm from the Bregma as it offers the ability to analyze the NAc core and shell subdivisions and the dStr from a single brain slice. For each mouse, 10 images were acquired in the NAc (5 images per hemisphere) and 10 images for the dorsal striatum (5 per hemisphere). The number of Npas4-postive cells (or Fos-positive in new Expanded View Figure 1) per mice has been averaged. Statistical analyses were performed using the number of mice as the n. This is now included in the figure legends and in the material and method section.

2. What is the p value for Fig 3b, Arc, +HiK, +TTX + verapamil. It is difficult to believe it is not statistically significant.

Response:
The p-value is p = 0.0795 (One-way-ANOVA, Bonferroni's multiple comparison test).

3. Fig 4e. Please specify if Npas4 overexpression alone (without HiK) causes the resulting upregulation in gene expression.

Response:
The upregulation is in the absence of an experimentally induced stimulus. We have now included the term "basal" in the Figure Legend to describe this fact: "Npas4 overexpression leads to basal upregulation of genes encoding for proteins targeted to glutamatergic synapse function and Ca^{2+} channels in MSNs."

4. Fig 4f y axis- is 'mRNA level' fold change vs control?

Response:
Yes. We have included the following sentence in the Figure legends to clarify this: "mRNA level = fold change in mRNA compared to shscrambled unstimulated control."

5. Fig 5d- Were all GFP+ cells counted, or does this represent a subset? How many cells (or images) were counted per mouse?

Response:
We quantified the number of GFP-positive and Npas4-positive neurons in the presence of the shscr and shNpas4-AAVs one-hour post cocaine treatment (Y-axis in Fig. 5d). Data were normalized so that the number of GFP+/Npas4+ cells in the control virus (shscr) is set to 100% in order to appreciate the percent decrease of Npas4+/GFP+ cells upon Npas4 knock-down. Twenty fields of view were analysed from n = 3 mice in the case of AAV-shscr-GFP and 30 fields from n= 3 mice for AAV-shNpas4-GFP. This information is now included in the legend of figure 5.

6. By eye, Npas4 knockdown seems to reduce the number of dendritic spines with larger heads in Fig 5f. How does Npas4 knockdown impact different MSN spine types (I.e. thin, stubby, mushroom). This information should be easy to obtain since the authors indicate they used neuronstudio to count dendritic spines, which automatically classifies spine type.

Response:
This is an excellent suggestion (see also our reply to reviewer 1, specific point 5). We added a spine head diameter analysis for D1- and D2-MSNs from the dataset from Fig. 5 (Extended View Fig. 2) and found no significant impact of Npas4 knockdown on spine morphology.

7. For dendritic spine density--How many dendrites from how many cells per mouse? Were these secondary dendrites?

Response:
Secondary dendrites were considered, with at least 40 microns of dendrite length being analyzed. We added this information in the methods section. A total of 40 dendrites (each from a different neuron) from 4 mice were analyzed for each experimental group. We added this information in the figure legend.

8. Fig 7b. Please provide units for the Y axis
We have decided to remove this Figure panel, as it was redundant. Please see our answer to reviewer 3, point 8, reproduced here.

"To enhance the clarity of the figure, we have decided to remove panel b) as it was merely a different visualization method of parts of the data from panel c). It was the locomotion of days -1 and 0 pooled according to AAV treatments (i.e. shscr vs shNpas4), disregarding what drug treatment (saline vs. cocaine) the animals will receive later on. However, as its relation to Figure 7c would have to be explained in a more detailed fashion while adding no additional information (the lack of a basal locomotor effect of

Npas4-knockdown can be seen in Fig 7c equally well), we decided to remove this visualization. All the relevant data on basal locomotion is contained in Figure 7c. The variability in that panel was large because it was plotted with the standard deviation instead of the standard error of mean. In response to point b), there is no statistically significant effect (Bonferroni post-hoc test, P>0.9999)."

Response:

9. Supplemental Table 1 Nasp instead of Npas. Please correct.

Response:
We corrected this spelling mistake.

10. Please specify if independent cultures are from independent mice, and specify % of neurons that are positive for DARPP32 in addition to representative images.

Response:
Independent cultures are from independent mouse litters. We included this in the figure legends as "n = 3 cultures from independent mouse litters." Quantification via cell counting revealed 68% +- 3% of the neurons in the culture are DARPP32-positive and we added this percentage in the text.

11. In the methods, please specify which samples are analyzed via Taqman vs traditional SybrGreen

Response:
We clarified this by adding the following sentence to the methods section: "For all qPCR experiments except those in forebrain organoids in Fig. 2f and g, the TaqMan platform (Applied Biosystems) was used. For qPCR experiments in forebrain organoids, a custom SYBR green assay was used."

12. The locomotor testing appears to be called "cyclotron" in several figures, but is not referenced in the methods. Please either specifiy the locomotor sensitization device is called a "cyclotron" or change the figure timelines to read "locomotion"

Response:
We now mention "cyclotron" as the method to quantify locomotor activity in the methods section.

13. References to "in vivo" MSN spine density is confusing, since spine density was determined in fixed brain slices, and not in an intact animal (say with 2p imaging, e.g.). Please remove "in vivo" to improve clarity.

Response:
In this context, *in vivo* refers to the fact that Npas4 function regarding spine density was assessed through perturbation in living animals. The AAV injections and all relevant physiological adaptations are happening in live animals, hence we think the term *in vivo* is justified here. While the experimental read-out happens in fixed brain slices, these brain slices, in our study and other studies in the field, are supposed to represent a snap-shot of a living brain state. Ongoing assessments in live animals (i.e. via two-photon microscopy) are usually specified further along the lines of "live imaging", "in living animals" etc. *Ex vivo* would be inaccurate in our opinion as this gives the impression of AAV infection of slices.

14. Locomotor sensitization does not equal drug-abuse liability. References to "how responsive an animal is to cocaine" or "behavioral sensitivity to drugs of abuse" is too general, as the only behavioral test employed is cocaine locomotor sensitization (and not conditioned place preference or self-administration).

Response:
We agree with this assessment. We now use a more precise phrasing that better matches the behavioral experiment we performed. We now use “cocaine-induced locomotor sensitization” or “behavioral sensitization”.

15. Please specify when MSNs are from striatal culture as opposed to in brain slices throughout the text.

Response:
All in vitro experiments, except the ex vivo preparations in Fig. 6 were performed in cultures. We now highlighted this by referring to *ex vivo* explicitly in the text, i.e. “in ex vivo slice preparations from adult mice injected with rAAV-shNpas4”.

16. The phrase "synapse to nucleus cascade" is inappropriate, since striatal cultured MSNs don't have the same glutamatergic inputs as in an intact brain, or even brain slice, (unless they are corticostriatal cultures, in which case, please clarify).

Response:
We believe that synapse to nucleus signaling is adequate in this case, as HiK application induces cellular signaling mechanisms with major overlaps to synaptic induction mechanisms, as evidenced by the use of HiK to study synapse to nucleus cascades over the last 20 years.

17. iPSC's are not human neurons. Please change the language on pg 10 "whether differential inducibility of Npas4 is conserved in human neurons."

Response:
The assessment is not done in iPSCs but in iPSC-derived cells. These cells express a neuronal phenotype similar to that which we previously characterized in (Pruunsild et al., 2017). They possess elaborate cellular processes and express the neuronal marker MAP2 as well as the forebrain marker FOXG1 (Fig. 2f). Moreover, they display a neuron-typical membrane potential and produce action potentials (data not shown, submitted elsewhere). We thus believe that the term human neurons is justified in this case.

18. Pg 20. "Npas4 induction is hence tightly coupled to membrane depolarization, which enables the cell to discriminate different circuit inputs." This was not tested. Please remove.

Response:
We agree that the formulation was too strong. We rephrased it to “…which might enable the cell to…” to make clear that this is a speculative statement.

19. Pg 22. "What is especially intriguing about Npas4 as a treatment target is that changing Npas4 levels leaves basal locomotor behaviour unchanged, as shown in or study, implying the possibility of targeted interventions with minimal side-effects on other behaviour." This is entirely too strong and an ill-supported statement. Please remove.

Response:
We have removed the second part and narrowed it down to basal locomotion. The sentence and the one following it are now: “What is especially intriguing about Npas4 as a treatment target is that changing Npas4 levels leaves basal locomotor behaviour unchanged, as shown in our study. This potential benefit of a more selective and context-dependent Npas4 action mechanism is also implied by a recent study

which showed that striatal Npas4-knockdown selectively decreased the rewarding properties of cocaine but not of sucrose, while also leaving aversion-learning intact (Taniguchi et al., 2017)."

20. Verapamil has also been shown to inhibit voltage-gated K+ channels. Please comment on this possibility.
The inhibition of K^+-channels is indeed a well-known side effect of verapamil. Blockade of K^+-channels through verapamil can lead to neuronal AP bursting and hence induce transcriptional mechanisms unrelated to the intended calcium channel blockade. This is why we added TTX in the experiments in Fig. 3b to suppress this well-known side-effect.

Referee #3:

In this manuscript, Lissek et al. provide evidence that Npas4 uniquely controls baseline striatal physiology and transcriptional networks essential for responsiveness to cocaine. Overall, this is a wide-ranging study including transcriptional analysis, slice physiology, ca imaging and behavioral approaches. These data are generally well-executed, the data analysis is largely appropriate, and the manuscript is well written with clear figures. In particular, Fig. 3 is an excellent investigation of the signaling pathways the induce Npas4 activity. Nevertheless, there are some remaining issues that should be done to strengthen these data prior to publication:

Major Points:

1. the use of organoids to complement the striatal cultures is viewed as a positive, however, what exactly are the identity of these "neurons"? Forebrain FoxG1+ neurons encompass all telencephalic neurons - does this imply that the authors see such clear Npas4 regulation because the same upregulation is happening in cortical neurons as well as striatal? Are there striatal neurons in this preparation?

Response:
The forebrain organoids used in this study are the best model we have available in our labs for investigating human telencephalic neuron transcriptional processes. It is true that FOXG1+ marks all telencephalic neurons and, therefore, we were deliberately not differentiating identities. The main rationale for performing the experiment was to determine if the differential inducibility of Npas4 is conserved in human neurons regardless of possible neuron type specificities. Our result that in the human iPSC-derived organoids the differential regulation of *NPAS4* was detected suggests that this is indeed the case. Since the organoids contain a heterogenous population of cells (including in various maturity states) we prefer to avoid speculations about the exact identity of the neurons.

2. regarding the transcriptional analyses, it would be good if the same candidates were shown in both hippocampal and striatal O/E experiments - these do not need to be identical but would help give a picture of the core transcriptional components regulated in O/E. In addition, it may be more meaningful to focus on the clear, consistent specific results (GRIN1; GRIN2b; Cacna1d) rather than focusing on GO groups that are vague.

Response:
We believe that the GO results are relevant because they led us to several subsequent investigations, such as spine density analysis and electrophysiological parameters. The microarray datasets are hypothesis-generating and confirmed with subsequent refined analyses. With regard to the additional representation of genes that are non-significantly upregulated in the microarray analysis, we believe that too many non-significant plots would distract from the main points of the figure, especially since at the time of the microarrays, we did not investigate the genes mentioned by the reviewer (i.e. Grin2b, Cacna1).

Rather, the results from the microarray analysis made us subsequently investigate certain gene families (NMDAR and VDCC subunits) and in this process we found Grin2b and Cacna1d upregulations.

3. furthermore, given the suggestion that Npas4 controls transcription in adults, the transcriptional data would be strengthened by validating some of these results in mice by qPCR from NAc tissue following NPas4 KD.

Response:
While a Npas4 knockdown in adult mice seems useful in this scenario, we believe its additional explanatory power would be limited. Our *in vitro* neurons, derived from postnatal animals (P0), express a clear MSN phenotype (Fig. 2) and provide a very clean neuronal dataset, free from by transcripts from other cell types, such as glial cells. Expression analysis from brain tissue *in vivo* generally has strong dilution associated with it and might hence miss many Npas4 target genes.

4. was the data for Fig4d done in a systematic way? or are these promoter elements anectodal in their description?

Response:
These are anecdotal and based on our hypothesis of Npas4 regulating glutamatergic synapses in MSNs. Due to the related point 5 by referee 2, we decided to remove the in silico analysis from the Figure. Our answer to this point is replicated here for clarity:

“We share the referee’s view that the in silico analysis by itself is limited in its explanatory power. Due to suggestions made by referee 1 regarding the clear focus of the manuscript, we decided to exclude the in silico analysis and not further investigate with additional ChIP experiments. In this case, a detailed dissection of transcriptional mechanisms would be diverging too much from the main points of the study (differential Npas4 induction, Npas4 as a regulator of MSN physiology and cocaine hyperlocomotion).”

5. in general, in addition to the posthoc tests, the results of the ANOVAs should be included as supplemental materials. For example, in Fig.5F, is the interaction term of the 2w ANOVA significant or is there just a main effect of NPas4-kd on spine density - these are two very distinct outcomes. one supports a role in cocaine-induced plasticity, the other in general synaptogenesis.

Response:
We have now included an additional supplementary table (Supplementary Table 5), which contains all ANOVA F-Stats and P-values used in the manuscript. In general, we decided beforehand to rely on post-hoc tests to perform targeted between group analyses. In these cases, the ANOVA P-values and F-Stats have a reduced relevance for the assessment of the experiments.

6. given prior publications of SPN subtype-specific differences in both NAc (Greuter, NN 2010) and DStr (Kreitzer, Nature 2007), it seems problematic to lump together all recorded neuronal subtypes, particularly given that the authors used a viral tool to distinguish cell types. Do these physiological phenotypes hold up when comparing neurons of the same subtype?

Response:
We distinguished the two MSN subtypes precisely to observe putative differences between direct and indirect pathway MSNs. As detailed in the Supplementary Table 4, regarding paired pulse ratio experiments, we did not observe significant differences between D1+ and D1- MSNs in term of facilitation magnitude for 50 and 250 ms interstimulus intervals; there was a tendency (but not significant) for higher PPF in D1- than D1+ MSN in scramble conditions. In the literature, results are quite variable: Grueter et

al., (2010) reported a higher PPF magnitude in D1 than D2 MSNs (for 20 and 50 ms interstimulus intervals but not for 100 and 200 ms), whereas Cepeda et al. (2008) reported opposite results, i.e. PPF was larger in D2 than in D1 MSNs (for 100 ms interstimulus intervals but not for 50 ms). In the dorsal and ventral striatum, data regarding paired pulse experiments differ across studies and seem to strongly depend on experimental conditions. For example, our experimental conditions differ when compared with these two studies: brain slices angle, the use (or the absence of as in our case) of GABAergic inhibitors (bicuculline, picrotoxin) and the location of the stimulation electrode (in layer V of the cerebral cortex in our study, in the corpus callosum in Grueter et al. and in Cepeda et al. studies).

7. what is the explanation for the increased firing rate given the other changes indicate a reduction in excitability?

Response:
We agree with reviewer that these two findings (firing rate and passive membrane properties) appear to be functionally contradictory. An explanation could be a homeostasis phenomenon: an increased input/output gain function can be observed with decreased excitability as an attempt for the neuron to re-establish an overall activity similar to its previous state. Another explanation is an opposite regulation of active (suprathreshold) and passive (subthreshold) MSNs properties; this would reinforce the coincidence detection properties of MSNs of incoming excitatory inputs, i.e. disfavor EPSCs summation, whereas once spikes are emitted and the suprathreshold mode is reached than firing activity is favored. It would actually exacerbate the physiological functioning of MSNs which act as efficient coincidence detectors in subthreshold regimes, and potent integrators in suprathreshold regimes. In the Discussion the following sentence has been added: “The reduction in neuronal excitability together with increased firing rate could result from homeostatic mechanisms and exacerbate the coincidence detection properties of MSNs.”

8. For the behavioral figure, the potential effects on basal locomotion seem important for determining the specificity of NPas4 effects for cocaine or just locomotion in general. Along those lines: a) is basal locomotion calculated the same way as the movement in 7C? why is the variability so large? b) is there an effect of the shRNA on the day prior to cocaine administration in the group that will receive cocaine (7C, last two bars in 0d time group)?

Response:
To enhance the clarity of the figure, we have decided to remove panel b) as it was merely a different visualization method of parts of the data from panel c). It was the locomotion of days -1 and 0 pooled according to AAV treatments (i.e. shscr vs shNpas4), disregarding what drug treatment (saline vs. cocaine) the animals will receive later on. However, as its relation to Figure 7c would have to be explained in a more detailed fashion while adding no additional information (the lack of a basal locomotor effect of Npas4-knockdown can be seen in Fig 7c equally well), we decided to remove this visualization. All the relevant data on basal locomotion is contained in Figure 7c. The variability in that panel was large because it was plotted with the standard deviation instead of the standard error of mean. In response to point b), there is no statistically significant effect (Bonferroni post-hoc test, $P>0.9999$)

Minor Points:

1. given the effects of NMDAR blockade on Npas4 induction, it seems likely that the striatal cultures have a reasonable number of glutamatergic neurons - this should be quantified in some way and mentioned b/c otherwise these effects are confusing.

Response:

As the referee suggests, the results of NMDAR blockade after HiK stimulation are intriguing in that striatal neuron cultures should not contain glutamatergic neurons that would provide the necessary glutamate for NMDAR activation. We have shown previously however (through either removal of endogenous glutamate (replacement by fresh medium or through enzymatic degradation of endogenous glutamate) that low endogenous levels of glutamate are able to stimulate NMDARs (Pascoli et al. Biol. Psych Fig. 2B 2011). We have also shown that the magnesium block is relieved from NMDAR in cultured MSN (see Pascoli et al. Biol. Psych 2011; Fig.S1). The NMDAR-dependence of HiK induced signaling in striatal neurons is furthermore described in the paper “Potassium chloride depolarization mediates CREB phosphorylation in striatal neurons in an NMDA receptor-dependent manner” by Macias et al, Brain Res, 2001. The authors dedicated a whole paper to investigating this phenomenon and came to the following conclusion: “Here, we show that in striatal neurons, but not in hippocampal neurons, CRE binding protein (CREB) phosphorylation and CRE-mediated gene expression after KCl-depolarization depends on functional NMDA receptors.” We have incorporated the above points into the main text in the results section to provide immediate clarification: “This NMDA receptor dependency of HiK mediated gene inductions has been described previously (Macias et al., 2001) (Pascoli et al., 2011a) and is due to the fact that NMDA receptors are activated by ambient glutamate from the culture medium.”

2. "This decreased facilitation indicates an increase in c-str transmission" should be softened as this single assay suggests an increase in release probability (although in isolation, it could also reflect changes to AMPAR desensitization).

Response:
We agree with Reviewer and we softened our previous sentence to: “This decreased facilitation indicates an increase of probability of glutamate release from pyramidal cells".

3. In Figure 1, details of quantification such as animal number, images collected per animal seem to be missing.

Response:
We agree with the referee and we revised the legend of Fig. 1 accordingly. “Quantifications of Npas4-positive cells (fold change normalized to the 30min saline control group) in the NAc and CPu; n = 4 mice per group; 10 images (5 per hemisphere) per mice for the NAc and 10 images (5 per hemisphere) for the dStr p** < 0.01, unpaired Student’s t-test.

4. In Figure 3h co-application of HiK and SKF had no additional effect on Npas4 induction, leading the authors to conclude that there is no crosstalk between Ca2+ and D1 related signaling. This seems at odds with Figure 3e in which they show that inhibition of PKA by H-89 (a downstream effector of D1R signaling) increases Npas4 induction. The authors conclude that this result indicates negative regulation of Ca2+ Npas4 induction by PKA. To me this seems like crosstalk between the two pathways.

Response:
These two experimental results indeed seem to contrast each other somewhat. The statement however refers specifically to D1 receptor activation via SKF, and not PKA. As pointed out by the referee, the two pharmacological treatments target different proteins (SKF targets D1 receptors, H89 targets PKA) and in different directions (SKF is an agonist, H89 a blocker). D1 receptor activation and PKA activation are not synonymous. D1 receptor activation in striatal neurons has been shown to activate additional PKA-independent downstream cascades such as phospholipase C activation (Kuroiwa et al., 2008), which could compensate for PKA-mediated Npas4 downregulation. It is also possible that Npas4 regulation by PKA happens only in one direction, i.e. blockade of basal levels of PKA activity leads to Npas4

superinduction but any elevation of PKA activity beyond basal levels by D1 agonists has no additional blocking effect on Npas4.

5. In Figure 5d, I am unclear on how the exact measure being displayed. It looks to be normalized so that the control condition is set to 100. If so this should be indicated for clarity. If the data is a raw percentage of Npas4+/GFP+ cells, this seems at odds with the images shown.

Response:
Yes, these quantifications were normalized so that the control condition is set to 100%. Please see also the answer to referee 1, other concern 5 on that specific issue for more details. To clarify this point, the corresponding figure legend has been revised: “Quantifications of GFP-positive and Npas4-positive cells 1h post cocaine treatment. Data were normalized so that the number of GFP- and Npas4- positive cells was set 100%. 20 fields of view were analysed from n = 3 mice in the case of AAV-shscr-GFP and 30 fields of view from n = 3 mice for AAV-shNpas4-GFP.

6. In Figure 5d, the figure legend says that 3 mice per group were used, but does not detail how many images were taken per mouse, or whether data is displayed with each image as a data point or with data from each mouse averaged together.

Response:
We included this information in the figure legends: “20 fields of view were analysed from n = 3 mice in the case of AAV-shscr-GFP and 30 fields of view from n = 3 mice for AAV-shNpas4-GFP.”

7. In Figure 5e, information about the number of animals, and number of cells imaged seems to be missing. Additionally, the figure legend says that normalization was done "to 10 um of dendrite for saline control and after cocaine injection". If this means that only 10 um of control dendrite was used for normalization (as opposed to the data simply being displayed as spines/10 um), this is not a large enough segment of dendrite to be taken as representative. Usually at least 50 um is counted.

Response:
The results on spine density have been obtained with 40 dendrites (each from a different neuron) from 4 mice for each experimental group. This information has been added to the figure legend. For every dendrite analyzed, at least 40microns of dendritic length was analyzed. This information has been added to the methods section.

8. In the Discussion section, suggestions of Npas4 as a potential drug target should be toned down. The data presented indicating that knockdown of Npas4 does not have non-specific effects on behavior is quite limited and manipulating the expression and/or function of a master transcriptional regulator is not likely to be a viable therapeutic strategy.

Response:
We agree with the sentiment that using Npas4 as an addiction diagnosis and treatment target is speculative at this point. We have now used more careful language to convey this fact in the discussion, i.e. “could potentially be used as markers”, “It is feasible”. However, as stated in the text, other papers such as (Taniguchi et al., 2017) have mirrored a selective impact of Npas4 on behavior: “This potential benefit of a more selective and context-dependent Npas4 action mechanism is also implied by a recent study which showed that striatal Npas4-knockdown selectively decreased the rewarding properties of cocaine but not of sucrose, while also leaving aversion-learning intact (Taniguchi et al., 2017)”. We do think that exploring Npas4 as a marker or target in addiction treatment could hence be a worthwhile strategy, even though it does regulate many target genes.

9. The authors mention that their results "imply that it (Npas4) controls overall stimulus-independent and -dependent synaptic input onto MSNs". This seems to be an overgeneralization. The data-presented show that Npas4 regulates spine density independently of experimentally induced stimuli (ex. Cocaine treatment) only. This does not rule out naturally occurring stimuli which are not being controlled for in this study.

Response:
We agree and replaced “overall” with “certain” in the text passage, making the statement more restrictive. It now reads: “… imply that it controls certain stimulus-independent and -dependent synaptic inputs onto MSNs.”

References

Besnard, A., Bouveyron, N., Kappes, V., Pascoli, V., Pages, C., Heck, N., Vanhoutte, P., and Caboche, J. (2011). Alterations of molecular and behavioral responses to cocaine by selective inhibition of Elk-1 phosphorylation. J Neurosci *31*, 14296-14307.
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Kuroiwa, M., Bateup, H.S., Shuto, T., Higashi, H., Tanaka, M., and Nishi, A. (2008). Regulation of DARPP-32 phosphorylation by three distinct dopamine D1-like receptor signaling pathways in the neostriatum. J Neurochem *107*, 1014-1026.
Pascoli, V., Cahill, E., Bellivier, F., Caboche, J., and Vanhoutte, P. (2014). Extracellular signal-regulated protein kinases 1 and 2 activation by addictive drugs: a signal toward pathological adaptation. Biol Psychiatry *76*, 917-926.
Pruunsild, P., Bengtson, C.P., and Bading, H. (2017). Networks of Cultured iPSC-Derived Neurons Reveal the Human Synaptic Activity-Regulated Adaptive Gene Program. Cell Rep *18*, 122-135.
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Spiegel, I., Mardinly, A.R., Gabel, H.W., Bazinet, J.E., Couch, C.H., Tzeng, C.P., Harmin, D.A., and Greenberg, M.E. (2014). Npas4 regulates excitatory-inhibitory balance within neural circuits through cell-type-specific gene programs. Cell *157*, 1216-1229.

Dear Prof. Bading,

Thank you for the submission of your revised manuscript. We have now received the enclosed reports from the referees who still have a few more minor suggestions that I would like you to incorporate before we can proceed with the official acceptance of your manuscript. Please address the last referee concerns also in a point-by-point response.

A few other editorial changes are also required:

- Please reduce the number of keywords to 5.

- Marie-Charlotte Allichon is missing from the author contributions, please add.

- Please add all funding info also into our online system when you upload the final manuscript.

- The Fig 5G callout is missing. There is a callout to 5i which doesn't exist.
The Fig EV1 panels are not called out, please add callouts.

- Suppl Tables 1-3 should be called Dataset EV1-EV3. Please add the legends to the first tab of the excel files. Suppl table 4 should be called Table EV1. Suppl table 5 is information which should be included in the figure legends and can therefore be removed as a table file.

- Please make sure that your deposited data is freely accessible upon online publication of your paper.

- Summary needs to be changed to Abstract. The Materials & Methods should come after the Discussion section. The supplementary table legends should be removed from the Article file and added into the table files. The expanded view figures should be named 'Figure EV#' throughout.

I attach to this email a related manuscript file with comments by our data editors. Please address all comments in the final manuscript.

I would like to suggest a few minor changes to the abstract. Please let me know whether you agree with the following:

We show here that the transcription factor Npas4 is an important regulator of medium spiny neuron spine density and electrophysiological parameters, and that it determines the magnitude of cocaine-induced hyperlocomotion in mice. Npas4 is induced by synaptic stimuli that cause calcium influx, but not by dopaminergic or PKA-stimulating input, in mouse medium spiny neurons and human iPSC-derived forebrain organoids. This induction is independent of ubiquitous kinase pathways such as PKA and MAPKs, and instead depends on calcineurin and nuclear calcium signaling. Npas4 controls a large regulon containing transcripts for synaptic molecules, such as NMDA receptors and VDCC subunits, and determines in vivo MSN spine density, firing rate, I/O gain function and paired-pulse facilitation. These functions at the molecular and cellular levels control the locomotor response to drugs of abuse, as Npas4 knockdown in the nucleus accumbens decreases hyperlocomotion in response to cocaine in mice while leaving basal locomotor behaviour unchanged.

I look forward to seeing a final version of your manuscript as soon as possible. Please use this link to submit your revision:
https://embor.msubmit.net/cgi-bin/main.plex

Best regards,
Esther

Esther Schnapp, PhD
Senior Editor
EMBO reports

Referee #1:

The authors have addressed most of my comments and concerns successfully. Congrats for the extra work that makes a difference with the original submission.

Referee #2:

The authors have addressed most of my concerns in their revision.

Since the mouse findings are from only males, this should be more clearly specified in the abstract and introduction. For example " ...hyperlocomotion in response to cocaine in mice while leaving basal locomotor behaviour unchanged." should be changed to "...in male mice".

Npas4 knockdown increases firing rate and decreases rheobase only in D1- cells. Dendritic spine changes are only happening on D1+ cells. In light of this I think it is inappropriate to show ephys data combined in the main figure, with the D1+ and D1- specific comparisons buried in supplemental. This discrepenacy between spine changes and ephys changes is also inadequately discussed.

Fig 1 legend says Cpu instead of dStr.

Referee #3:

I apologize for the long delay.
overall, the authors have done a good job at addressing my comments from the previous version.

the only outstanding/puzzling issue is found in Fig.5F. at my request, the authors have included a table showing all of the ANOVA values (support table 5). I am unclear how these values exactly relate to Figure 5 though - I assume the D1-MSN relates to 5F? if so, these numbers seem weird - there should be much stronger evidence of a main effect of shNPas4 (the red bar goes down a lot in both). I understand the ability to go directly to postdoc testing in some cases, but this seems like a situation where the interaction term of the ANOVA would be the most important read-out. right now, the data looks like there is a synapse-reducing effect of shNpas4 in the control condition and that same amount of suppression is just added to the cocaine group (the cocaine red bar goes up from the saline). directly testing that would require a measurement of the relative suppression of shRNA in both conditions (not for example comparing the number of stars between the 2 groups, 1 vs 3). maybe the authors feel this is semantics but it seems that there is a difference between a basal effect on synapses and a specific effect on cocaine-induced spine growth.

Point-by-point response after revision round 1 – Lissek et al, EMBOR-2020-51882V2, 2021

We would like to thank the reviewers for their valuable comments and suggestions, which have made the manuscript considerably stronger.

Referee #1:

The authors have addressed most of my comments and concerns successfully. Congrats for the extra work that makes a difference with the original submission.

We thank the reviewer for the insightful comments that improved our study noticeably.

Referee #2:

The authors have addressed most of my concerns in their revision.

Since the mouse findings are from only males, this should be more clearly specified in the abstract and introduction. For example " ...hyperlocomotion in response to cocaine in mice while leaving basal locomotor behaviour unchanged." should be changed to "...in male mice".

We have included the term „male“ in the abstract.

Npas4 knockdown increases firing rate and decreases rheobase only in D1- cells. Dendritic spine changes are only happening on D1+ cells. In light of this I think it is inappropriate to show ephys data combined in the main figure, with the D1+ and D1- specific comparisons buried in supplemental. This discrepenacy between spine changes and ephys changes is also inadequately discussed.

We have revised Figure 6 and now show the different ephys parameters also sperated in to D1+ and D1- MSNs in addition to the pooled population and describe this in the results section (starting in line 28)7. We have also included a discussion section on the discrepancy between the spine density and the ephys data (starting in line 422).

Results section:

> We have differentiated D1-positive and D1-negative MSNs through the D_1-tdTomato rAAV labelling approach used in the dendritic spine analysis and analysed these two neuron populations seperately and pooled to study overall effects on MSN physiology. We examined 15 active and passive membrane properties (Table EV1) and found an increased rheobase (Fig. 6a) and firing rate (Fig. 6b) and a decreased input/output gain function (Fig. 6c) in the Npas4-knockdown mice compared to control. The rheobase and firing rate increases were primarily present in D1-negative MSNs and pooled cells, whereas the I/O gain decrease had similar effect sizes in D1-positive and D1-negative MSNs, although only the pooled cell group was statistically significant in this experimental set. Together, these findings indicate a lower MSN excitability in the AAV-shNpas4-injected mice. We observed no difference for membrane resting potential, input resistance or other AP features (Table EV1). We next recorded EPSCs evoked by paired-pulse

stimulations at 20 and 4 Hz to assess the probability of release (Fig. 6d). Paired-pulse ratio (PPR) analysis revealed that in control mice, the corticostriatal short-term plasticity was facilitated for 20 and 4 Hz paired-pulse stimulations (Fig. 6d and Table EV1). In the shNpas4 injected mice, facilitation was still observed but was significantly decreased at both 20 and 4 Hz stimulations (Fig. 6d). The observed effects were due to significant changes in D1-negative MSNs, whereas D1-positive MSNs did not show changes in short-term plasticity. This decreased facilitation indicates an increase in the probability of glutamate release from pyramidal cells.

Discussion section:

To understand how Npas4 can already affect behaviour on the first day of cocaine administration, we analysed MSN electrophysiological parameters in naive animals in D1-positive, D1-negative and pooled MSNs. Npas4 controls MSN membrane excitability (as inferred from an increased rheobase and a decreased input/output gain function in the Npas4-knockdown animals) and firing rate. The rheobase and firing rate effect was only present in D1-negative MSNs, while the decrease in I/O gain function was seen in both D1-positive and D1-negative MSNs with however only the pooled cell group showing statistically significant differences. Npas4 also negatively regulates cortico-striatal transmission, as seen by a decreased paired-pulse facilitation in the Npas4-knockdown animals, with a more pronounced effect in D1-negative MSNs. In this electrophysiologal dataset some of the effects seem to be dominant in D1-negative MSNs (especially rheobase and PPF), while others (I/O gain) occur in both D1-positive and D1-negative MSNs with similar magnitude. None of the effects are significant in D1-positive MSNs, which might either indicate an absence of a biological effect or it might be due to lower sample sizes in this dataset. The observation that some effects are stronger in D1-negative MSNs stands in an interesting contrast to our findings on dendritic spines, where the Npas4-knockdown effect was restricted to D1-positive MSNs. It could be that Npas4 regulates certain cellular parameters in different ways in different MSN subtypes and that adaptations in D1-negative MSNs are either synergistic or homeostatic (e.g. D1-negative MSNs change their activity in response to synaptic alterations in D1-positive MSNs to maintain circuit balance).

Fig 1 legend says Cpu instead of dStr.

We have corrected this mistake.

Referee #3:

I apologize for the long delay.
overall, the authors have done a good job at addressing my comments from the previous version.

the only outstanding/puzzling issue is found in Fig.5F. at my request, the authors have included a table showing all of the ANOVA values (support table 5). I am unclear how these values exactly relate to Figure 5 though - I assume the D1-MSN relates to 5F? if so, these numbers seem weird - there should be much stronger evidence of a main effect of shNPas4 (the red bar goes down a lot in both). I understand the ability to go directly to postdoc testing in some cases,

but this seems like a situation where the interaction term of the ANOVA would be the most important read-out. right now, the data looks like there is a synapse-reducing effect of shNpas4 in the control condition and that same amount of suppression is just added to the cocaine group (the cocaine red bar goes up from the saline). directly testing that would require a measurement of the relative suppression of shRNA in both conditions (not for example comparing the number of stars between the 2 groups, 1 vs 3). maybe the authors feel this is semantics but it seems that there is a difference between a basal effect on synapses and a specific effect on cocaine-induced spine growth.

As the reviewer states, the data in Figure 5F shows several pieces of information with regard to treatment groups. First, it shows that Npas4 knockdown decreases spine numbers in both saline- and cocaine-treated animals (i.e. comparing shscr to shNpas4 in both conditions). In addition, there is a blockade of cocaine induced spine-growth: in the shscrambled condition (AAV control) there is a significant increase from the saline to the cocaine condition. However, in the shNpas4 group this increase is not significant and smaller (i.e. using the relative measurement as suggested by the reviewer gives a 18.5% cocaine-dependent spine number increase in the shscr condition (17.9852 / 15.1755) but only a 8.4% increase in the shNpas4 condition (14.4152 / 13.2965). The reviewer is correct in that Suppl. Table 5 5F refers to the D1-MSN data. Here, it can be seen that the P-value for the interaction is 0.087 (i.e. small but not below 0.05). However, as stated, we rely primarily on post-hoc tests to test our hypotheses because of increased sensitivity. In this case, we see that the comparison between shNpas4-saline and shNpas4-cocaine is not significant. Based on these results, we deem it justified to conclude that Npas4 regulates basal spine density as well as the cocaine dependent increase in spine density.

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Corresponding Author Name: Prof. Dr. Hilmar Bading, Dr. Peter Vanhoutte, Thomas Lissek
Journal Submitted to: EMBO reports
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes and estimations of variances were based on previous similar experiments from our labs and others.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample sizes were based on previous similar experiments either performed and published by the authors or published by other reserach groups.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	For in vitro experiments, the pre-established guidelines were to exclude samples when there were technical errors (i.e. no amplification in a well in a qPCR run). These would then be reanalyzed. For in vivo studies, mice were excluded from the study when they showed poor quality of AAV infection in the post-behavior histological assessment (e.g. non-bilateral expression, off-target diffusion, excessive backflow or low GFP expression). This assessment was performed by a different person than the one who performed the injections. For behavioral experiment outlier detection, we used GraphPad Prism's "Identify outliers" function.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	No blinding was performed for in vitro or in vivo experiments.
For animal studies, include a statement about randomization even if no randomization was used.	Mice subjected to distinct pharmacological treatments were randomly assigned to cages.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Mice were randomly assigned to a given group and subjective bias was avoided through automated analysis methods (i.e. locomotion measurements in cyclotron).
4.b. For animal studies, include a statement about blinding even if no blinding was done	Blinding was not performed for behavioral studies because mice needed to be marked according to the respective experimental condition (i.e. treatment).
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We followed established practices in the field.
Is there an estimate of variation within each group of data?	Yes.

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Is the variance similar between the groups that are being statistically compared?	Yes.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies: Darpp-32 (Cell Signaling, mAb 2306), Map-2 (Millipore, MAB3418), GFP (Molecular Probes, A11120), Npas4 (gift from Dr. Michael Greenberg, Harvard, Lin et al, Nature, 2008); Fos/FosB antibody (Santa Cruz, sc-166940).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used, other than HEK cells for AAV production where cell line identification is not necessary.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Six-week old male C57BL/6 mice were purchased from Janvier (Le Genest, St. Isle, France) and maintained in a 12h light/dark cycle in stable conditions of temperature (21 +/-1°C) and humidity (60%) with ad libidum access to food and water. Animals were habituated to the animal facility for one week prior to the experiments.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Animal care was conducted in accordance with standard ethical guidelines (NIH publication no. 85-23, revised 1985 and European Community Guidelines on the Care and Use of Laboratory Animals (86/609/EEC)). The experiments were approved by the local ethics committee "Comité d'Ethique en Experimentation Animale Charles Darwin
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirmed. The material and methods section includes all relevant information

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
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14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The microarray data (Fig 4) is deposited at ArrayExpress with the accession E-MTAB-10706.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No, it does not fall under dual use restrictions.