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KAT2A REGULATES A HIPPOCAMPAL GENE EXPRESSION NETWORK LINKED TO MEMORY FORMATION

Roman M. Stilling, Raik Rönicke, Eva Benito, Hendrik Urbanke, Vincenzo Capece, Susanne Burkhardt, Sanaz Bahari-Javan, Jonas Barth, Farahnaz Sananbenesi, Anna-Lena Schuetz, Jerzy Dyczkowski, Ana Martinez-Hernandez, Cemil Kerimoglu, Sharon Y.R. Dent, Stefan Bonn, Klaus Reymann and André Fischer

Corresponding author: André Fischer, German Center for Neurodegenerative Diseases DZNE , Site Göttingen

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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 13 February 2014

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

While both referees appreciate that there is an interest in the reported findings, there are also a number of concerns that have to be addressed in order to consider publication here. Both referees find that you need to strengthen the link between Kat2a and its target genes. Other concerns are also raised, but these are issues that you should be able to resolve. Should you be able to address the concerns raised in full then I would like to invite you to submit a revised manuscript. As you know it is our policy to allow for one major round of revision and it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may

be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The manuscript by Stilling and colleagues presents the analysis of a mouse model with a neuron specific deletion of the K-acetyltransferase (Kat2a) gene. Using this mouse model, the authors present a wide range of data documenting behavioral alterations, changes in synaptic function and gross alterations in neuronal gene expression. The findings, overall, point towards a rather global regulatory role for this histone acetyltransferase in neuronal chromatin function. Although, the data covers a fairly large number of measures, the manuscript stands as rather descriptive and its unique contribution to the field remains obscure. Over the last decade, impairments in several epigenetic regulatory pathways have been shown to alter behavioral and synaptic measures as shown here. However, the exact mechanistic bases of these observations remain unclear.

1. The authors show that the loss of Kat2a affects 'neuroactive-ligand receptor signaling' and specifically downregulates several key genes, including specifically several serotonin receptors which the authors then speculate lead to the observed phenotype. It is important to validate this link to be able to fully establish the mechanistic basis of the phenotypes observed.

2. In the CA1 specific deletion of Kat2a (Kat2a fl/fl -AAVCre), the use of WT (Kat2a +/+ -AAVCre) mice injected with Cre allows one to assess the off-target effects of Cre. However, the control that should have been used was to inject AAV-GFP into floxed Kat2a mice. As it stands, the authors cannot rule out that some of the reported behavioral phenotypes in the localized hippocampal deletion of Kata2a could be do to the comparison to WT mice.

3. In the mice with the localized deletion of Kata2a in the hippocampus, how were the placements confirmed? A representative figure should be shown.

4. Is Kat2a expressed in other brain regions such as the amygdala and striatum that can be targeted by most CaMKII-Cre driver lines? Was Kat2a expression altered in these brain regions in the conditional knockout mice? Additionally, the authors should specify which CaMKII-Cre driver line was used.

5. In the novel object recognition task (Figure 2G) the Kata2a cKO mice show a significant increase in long-term memory compared to chance suggesting there is not a deficit in this behavioral measure. The comparison to the control mice is interesting but the argument that the Kat2a cKO have less of a significant learning enhancement compared to wildtype control mice is hard to claim as a significant learning and memory deficit.

6. From Figure 1A, Kat5 appears to be as highly expressed in the hippocampal CA1 region as Kat2a. Why was Kat2a selected over Kat5 for further study?

7. The number of platform crossings of the control mice in Figure 3H is surprisingly low. The authors should comment on this point.

Referee #2:

In the manuscript by Roman Stilling et al., the authors explore the involvement of histone acetylation in memory formation by knocking out K-LYSINE ACETYLTRANSFERASE 2A (KAT2A) in mouse brain.
Starting from comparative analysis of the expression level of 18 mammalian HATs in hippocampus, the authors identified K-LYSINE ACETYLTRANSFERASE 2A as the highest transcribed HAT within the hippocampal CA1 region, the brain area principally involved in memory formation.

As predicted by the authors, a mouse conditional KO lacking Kat2a in neurons, showed impaired hippocampal synaptic plasticity and long-term memory consolidation. To characterize the transcriptional network controlled by KAT2A in resting conditions and upon training, the authors analyzed the Kat2a-dependent gene expression program in the hippocampal CA1 region in both resting conditions (unstimulated) and upon novelty exposure and highlighted and interesting network of genes linked to neuroactive ligand receptor signaling. In parallel the authors describe how impairment in memory consolidation is paralleled by impaired hippocampal plasticity, analyzing LTP at the CA3-CA1 synapse.

As is, the work is good and interesting but few points need to be answered and some clarification are required to make it more understandable

Major point
1
-The authors identified among possible KAT2A-regulated genes, several NFkB targets, and indicate KAT2A as NFkB interactor by coimmunoprecipitation experiments. Does the interaction between NFkB and KAT2A requires p65 to be acetylated? In the coimmunoprecipiation experiment interaction between KAT2A and p65 should be checked also using an antibody that does not discriminate between acetylated and non acetylated p65 and compare the amount of the coimmunoprecipitation.
-In figure 4G CoIP-IP-PreImmuneIgG and Input should all be shown as part of the same gel and film.
-In the experiment is not shown if overall the level of p65K310ac is reduced. The authors should show it in the input together with p65.

2
To strengthen the functional implication of KAT2A with NFkB-mediated gene regulation, it would be important to show that KAT2A is associated to the same NFkB targets by chromatin immunoprecipitation.

3
-How the chromatin immunoprecipitation was quantified? This is a very tricky matter and without any information on how the analysis was performed is difficult for me to assess the relevance. Furthermore, no negative controls to validate specificity of the immunoprecipitated chromatin are mentioned (known non-NFkB target)

Minor concern
The choice of Kat2a as possible HAT involved in memory formation was based on its high expression level within a brain area crucial for memory formation and consolidation. Strategically to choose the most abundantly expressed HAT as promising candidate in memory-related functions is a good starting point, but in general the expression level of a specific gene does not necessarily have to correlate with its biological relevance. For this reason I do not understand when the authors refer to Kat3a as another example of HAT involved in memory formation but expressed at rather low level in the hippocampal CA1 region for which "...consistently demonstrated conflicting results regarding spatial learning in the water maze test while more consistent findings were observed for as object recognition learning or fear conditioning were more robustly impaired". Could the authors better explain this concept?

1st Revision - authors' response 11 May 2014

Response to referee 1:

Referee #1 says: "*The manuscript by Stilling and colleagues presents the analysis of a mouse model with a neuron specific deletion of the K-acetyltransferase (Kat2a) gene. Using this mouse model, the authors present a wide range of data documenting behavioral alterations, changes in synaptic function and gross alterations in neuronal gene expression. The findings, overall, point towards a rather global regulatory role for this histone acetyltransferase in neuronal chromatin function. Although, the data covers a fairly large number of measures, the manuscript stands as rather descriptive and its unique contribution to the field remains obscure. Over the last decade,*

impairments in several epigenetic regulatory pathways have been shown to alter behavioral and synaptic measures as shown here. However, the exact mechanistic bases of these observations remain unclear."

We really appreciate this comment. However, we feel that there must have been a misunderstanding regarding the statement that our data is descriptive and that its contribution to the field is obscure. We can only imagine that the introduction was written in a misleading way and apologize for that. It is true that the role of histone-acetylation in memory function has been studied previously, but mechanistic insight is still limited. In fact, while the mammalian genome encodes 18 histone-acetyltransferases (HATs) only CBP has been studied in greater detail for its role in the adult brain and this is – at least to some extend - due to historical reasons that link CBP directly to CREB signaling, a major pathway linked to memory formation. Only one additional paper investigated PCAF and another one P300. Therefore we used an unbiased approach to study – for the first time – the expression of all HATs in a brain region essential for memory consolidation, namely the CA1 region. On this basis we decided to investigate *Kat2a* for its role in memory formation. We show for the first time that KAT2a regulates memory function and provide mechanistic insight by deciphering the underlying gene-expression network that is controlled by KAT2a function. To the best of our knowledge there is no published data in which lack of a HAT in the adult mouse brain has been studied on the level of whole gene expression using RNA-sequencing. Only two studies employed microarray approaches to study gene-expression within the entire hippocampus in CBP knock out mice (Chen et al, 2010) (Lopez-Atalaya et al, 2011). Of note, the genes regulated by KAT2a appear to differ completely form genes regulated by CBP and therefore our findings set the stage for creating a database of regulatory gene-expression networks affected by the various HATs. We furthermore provide first evidence that KAT2a associates with NF-κB to regulate learning-associated genes.
Thus, in our opinion we provide multiple lines of important new insight. We apologize if this point might have been presented in a misleading way. At the same we argue that it is very likely that no only one mechanism underlies the action of an epigenetic enzyme such as KAT2a on memory function. To support this view we now also present novel data to suggest that KAT2a affects mRNA editing.

We now address all these issues in the revised version of the manuscript. See pages 2, 13 & 14 as well as novel Fig. S6 &Table S6 of the revised manuscript.

Referee #1 states:
"1. *The authors show that the loss of Kat2a affects 'neuroactive-ligand receptor signaling' and specifically downregulates several key genes, including specifically several serotonin receptors which the authors then speculate lead to the observed phenotype. It is important to validate this link to be able to fully establish the mechanistic basis of the phenotypes observed.* "

This is excellent suggestion and essentially repeats the previous statement. However we feel that it is beyond the scope of a single manuscript to *fully establish* the underlying mechanisms of a phenotype that is as complex and as memory formation. The data provided in our study is based on an unbiased screening approach and subsequently combines mouse genetics, behavior biology and electrophysiology to demonstrate a role for KAT2a in hippocampus-dependent memory formation. In the next step we used RNA-sequencing to study the transcriptome and the microRNAome in order to elucidate the hippocampal gene-expression programs controlled by Kat2a. We find that KAT2a levels do not affect the microRNA, which is – albeit being "negative data" very interesting. Especially, since we find that within the *Kat2a*-dependent genes the "neuroactive-ligand-receptor signaling pathway" is overrepresented. We then selected genes of this pathway which have been clearly linked to memory formation on the basis of pharmacological and/or genetic studies for ChIP analysis. We would argue that the reduced expression of genes that have been linked to memory impairment in a variety of previous studies provide substantial evidence that loss of Kat2a – at least in part – leads to memory impairment via the regulation of such genes. We are however aware that we have most likely not discovered all mechanisms by which Kat2a controls memory formation. In fact it is in our view not plausible to expect that only one mechanism underlies the complex actions of HATs. To further support this view we now also present new data to suggest that KAT2a affects mRNA editing. We address this issue now in greater detail on pages page 14 and Fig S6 of the revised manuscript.

Referee #1 says that:
"*2. In the CA1 specific deletion of Kat2a (Kat2a fl/fl -AAVCre), the use of WT (Kat2a +/+ -AAVCre) mice injected with Cre allows one to assess the off-target effects of Cre. However, the control that should have been used was to inject AAV-GFP into floxed Kat2a mice. As it stands, the authors cannot rule out that some of the reported behavioral phenotypes in the localized hippocampal deletion of Kata2a could be do to the comparison to WT mice.* "

We understand that referee 1 is concerned about the control mice used in the experiment in which we deleted *Kat2a* specifically from the CA1 region using viral-mediated expression of CRE. He/she is specifically concerned that we compared *Kat2a fl/fl* –AAVCre mice to wild type –AAVCre mice.
As in any experiment that uses a dual genetically modified system, in this experiment, there are two potential confounding factors that one can control for: 1. the loxP sites, and 2. the CRE protein that will be overexpressed. Based on experience and published data we felt that controlling for the effect of viral overexpressed CRE is more important than to control for the loxP sites. This is because extreme overexpression of CRE can be cytotoxic, while the integration of the relatively short loxP sites in two introns of the *Kat2a* locus does to affect the expression level of the gene or causes any other adverse effects. At the same time the German animal protection law, which also regulates experimental work with mice and has become even more strict in the recent past is especially emphasizing strict application of the 3 R's (Replacement, reduction and refinement) in all animal research activities. Thus we had chosen the current control condition (i.e. *Kat2a*+/+ mice overexpressing CRE) to accommodate any potential confounding due to CRE expression.

However, appreciating this reviewers concerns we now have designed a further experiment to control for the integrated loxP sites. Here, we injected an AAV overexpressing GFP into wild type ($Kat2a^{+/+}$) mice and $Kat2a^{fl/fl}$ mice and tested them in the Morris water maze. We do not find any significant differences among groups suggesting that the floxed allele has no effect on cognitive function. This data has now been included as novel panels in **Figure S2 G-H** and is discussed on page 6 of the revised manuscript.

Referee #1 states that:

"*3. In the mice with the localized deletion of Kata2a in the hippocampus, how were the placements confirmed? A representative figure should be shown*."

We appreciate the reviewers comment. Our lab has documented ample experience in *in vivo* manipulation of the adult brain using either microcannulae or guided injection via glass capillaries. We addressed this issue to some extend in previous FigS2 and we apologize that this data was incomplete. In addition to showing the specific deletion of *Kat2a* from the CA1 region we now include an overview image showing that *Kat2a* is expressed in the cortex and the hippocampus (Fig S2A) along with an overview image showing the expression of CRE-GFP specifically in the CA1 region but not in any other hippocampal region nor in the cortex. This data is now presented as novel panels A & B in Figure S2 of the revised manuscript.

Referee #1 remarks that:
"4. *Is Kat2a expressed in other brain regions such as the amygdala and striatum that can be targeted by most CaMKII-Cre driver lines? Was Kat2a expression altered in these brain regions in the conditional knockout mice? Additionally, the authors should specify which CaMKII-Cre driver line was used*."

We had mentioned details about the CRE line used in the methods section of the original manuscript. We now describe this even more specifically in the revised version of the manuscript. See page 15. Furthermore, in the original manuscript in Figure 1D we showed expression of *Kat2a* in (neo)cortex (NC), hippocampus (including subregions) and cerebellum. In all forebrain tissues we find a dramatic reduction of *Kat2a* in conditional knockout mice, but not in cerebellum – as expected for this driver line. We have now additionally added data for another brain region, namely the prefrontal cortex, a region that is critically involved in remote memory storage (see revised **Figure 1B**).
We understand however that this comment is mainly referring to the possibility that the phenotypes observed in *Kat2a* cKO mice are also due to the deletion of *Kat2a* in other brain regions than the

hippocampus. It is true that this cannot be excluded when using our CamKII-CRE driver line. This is the reason why we have chosen an additional viral approach to express CRE specifically in the CA1 region. To address this reviewer's concern we now specifically mention that – while the specific deletion of *Kat2a* from the CA1 region confirms that KAT2A in the hippocampus is essential for memory formation – it does not exclude the possibility that KAT2A in other brain regions also plays a role in memory function. See page 10 of the revised manuscript.

Referee #1 says:
"*5. In the novel object recognition task (Figure 2G) the Kata2a cKO mice show a significant increase in long-term memory compared to chance suggesting there is not a deficit in this behavioral measure. The comparison to the control mice is interesting but the argument that the Kat2a cKO have less of a significant learning enhancement compared to wildtype control mice is hard to claim as a significant learning and memory deficit."*

We appreciate this comment and have addressed this issue accordingly. See page 5.

Referee #1 says:
"*6. From Figure 1A, Kat5 appears to be as highly expressed in the hippocampal CA1 region as Kat2a. Why was Kat2a selected over Kat5 for further study?* "

We agree that KAT5 would also have been an interesting protein for further studies. Indeed, a PhD student has recently started to work on this project, which is however very far away from being published in a scientific manuscript. Additional reasons for choosing *Kat2a* for further investigation were data reporting its up-regulation during memory formation in the fear-conditioning paradigm (see Peleg et al. Science, 2010) and its role in stimulus-mediated gene expression, as demonstrated in diverse tissues and organisms (see Hargreaves et al. 2009 in Cell, Johnsson et al. 2009 in EMBO reports). To further address this reviewers concern we now describe this issue more clearly in the description of the experimental design and in the discussion of the revised manuscript. See page 3 of the revised manuscript.

Referee #1 says:
"*7. The number of platform crossings of the control mice in Figure 3H is surprisingly low. The authors should comment on this point.*"

This is an excellent point and we apologize that we did not address this issue more specifically. It is true that on average the platform crossings were higher when comparing the experiment depicted in Fig3D (deletion of *Kat2a* by crossing with CamKII-Cre driver line) and Fig3H (*Kat2a* was deleted by injection of AAV-CRE particles). It is well known that behavior experiments are variable and depend on multiple factors such as the day of testing or the experimental set up. A recent study even suggests that the "smell" of the experimenter has direct influence on behavior (Crabbe et al., Science, 284,1999; Lewejohann et al., Genes Brain & Behav., 5, 2006; Grimm, Science, 344, 2014; Sorge et al., Nature Methods, 2014). Although we try to control such factors as much as possible it is generally difficult - if not impossible - to compare two independent behavior experiments quantitatively. Thus, each experimental group can only be quantitatively compared to its own experimental control group that has been tested an treated at the same time. It is however possible to compare different experiments qualitatively and in this case our data fits very well in that deletion of *Kat2a* using a CamKII-CRE driver mouse line or AVV-mediated expression of CRE both impairs memory function and platform crossings. We now address this issue in the revised manuscript See page 17.

Response to eferee #2:

Referee #2 says that our " *work is good and interesting but few points need to be answered and some clarification are required…*"

We appreciate this very encouraging comment.

Referee #2, point 1:
"*The authors identified among possible KAT2A-regulated genes, several NFkB targets, and indicate*

KAT2A as NFkB interactor by coimmunoprecipitation experiments. Does the interaction between NFkB and KAT2A requires p65 to be acetylated? In the coimmunoprecipiation experiment interaction between KAT2A and p65 should be checked also using an antibody that does not discriminate between acetylated and non acetylated p65 and compare the amount of the coimmunoprecipitation"
-In figure 4G CoIP-IP-PreImmuneIgG and Input should all be shown as part of the same gel and film.
-In the experiment is not shown if overall the level of p65K310ac is reduced. The authors should show it in the input together with p65. "

We appreciate this very insightful comment and have now revised Figure 4G accordingly to show all samples on one western blot. At the same time we like to mention that the message to be communicated by the data may be more difficult to be appreciated in the revised version of the figure. Thus, we would leave if to the editor to use the revised version of Fig 4 or alternatively keep the original version for Fig. 4 and move the new data showing the original blot to the supplement. In any case the newly presented image now also includes data suggesting that *Kat2a* also co-precipitates with non-acetylated p65. From the image it appears that the association between KAT2a and acetylated p65 is stronger as for the non-acetylated form of p65. Nevertheless we like to point out that although an equal amount of input material was used for all immunoprecipitation reactions, differences in antibody affinity make it impossible to draw quantitative conclusions. We now discuss this issue in the revised version of the manuscript. See revised Fig 4 and page 9 of the revised manuscript.

Furthermore, the reviewer raises the concern that p65 levels might be altered. While we don't see any indication for changes of p65 expression in our RNA-seq data, we have further investigated this possibility on the protein level and have added this data in the revised Supplementary Figure S4C. We find no difference in hippocampal p65 levels between *Kat2a* cKO and control mice.

Referee #2, point 2:
" *To strengthen the functional implication of KAT2A with NFkB-mediated gene regulation, it would be important to show that KAT2A is associated to the same NFkB targets by chromatin immunoprecipitation."*

We completely agree with this suggestion. We tried extensively to establish KAT2a ChIP using various antibodies (NBP1-00845, Novus Biologicals; ab1831, Abcam; 07-1545, Millipore; 3305, Cell Signaling; sc-20698 (H-75), Santa Cruz; 607201 Biolegend). Using our knock out mice as control we were able to identify antibodies that reliably detected KAT2a in immunoblots and immunostaining. However, we failed to obtain an antibody that would specifically prepiciptate KAT2a in various ChIP protocols we have established in the lab.

Thus, regardless of our established protocols and extensive experience in performing ChIP on limited amounts of brain tissue we were not able to obtain specificity for KAT2a. Interestingly, to the best of our knowledge, there are no published reports of any successful application of ChIP on tissue samples for Kat2a/Gcn5, suggesting that either antibody quality or target presentation in the multiprotein complex that Kat2a is known to work in is insufficient for ChIP experiment. We like to mention that the monoclonal rabbit antibody (Cell signaling) used in this study was the only of 11 tested Kat2a antibodies that showed sufficient specificity in both, our western blot and immunohistochemistry experiments. The polyclonal rabbit antibody sc-20698 (H-75), (Santa Cruz) worked for western blot but showed the same staining in IHC in both cKO and control sections.

However, since western blot works with linearized, denatured proteins it is impossible to predict ChIP performance of these antibodies and both of them failed to provide satisfactory results in ChIP experiments.
We now address this issue on page 18 of the revised manuscript.

Referee #2, point 3:
"*How the chromatin immunoprecipitation was quantified? This is a very tricky matter and without any information on how the analysis was performed is difficult for me to assess the relevance. Furthermore, no negative controls to validate specificity of the immunoprecipitated chromatin are mentioned (known non-NFkB target)*"

We now describe in detail how ChIP experiments were quantified in the material and methods sections. See page 20 of the revised manuscript.

For the second part of this comment, we feel there has been a misunderstanding. If a gene is not regulated by NFkB we would not expect to find NFkB at the promoter of this gene and thus would need to show “no amplification” during qPCR, which does not provide useful insight.

However we understand that this reviewer is asking for some kind of negative control. To address this issue we therefore selected two known NFkB target genes that have been verified in other tissues than brain but were - according to our RNA-seq data - expressed in the hippocampal CA1 region, but were not differentially regulated in our experimental system. We show that there is no difference in the enrichment for p65 and histone acetylation at the promoters (around the predicted NFkB target site) for these genes. See revised supplementary Figure S4 D and discussion on page 9 of the revised manuscript.

Minor concern
The choice of Kat2a as possible HAT involved in memory formation was based on its high expression level within a brain area crucial for memory formation and consolidation. Strategically to choose the most abundantly expressed HAT as promising candidate in memory-related functions is a good starting point, but in general the expression level of a specific gene does not necessarily have to correlate with its biological relevance. For this reason I do not understand when the authors refer to Kat3a as another example of HAT involved in memory formation but expressed at rather low level in the hippocampal CA1 region for which "...consistently demonstrated conflicting results regarding spatial learning in the water maze test while more consistent findings were observed for as object recognition learning or fear conditioning were more robustly impaired". Could the authors better explain this concept?

We apologize for this confusion and have now reworded this passage. See page 11 of the revised manuscript.

2nd Editorial Decision 12 June 2014

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been re-reviewed by the two referees. Both referees appreciate the introduced changes and support publication here. Referee #2 has no further comments to the authors. I am therefore very pleased to accept the manuscript.

REFEREE REPORT

Referee #1:

The authors have addressed my previous concerns.

