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Calcium-permeable AMPA Receptors and Silent Synapses in Cocaine-conditioned Place Preference

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 14 September 2016

Thank you for submitting your manuscript on silent synapses in cocaine-conditioned place preference to The EMBO Journal. We have now heard back from three expert referees, whose comments are copied below. I am pleased to inform you that all of them find your study interesting, important and generally well-conducted. We shall therefore be happy to consider a revised manuscript further for publication, pending addressing of a limited number of specific concerns. As you will see, the only real issue arises from the proposed link between silent synapses and insertion of CP-AMPARs, and while clarification through additional data would certainly be helpful, it appears that the referee comments may partly also be addressable via further explanations and more careful wording of claims in the results and discussion sections and possibly also the title.

When modifying the manuscript, I would appreciate if you could also slightly revise the abstract and introduction with the broader, non-specialist readership of The EMBO Journal in mind, in particular by better explaining early on the significance of the CPP paradigm as as compared to other already studied drug-related behaviors. Please also pay attention to EMBO Journal article format as stipulated in our Guide to Authors, e.g. by adjusting the in-text referencing style, re-ordering manuscript sections, and including author contribution and conflict of interest sections. Finally, please revise the various incomplete entries in the reference section and adjust its format as well.

REFEREE REPORTS

Referee #1:

In the manuscript titled "The role of accumbens silent synapses in cocaine-conditioned place preference", Shukla and colleagues investigate molecular mechanisms underlying a mouse model of non-contingent cocaine-mediated learning and memory. First the authors demonstrate that, like cocaine sensitization paradigms previously published by this group, there is an early increase in silent synapse expression followed by insertion of calcium-permeable AMPA receptors in the CPP paradigm. The authors then utilize knockout and knockdown approaches to investigate the divergent roles of MAGUK's in the formation of silent synapses and expression of CP-AMPA receptors. Interestingly, the authors then addressed the role of mGluR1 in the development of silent synapses and CP-AMPA receptor expression. This is a nice link to fill the gap between this groups published work and that of Wolf lab and the role of CP-AMPA receptors in incubation of drug craving. The claims presented here are both significant to the field and convincing. In my opinion, this is a solid piece of work that advances the field of neuroscience in general and addiction research in particular.

The manuscript is concisely written and exhibits a commendable amount of work. The authors thoroughly represent the current literature and utilize well -controlled, cutting edge methodologies.

Referee #2:

This manuscript describes experiments designed to assess the role of silent synapses in the nucleus accumbens (NAc) during cocaine-conditioned place preference. This is topic is highly relevant as addiction associated with cocaine and other drugs of abuse is a challenging societal problem. Long-term changes in synaptic function and neuronal circuits in the NAc are thought to underlie certain forms of addiction. Transitioning synapse between silent and active states have been shown to correlate the acquisition and maintenance of the long-term circuit changes. To explore the contribution of silent synapses to cocaine-conditioned place preference in the NAc, the authors have taken advantaged of mice lacking members of the PSD-95 family (PSD-95, PSD-93 and SAP102) known to regulate synaptic strength and the accumulation of AMPA-type glutamate receptors at excitatory synapses. Together this analysis shows that PSD-95, PSD-93, and SAP102 differentially regulate the maturation of cocaine-generated silent synapses in the NAc and that disrupting silent synapse maturation does not disrupt CPP retention.

Overall, this is an excellent study. The data are of very high quality and support the author's claims. The manuscript is clearly and logically written with a balanced discussion that fairly interprets data in the context of published work. The topic is timely and well suited for EMBOJ. I thus recommend publication without revision.

Referee #3:

In this study the authors report the presence of silent synapses following ten days of cocaine CPP training and subsequent insertion of CP-AMPARs following withdrawal from cocaine CPP. The authors further investigate the role of scaffolding proteins PSD-95, PSD-93, and SAP-102 using a global knockout strategy. PSD-95 is further investigated using a shRNA knockdown in the brain region of interest (nucleus accumbent in this case). The authors suggest a dissociation between the presence of CP-AMPARs and the retention of cocaine CPP memory. This is a thorough and interesting study that will be of interest to readers, however some issues arise with the interpretation and conclusions drawn.

Major comments:

1. Although the title of the paper is "The role of silent synapses in cocaine conditioned place preference", the more thorough investigation is in the role of CP-AMPARs in cocaine CPP. The title should be changed to reflect the content of the paper

2. The authors show that silent synapses and CP-AMPARs appear following a timeline that could coincide with CP-AMPARs being inserted into newly generated synapses. However, there is no real

link between silent synapses and the appearance of CP-AMPARs, and indeed these could be separate phenomena that occur in separate synapses. There is no demonstration that silent synapses are required for the insertion of CP-AMPARs which would be helpful in supporting this assertion.

3. Control experiments are performed that show CPP training to be a requirement for the insertion of CP-AMPARs, but no controls are performed to demonstrate that this is true for silent synapses as well. Indeed the presence of silent synapses based on the data in this paper could simply be a result of pharmacological exposure to cocaine. If minimal stimulation assays were performed in the cohorts shown in Figure 1H, it would be of great utility to show these data. If possible, a control should be added in which animals are given either saline or cocaine in the home cage and then silent synapses are measured to determine if CPP training is a requirement for the appearance of silent synapses.

4. The authors show a dissociation between the presence of CP-AMPARs and retention of the CPP memory. However, the CPP training regimen is very strong. In one experiment, a "weaker" training paradigm is used in which rats only received 5 days of CPP training. It would be informative to determine if there is any impairment when insertion of CP-AMPARs is prevented when the memory is less thoroughly stamped in.

Minor comments:

1. The discussion of silent synapse-based circuit remodeling and silent synapse maturation should be edited as the data do not prove that CP-AMPAR insertion is causally linked to silent synapses.

2. It would be helpful to view behavioral data additionally in the form of time in minutes or seconds rather than the normalized place preference score. At the very least a rationale for presenting the data this way should be discussed.

3. There are several grammatical errors and typos scattered throughout the manuscript, please edit.

1st Revision - authors' response 04 November 2016

Response to Referees

We thank the editor and three referees for appreciating our work and their positive comments, such as "…*this is a solid piece of work that advances the field of neuroscience in general and addiction research in particular*" (referee 1), "...*this is an excellent study*." (referee 2), and "*This is a thorough and interesting study that will be of interest to readers*" (referee 3).

Referees 1 and 2 did not suggest modifications.

Referee 3:

1. *Although the title of the paper is "The role of silent synapses in cocaine conditioned place preference", the more thorough investigation is in the role of CP-AMPARs in cocaine CPP. The title should be changed to reflect the content of the paper.*

We agree with the referee and editor and changed the title to: "Calcium-permeable AMPA Receptors and Silent Synapses in Cocaine Conditioned Place Preference".

2. *The authors show that silent synapses and CP-AMPARs appear following a timeline that could coincide with CP-AMPARs being inserted into newly generated synapses. However, there is no real link between silent synapses and the appearance of CP-AMPARs, and indeed these could be separate phenomena that occur in separate synapses. There is no demonstration that silent synapses are required for the insertion of CP-AMPARs which would be helpful in supporting this assertion.*

We agree with the referee that silent synapses and CP-AMPARs could be separate phenomena.

Indeed, our previous results (Lee et al., 2013) show inhibiting AMPARs after 45d cocaine withdrawal recovers ~45% of silent synapses within the amygdala-to-NAc projection, suggesting that i) a portion and only a portion of cocaine-generated silent synapses are matured by recruiting CP-AMPARs; and ii) the pronounced upregulation of CP-AMPARs after cocaine withdrawal (Conrad et al., 2008) occurs at most excitatory synapses including both pre-existing and silent synapses. We modified the related sentence to reflect the referee's point:

"Thus, genetic, pharmacological and optogenetic manipulations consistently reveal a correlation between silent synapse reduction and CP-AMPAR expression and vice versa. The most parsimonious explanation is that cocaine-generated silent synapses are at least partially unsilenced by CP-AMPAR accumulation (Huang et al., 2015b)."

3. *Control experiments are performed that show CPP training to be a requirement for the insertion of CP-AMPARs, but no controls are performed to demonstrate that this is true for silent synapses as well. Indeed the presence of silent synapses based on the data in this paper could simply be a result of pharmacological exposure to cocaine. If minimal stimulation assays were performed in the cohorts shown in Figure 1H, it would be of great utility to show these data. If possible, a control should be added in which animals are given either saline or cocaine in the home cage and then silent synapses are measured to determine if CPP training is a requirement for the appearance of silent synapses.*

We performed the suggested experiment, and the data are included in Figure S1 and in the Results.

4. *The authors show a dissociation between the presence of CP-AMPARs and retention of the CPP memory. However, the CPP training regimen is very strong. In one experiment, a "weaker" training paradigm is used in which rats only received 5 days of CPP training. It would be informative to determine if there is any impairment when insertion of CP-AMPARs is prevented when the memory is less thoroughly stamped in.*

In an attempt to address this point, we modified our statement: "While our results show that CP-AMPARs are not required for CPP acquisition and retention, it is possible that our currently used robust CPP procedure might mask some subtle contribution of CP-AMPARs to CPP. However, after relatively weak 5-day procedure, although CPP was not established in wild-type mice, it was established in PSD-95 KO mice, but without inducing subsequent CP-AMPAR insertion after cocaine withdrawal.

Minor 1. *The discussion of silent synapse-based circuit remodeling and silent synapse maturation should be edited as the data do not prove that CP-AMPAR insertion is causally linked to silent synapses.*

We have modified the discussion as suggested.

Minor 2. *It would be helpful to view behavioral data additionally in the form of time in minutes or seconds rather than the normalized place preference score. At the very least a rationale for presenting the data this way should be discussed.*

As suggested, we have provided an explanation and rationale for presenting the normalized PPS: "The PPS takes the time spent in the neutral area into account and as such decreases the variance of the measures compared to the measures of time in the conditioning compartments (Roux et al., 2003). In the normalized PPS, the PPS was subtracted by the baseline PPS, for the purpose of illustration, so that positive normalized PPS depict preference and negative normalized PPS depict aversion."

Minor 3. *There are several grammatical errors and typos scattered throughout the manuscript, please edit.*

We apologize for these errors and have carefully edited the manuscript.

2nd Editorial Decision 12 December 2016

Thank you for submitting your revised manuscript for our consideration. It has now been seen once more by one of the original referees (see comments below), and I am happy to inform you that there are no further objections towards publication in The EMBO Journal.

Referee #3

Authors have done an excellent job of addressing the comments and have gone so far as to provide additional control experiments to complete the study. The study is ready for publication in my opinion.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Oliver Schlüter
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ201695465

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

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 χ2 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.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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?	Power analysis with estimated effect size, which was determined by pilot experiments.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	comparison with published literature with similar methods; previous experience
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Mice with a biased basal preference of >75% for either of the two chmabers were excluded. Recordings with less than 80 data points for minimal stimulation or an increase in series resistance of more than 20% were excluded. Yes.
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.	The assignement of saline or cocaine compartment was pseudo-randomized. Animals were randomly assigned to control or experimental groups.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were randomly assigned to different groups. Both control and experimental groups were run in parallel or interleaved.
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.	The analysis of data was partially performed by two experimenters to avoid bias.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Experimenter was not blinded to the condition of the animal, but the analysis was performed with an automated computer based analysis system.
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.	biological samples are at best pseudo normally distributed. However, it is a good assumption that absolute numbers can be treated as normally distributed and were therefore analyzed with parametric statistics.
Is there an estimate of variation within each group of data?	We calculated the standard error of the mean, which includes the variation.
Is the variance similar between the groups that are being statistically compared?	We did not assume similar variance.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	We validated the used antibodies against the recombinant protein and respective KO animals.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	ATCC

* 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.	Previously described KO lines were backcrossed in C57Bl6J mice and used either as littermates or controls from same background. References for animals are given in the manuscript. Mice were kept in a 12 hour dark/light cycle with food and water at libitium. All procedures were performed during the light cycle.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experimental procedures were approved by the Animal Care and Use Committees of the University Medical School Göttingen, the Lower Saxony State Office for Consumer Protection and Food Safety, or the University of Pittsburgh.
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.	yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
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.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	N/A
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).	N/A
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).	N/A
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	N/A
22. 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.	N/A

G- Dual use research of concern

23. 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