Uncovering the role of G9a modulatory landscape promoting neuronal plasticity and neuroprotection in Alzheimer's disease

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Article

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Uncovering the role of G9a modulatory landscape promoting neuronal plasticity and neuroprotection in Alzheimer's disease

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Abstract

Epigenetic alterations are a fundamental pathological hallmark of Alzheimer’s disease (AD). Herein, we uncover the unknown G9a modulation pathways involved in AD, showing the upregulation of G9a and H3K9me2 in the brains of AD patients. Likewise, treatment with a G9a inhibitor in SAMP8 mice reversed the high levels of H3K9me2 and rescued the cognitive decline. Interestingly, a transcriptional profile analysis revealed induction of neuronal plasticity and a reduction of oxidative stress and neuroinflammation; the latter being also validated in cell cultures. Furthermore, an exploratory H3K9me2 ChIP-seq analysis demonstrated that during G9a inhibition treatment, the H3K9me2 mark is enriched at the promoter of genes associated with neural functions. Lastly, we showed in Caenorhabditis elegans (C. elegans) AD transgenic strains, similar epigenetic modifications and modulated pathways were altered with increased β-amyloid levels, which were reverted by the set-25 (in C. elegans is similar to the mammalian G9a protein) knockout, including the cognitive impairment. Therefore, our findings confirm that RNAi suppression of set-25 or pharmacological G9a inhibition promotes a positive outcome in AD, being a promising therapeutic strategy.
Introduction

Aberrant epigenetic mechanisms represent one of the key pathophysiological drivers of aging and neurodegeneration\(^1,2\), and are relevant to the progression of age-related cognitive decline, including Alzheimer's disease (AD)\(^2\).

Transcriptionally activating and repressing global histone changes in different regions have been observed in postmortem AD brains\(^3\). Despite enormous research, none of the clinically approved drugs for AD is effective, being all of them only symptomatic treatments, thereby there is an urgent need to identify new targets based on epigenetic therapies for neurodegenerative diseases\(^4\).

Of note, growing evidence suggests that lysine methyltransferases (KMTs), such as G9a, act as a crucial regulator in human diseases\(^5\). G9a is a KMT able to mono- and di-methylate K9 of histone H3 (H3K9me1 and H3K9me2), which are marks linked to the repression of genes implicated in synaptic plasticity\(^6\)–\(^9\), learning, and memory formation\(^3,10\)–\(^12\). However, until now, G9a has been widely explored as an anti-cancer and an anti-malarial target\(^13\). Recently, it has been demonstrated overexpression of G9a in the brain from late-stage familial AD (5XFAD) mice and AD patients\(^3\). Similarly, another study showed elevated levels of H3K9me2 in the occipital cortex of postmortem AD brain compared to the non-demented (ND) group\(^14\). Besides, H3K9me2 seems to participate in the pathogenesis of neurodegenerative disorders, such as posttraumatic stress disorder (PTSD)\(^15\) and anxiety-like behavior\(^16\). In Caenorhabditis elegans (C. elegans), the putative methyltransferase that targets H3K9me2 is SET-25\(^17\), which is similar to the mammalian G9a protein (28.8% identity, 44.6% similarity)\(^18\), and it has only been implicated in the transgenerational epigenetic
inheritance of RNAi, thereby SET-25 is required for RNAi-initiated heritable
gene silencing\textsuperscript{18,19}.

Over the last decade, several \textit{in vitro} and \textit{in vivo} studies demonstrated that G9a
inhibition reduces neurodegeneration and cognitive impairment\textsuperscript{3,12}. Indeed, the
G9a inhibition by BIX01294 prevented the β-amyloid (Aβ) oligomer-induced
late-long term potentiation (LTP) and induced synaptic plasticity by increasing
the gene expression of \textit{brain-derived neurotrophic factor} (\textit{Bdnf}), previously
repressed\textsuperscript{3,7,12,16}. Subsequently, three selective G9a inhibitors such as A-366,
UNC0638, and UNC0642 demonstrated neuroprotective effects\textsuperscript{20}. Notably, our
group demonstrated that G9a inhibition by UNC0642 produced significant
neuroprotective effects in an early-onset AD mouse model\textsuperscript{12}.

Given that, several pieces of evidence underline a broad involvement of
H3K9me2 in the central nervous system (CNS) function and disease. In the
present study, we confirmed the high levels of H3K9me2 in samples of AD
human patients. Importantly, we focused on the new modulated pathways by
G9a inhibition that underlie how H3K9me2 promotes changes in the
transcriptome of the CNS. Then, an integrative sequencing data analysis using
RNA- and miRNA-seq was performed to further explore differential gene
expression under G9a inhibition. Besides, the inspection of H3K9me2
chromatin immunoprecipitation assays with sequencing (ChIP-seq) revealed
that H3K9me2 is enriched at genes associated with neural functions in cells
treated with a G9a inhibitor. Unexpectedly, our analysis showed that
pharmacological G9a inhibition induces changes in different molecular
pathways. Strikingly, our findings were validated in primary cell cultures and \textit{C.
elegans} set-25 knockout, clarifying precise mechanisms underlie by G9a
inhibition. Thus, these results provide new insights about the transcriptional
modulatory capacity of the G9a as a trigger of neurodegeneration and the
beneficial effects of RNAi suppression of set-25 or pharmacological targeting of
G9a as a treatment for AD.

Results

Overexpression of G9a and its repressive mark H3K9me2 in AD patients’
brains correlates with increased levels of Aβ amyloid.

Alterations in the histone methylation marks and their enzymes are implicated in
senescence, age-related cognitive decline and AD, but as far as we know,
their direct link is not yet well described. To characterize G9a as a novel target
for AD, we carried out several experiments and bioinformatic analyses (Fig 1a).
Firstly, we examined G9a protein levels by western blot (WB) in human AD
patients' brains. Of relevance, we found higher levels of this protein in AD
patients' brains compared to the human ND patients' brains (Figs. 1b and c,
Supplementary Table 1). Since G9a is a KMT enzyme responsible for
depositing methyl groups in H3K9, we also evaluated H3K9me2, a repressive
mark. Higher H3K9me2 levels were statistically significant in human AD patients
when compared to ND group (Figs. 1d and e). Furthermore, we found that the
ratio of Aβ42/Aβ40 was significantly increased in AD patients in comparison with
the ND human group (Fig. 1f). Of relevance, we also found a strong positive
correlation between H3K9me2 levels and Aβ concentration in AD patients (Fig.
1g). Thus, the data reveal that high H3K9me2 are correlated with increased
levels of Aβ in human AD patients’ brains, indicating the role of H3K9me2 in the
neurodegenerative process of AD.
Figure 1. Overexpression of G9a and its repressive mark H3K9me2 in AD patients' brains correlates with increased levels of Aβ amyloid. a. Scheme of experimental procedures for in vitro and in vivo experiments. b. and c. Representative WB, and quantification for G9a (EHMT2) of human patients’ brains. d. and e. Representative WB, and quantification for histone H3K9me2 of human patients’ brains. f. The ratio of Aβ42/Aβ40 by ELISA in human patients’ brains. Values represented are mean ± Standard error of the mean (SEM); (n = 14 (ND n = 6, AD n = 8)). Groups were compared by Student’s t-test analysis (significant at *p < 0.05; ***p < 0.001). g. Correlation between Aβ42/Aβ40 ratio and H3K9me2 (slope = 0.2394). R² and p-values are indicated on graphs (significant at *p < 0.05). Source data are provided as a Source Data file.

Inhibition of G9a with UNC0642 leads to a reduction in H3K9me2 and Aβ levels as well as behavioral and cognitive improvements in SAMP8 mice.

Because aging is the main risk factor for AD, we determined the G9a protein in the SAMP8 strain, a well-established AD model to investigate the key mechanisms of age-related cognitive decline with accelerated aging and generated through phenotypic selection from AKR/J strain of mice. Then,
we examined protein levels in the hippocampus by WB, including in SAMR1, a normal aging control. G9a protein expression was higher in the SAMP8 control group in comparison with the SAMR1 Control group (Figs. 2a and b). Likewise, significantly higher levels of the ratio H3K9me2 were only observed in the SAMP8 Control group compared to the SAMP8 treated with UNC0642 mice group (Figs. 2c and d). Increased Aβ42/Aβ40 ratio levels in the hippocampus of SAMP8 were reduced by G9a inhibition (Fig. 2e). Notably, we found a strong positive correlation between H3K9me2 levels and Aβ concentration in SAMP8 mice (Fig. 2f). Thus, the data suggest that high H3K9me2 is correlated with increased levels of Aβ in SAMP8, suggesting the contribution of the G9a and H3K9me2 in the age-related cognitive decline presented by this AD rodent model.

Aβ levels are associated with behavioral abnormalities and cognitive decline, so we next performed behavioral studies to determine whether G9a inhibition with UNC0642 could revert them in SAMP8. Firstly, we used the three-chamber test (TCT) to assess the general sociability behavior in mice. In the sociability phase, in all mice groups, the presence of an intruder increased the time significantly spent in the intruder chamber instead of the empty cup chamber (Fig. 2g). Remarkably, we only found a significant increase in the time sniffing the intruder mouse in the SAMP8 treated mice group, confirming the improvement in social behavior after G9a inhibition by UNC0642 at 5mg/Kg (Fig. 2h). To evaluate the working and spatial memories, mice were assessed in the novel object recognition (NORT) and object location (OLT) tests, respectively. During the familiarization phase of the NORT task (Fig. 2i), the exploration time was unchanged by UNC0642 treatment. The task results
revealed that SAMP8 treated with UNC0642 exhibited a significant reduction in
cognitive deficits in both short- and long-term memories (Figs. 2j and k),
presented by the SAMP8 Control mice group.

Regarding the OLT results, the exploration during the habituation phase (Fig. 2l) was unaffected by the treatment. The task results showed that SAMP8 treated with UNC0642 exhibited an increase of the discrimination index (DI) compared to the SAMP8 Control mice group (Fig. 2m), suggesting an improvement in spatial memory. Finally, the polygonal graph depicts differences among SAMR1 and SAMP8 Control, and SAMP8 treated mice groups by graphing several TCT parameters, DI of NORT and OLT, and molecular ratios of H3K9me2 and Aβ_{42}/Aβ_{40} (Fig. 2n). This data suggests that the G9a inhibition promotes beneficial effects on behavior and cognition.
Figure 2. Inhibition of G9a with UNC0642 leads to a reduction in H3K9me2 and Aβ levels as well as behavioral and cognitive improvements in SAMP8 mice. a. and b. Representative WB, and quantification for G9a in the hippocampus of SAMR1 and SAMP8 mice. c. and d. Representative WB, and quantification for H3K9me2 of SAMR1, SAMP8 and SAMP8 treated with UNC0642 (5mg/Kg) mice groups. e. The ratio of Aβ42/Aβ40 by ELISA. f. Correlation between Aβ42/Aβ40 ratio and H3K9me2 (slope = 0.8168). R² and p-values are indicated on graphs (*significant at p < 0.05). Values represented are mean ± SEM; (n = 18 (SAMR1 n = 6, SAMP8 Control n = 6, and SAMP8 UNC0642 (5mg/Kg) n = 6)). Groups were compared by Student’s t-test analysis or One-Way ANOVA and post-hoc Tukey’s test (significant at *p < 0.05; **p < 0.01; ***p < 0.001 vs SAMP8 Control). g. Time spent in chamber and h. sniffing time: sociability with the intruder animal. i. Familiarization phase. j. Short-term memory evaluation after 2 h of the acquisition trial by Discrimination Index and k. long-term evaluation after 24 h of the acquisition trial by Discrimination Index.
after exposure to novel objects of SAMR1, SAMP8 and SAMP8 treated with UNC0642 (5mg/Kg) mice groups. I. Habituation phase. m. OLT evaluation by Discrimination Index after changing the location of one object. n. The polygonal graph presented complete parameters obtained by TCT, NORT, WB and ELISA. Values represented are the mean ± SEM; (n = 30 (SAMR1 n = 10, SAMP8 Control n = 10, and SAMP8 UNC0642 (5mg/Kg) n = 10)). Groups were compared by One-Way ANOVA and post-hoc Tukey’s test (significant at *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 vs SAMP8 Control). Source data are provided as a Source Data file.

G9a inhibition with UNC0642 induces a transcriptional profile that allows beneficial effects on cognitive performance. To characterize the transcriptional profile associated with UNC0642 treatment, we analyzed the transcriptome by RNA-seq in SAMP8 control and SAMP8 UNC0642 (Fig. 3a, Supplementary Table 2). Differential expression analysis identified 697 differentially expressed genes (DEG) (fold change cutoff of ≥ 1.3, p-value < 0.05). Of which 217 are reduced, and 480 are increased in SAMP8 UNC0642 (Fig. 3b).

Functional analysis shows that DEGs regulate processes such as neuroactive ligand-receptor interaction, calcium signaling pathway and G–protein coupled receptor signaling pathway (Figs. 3c and d, Supplementary Figs. 1a-c), both important in regulating the function and plasticity of neural networks in the CNS²⁴. Interestingly, we identified an increase in the expression of genes associated with the sensory perception of mechanical stimulus (Fig. 3d, Supplementary Fig. 1d), which allows us to explain the beneficial effects on behavior and cognition induced by UNC0642 in the SAMP8 mouse model. On the other hand, we found a reduction in genes associated with the NF-κB signaling pathway (Fig. 3c), hinting that G9a inhibition could reduce neuroinflammation in the LOAD mouse model.
A more detailed analysis performed with Gene Set Enrichment Analysis (GSEA) shows enrichment of processes associated with sensory perception in SAMP8 treated with UNC0642 (Supplementary Figs. 2a and b). Among these processes, we can find sensory processing of sound, sensory perception of the light stimulus, and phototransduction visible light (Supplementary Figs. 2a and b), supporting the idea that UNC0642 treatment in the SAMP8 model activates genes that promote beneficial effects on behavior and cognition. Interestingly, we found an increase in interleukin 37 (IL-37) signaling in SAMP8 UNC0642 (Supplementary Figs. 2a and b). IL-37 is an anti-inflammatory cytokine that suppresses immune responses and inflammation in different tissues such as the brain. Of note, we identified an enrichment of processes associated with chromatin methylation, such as regulation of histone modification and histone H3K9 methylation (Supplementary Fig. 2) confirming an effect of UNC0642 on histone methylation status.

To validate the RNA-seq, we performed RT-qPCR for some DEGs such as Gmfb, synaptosome associated protein 25 (Snap25), T-box brain transcription factor 1 (Tbr1), and calcium/Calmodulin dependent protein kinase II gamma (Camk2g), which were significantly deregulated (Figs. 3e-g). Taken together, these data suggest that treatment with UNC0642 induces a transcriptional profile that allows beneficial effects on behavior and cognition.
Figure 3. G9a inhibition treatment induces a transcriptional profile that confirms the neuroprotective effects in the hippocampus of SAMP8 mice. 

a. Treatment, hippocampal extraction, and RNA-sequencing in SAMP8 Control and SAMP8 treated with UNC0642 (5mg/Kg). 
b. Volcano plot showing the DEG (fold change cutoff of ≥ 1.3, p-value < 0.05). Downregulated in green and upregulated in red. c. KEGG pathways regulated by SAMP8 treated with UNC0642 (5mg/Kg). 
d. Biological processes altered by DEG in SAMP8 treated with UNC0642 (5mg/Kg). 
e. Gene expression levels of Gmfb, f. Snap25, g. Tbr1, and h. Camk2g. Gene expression levels were determined by RT-qPCR. Values represented are the mean ± SEM; (n = 12 (SAMP8 Control n = 6, and SAMP8 UNC0642 (5mg/Kg) n = 6)). Groups were compared by Student’s t-test.
analysis (significant at *p < 0.05; **p < 0.01). Source data are provided as a Source Data file.

**G9a inhibition treatment increases the GMFB transduction pathway, restoring dendritic spine density in SAMP8 mice.**

Pathologically, it is known that SAMP8 presents a reduction in neuronal spine density\textsuperscript{26}. In addition, GMFB is a highly conserved brain-enriched protein implicated in neuroplasticity since a peak of these protein levels were correlated with learning and memory formation\textsuperscript{27}. Then, to further confirm GMFB pathway activation after the G9a inhibition in SAMP8, we investigated the GMFB protein levels and the downstream effector proteins using WB. Strikingly, a significant increase of GMFB was observed in the SAMP8 treated with UNC0642 in comparison with the SAMP8 Control mice group (Figs. 4a and b). Further, we evaluated ratio p-p38/p38, and cAMP-response element binding protein (CREB) protein levels. A significant increase in p-p38 protein levels were found in SAMP8 treated group with UNC0642 (5 mg/Kg) (Figs. 4a and c). Likewise, the ratio p-CREB/CREB was also augmented but did not reach significance between groups (Figs. 4a and d). Finally, we also evaluated the protein levels of BDNF and its receptor, tropomyosin-related kinase B (TrkB). Regarding the ratio of protein levels of p-TrkB/TrkB, we observed a significant increase in SAMP8 treated compared to the SAMP8 Control group (Supplementary Figs. 3a and b). Accordingly, an increase of BDNF protein levels in SAMP8 after UNC0642 treatment was found (Figs. 4a and e, Supplementary Figs. 3c).

Simultaneously, we found a significant increase in the dendritic spine length (Figs. 4f and g) and density in the brain treated with UNC0642 SAMP8 compared to the SAMP8 Control group, showing the potential of G9a inhibition for improving neuronal plasticity (Figs. 4h and i). Additionally, our results
suggest, for the first time, that G9a inhibition promotes neuronal plasticity through GMFB molecular pathway modulation (Fig. 4j).

Figure 4. G9a inhibition with UNC0642 treatment increases the GMFB transduction pathway, restoring dendritic spine density in SAMP8 mice. a. and b. Representative WB, and quantifications of GMFB. a. and c. Representative WB and quantifications for the ratio of p-p38/p38. a. and d. Representative WB and quantifications for the ratio of p-CREB/CREB. a. and e. Representative WB and quantifications of BDNF. Values represented are the mean ± SEM; (n = 12 (SAMP8 Control n = 6, SAMP8 treated with UNC0642 (5mg/Kg) n = 6)). Groups were compared by Student's t-test analysis (significant at *p < 0.05; ***p < 0.001). f. Representative images and tracings of Golgi-stained neurons from the SAMP8 Control group (top left) and the SAMP8 treated with UNC0642 (5mg/Kg) group (top right); (scale bar = 100 µm) g. SAMP8 mice treated with UNC0642 showed an increase in the number of neuronal intersections compared to the SAMP8 Control group, and this increase is most apparent at intermediate and more distal distances from the soma in a Sholl analysis. Values represented are the mean ± SEM; (SAMP8
Control n = 98, and SAMP8 treated with UNC0642 (5mg/Kg) n = 100). Groups were compared by Student’s t-test analysis (significant at *p < 0.01). h. Representative images of the spine density of Control (bottom left) and treated (bottom right) groups in SAMP8 mice by Golgi staining. i. Spine density quantification of neurons from SAMP8 Control and SAMP8 UNC0642 treated mice. Values represented are the mean ± SEM; (SAMP8 Control n = 60, and SAMP8 UNC0642 (5mg/Kg) n = 56 dendrites (of different neurons) were analyzed from 6 mice per group). Groups were compared by Student's t-test analysis (significant at *p < 0.05). j. Representative scheme of the GMFB molecular signaling modulated after UNC0642 treatment in SAMP8. Values in bar graphs are adjusted to 100% for protein levels of the SAMP8 Control. Source data are provided as a Source Data file.

The inhibition of the G9a ameliorates neuroinflammation, modulating molecular changes in the NF-κB pathway in SAMP8 mice.

We had previously shown an increased neuroinflammatory process in SAMP828. Of note, the RNA-seq showed a reduction in the NF-κB signaling pathway (Fig. 5c) and an increase in the anti-inflammatory cytokine Il-37 in SAMP8 UNC0642 (Supplementary Figs. 2a and b), which suggests that the treatment with UNC0642 could reduce the levels of neuroinflammation in the SAMP8 model. To demonstrate this, we evaluated the neuroinflammation status after treatment with UNC0642. NF-κB is a transcription factor related to inflammatory response and a master commander in the expression of pro-inflammatory genes, and its signaling is an important mediator of brain inflammation in AD29. To investigate the activation levels of NF-κB in the SAMP8 treated group, we evaluated its protein level and the expression of its target genes after treatment with UNC0642. Strikingly, a significant reduction in NF-κB protein levels was found in SAMP8 treated group (Figs. 5a and b). Next, we assessed the gene expression of some pro-inflammatory markers, such as interleukin-6 (Il-6), Cxcl10, and Tnf-α30. A significant decrease in the expression of these pro-inflammatory genes was observed between SAMP8 treated with
UNC0642 and the SAMP8 control mice group (Fig. 5c). Additionally, a GSEA analysis demonstrated a reduction in the enrichment of genes associated with the neuroinflammatory response in SAMP8 UNC0642 (Fig. 5d). By analyzing cell death in a primary mixed culture of neurons and microglia, it has been observed that pretreatment with UNC0642 (1 µM) significantly decreases cell death after activation with lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) from the cortex and striatum (Figs. 5e and f). Finally, it has been observed that LPS and IFN-γ induce microglial activation, detected as a fluorescence increase after one week of treatment. Interestingly, pretreatment with UNC0642 (1 µM) blocked this effect (Figs. 5g and h, Supplementary Fig. 4). Taken together, these data suggest that treatment with UNC0642 ameliorates neuroinflammation by regulating the transcriptional changes in the NF-kB pathway in brain cells (Fig. 5i).
Figure 5. G9a inhibition treatment reduces the inflammatory markers through the modulation of the NF-κB molecular pathway in SAMP8 mice. 

a. and b. Representative WB, and quantification of NF-κB protein levels. c. Representative gene expression of pro-inflammatory markers for Il-6, Cxcl10, and Tnf-α. Gene expression levels were determined by real-time PCR. Values represented are the mean ± SEM: (n = 12 (SAMP8 control n = 6, SAMP8 UNC0642 (5mg/Kg) n = 6)). Groups were compared by Student’s t-test analysis (significant at **p < 0.01; ***p < 0.001; ****p < 0.0001). d. GSEA plot of neuroinflammatory response up-regulated in SAMP8 control. e. Primary mixed culture of neurons and microglia from cortex and f. striatum was stimulated with UNC0642 (1 µM) or vehicle, and subsequently treated every 48 h with 1 mM LPS and 200 U/mL IFN-γ for two weeks. Neuronal viability was quantified by a TC20™ Automated Cell Counter (Biorad, 1450102). g. and h. IHC of Iba1 was performed in mice microglial primary cultures treated or not with 1 µM of the G9a inhibitor, UNC0642. The confocal microscopy images are shown (superimposed sections) in which Iba1 appears as red over Hoechst-stained nuclei. Scale bars = 30 µm. The bars graph shows the number of the red dots/cell (r). The cell nuclei were stained with Hoechst (blue) in all cases.
Values represented are the mean ± SEM (n = 10). Groups were compared by One-Way ANOVA and post-hoc Tukey’s test (significant at *p < 0.05; **p < 0.001; ****p < 0.0001). I. Representative scheme of the neuroinflammatory pathway modulated after UNC0642 treatment in SAMP8. Source data are provided as a Source Data file.

G9a inhibition treatment induces changes in the microRNA expression profile that confirm neuroprotective effects through the antioxidant capacity modulated by FOXO molecular pathway in SAMP8 mice.

Alterations in microRNAs have been associated with aging, cognitive decline as well as specific abnormal levels have been detected in several areas of the AD brain. Thus, to gain insight into the biology of G9a inhibition treatment, we analyzed the global microRNAs expression between SAMP8 control and SAMP8 treated with UNC0642. We identified 44 differentially expressed microRNAs (fold change cutoff of ≥ 1.9, p-value < 0.05), of which 27 were up-regulated and 17 were down-regulated in UNC0642 treatment (Fig. 6a). The top 10 up- and down-regulated miRNAs are shown in Figure 6b. Then, we validated the increase in the expression of mmu-miR-129 and the mmu-miR-669 and the reduction of the mmu-miR-677 by RT-qPCR (Figs. 6e-g). These results suggest that UNC0642 treatment induces an alteration in microRNAs expression.

To determine co-regulation between microRNAs and mRNAs we used the miRTarVis tool, and we found several nodes with a high number of microRNAs-associated mRNA molecules in our SAMP8 model (Figs. 6c and d). On the one hand, the up-regulated microRNAs, mmu-miR-92b-5p, mmu-miR-144-3p, mmu-miR-34c-5p, mmu-miR-204-3p, mmu-miR-539-5p, mmu-miR-34c-3p, mmu-miR-204-5p, mmu-miR-208b-3p, mmu-miR-148a-5p, mmu-miR-34b-5p, mmu-miR-448-3p, and mmu-miR-187-5p are associated with the down-regulation of 145 mRNAs (Fig. 6c, Supplementary Table 2b and c). On the other
hand, the down-regulated microRNAs, mmu-miR-34a-3, mmu-mir-128-1, and mmu-mir-128-2 are associated with the up-regulation of 124 mRNAs (Fig. 6d, Supplementary Table 2b and c) according to miRanda algorithm. Together, these data show that G9a inhibition with UNC0642 treatment causes a transcriptional dysregulation of microRNAs, which could alter biological processes in the SAMP8 model.

To analyze the pathways regulated by microRNAs, we determined the processes associated with the target mRNAs. Interestingly, we identified that the increase in mmu-miR-208b-3p is associated with the down-regulation of adenylate cyclase activating polypeptide 1 (Adcyap1) and cholinergic Receptor Nicotinic Beta 3 Subunit (Chrm3), both associated with neuroactive ligand-receptor interaction (Supplementary Fig. 5a). Besides, mmu-miR-92b-5p, mmu-miR-204-3p, and mmu-miR-204-5p are associated with the FOXO signaling pathway, as well as an increase in genes associated with Axon guidance (Pak7, Efna5, and Ephb6) (Supplementary Fig. 5a). Contrastingly, the reduction of mmu-miR-34a-3, mmu-mir-128-1, and mmu-mir-128-2 are associated with the regulation of the Wnt signaling pathway and PI3K-Akt signaling pathway (Supplementary Fig. 5b). Collectively, these observations suggest a possible role for microRNAs in regulating mechanisms associated with neurological processes.

The FOXO family of transcription factors is implicated in cognitive performance in healthy, aged and AD brain, promoting stem cell regeneration and neuroprotection against oxidative stress (OS). Then, to confirm the upregulation of FOXO signaling pathway one of the KEGG pathways obtained after the microRNA analysis (Supplementary Fig. 5a), we assessed the
FOXO1a protein levels as well as the OS status by gene expression of some FOXO1a target enzymes after treatment with UNC0642 in the hippocampus SAMP8 mice. Importantly, a major signaling pathway, regulating cellular aging is the FOXO transcription factor axis\(^{36}\). Indeed, reduced expression of FOXO transcription factors is associated with susceptibility to cell death induced by OS\(^{35}\). Here, we found increased protein levels of FOXO1a in SAMP8 treated with UNC0642 (5mg/Kg) compared to the SAMP8 control group (Figs. 6h and i). Furthermore, we also found in treated SAMP8 with UNC0642 increased gene expression of deacetylase sirtuin 1 (Sirt-1), which regulates the activity of FOXO transcription factors\(^{37}\) (Fig. 6j). Next, we evaluated the gene expression of some antioxidant markers such as superoxide dismutase-1 (Sod-1) and Hmox-1\(^{38}\) (Fig. 6k). A significant increase in both gene expressions was found in SAMP8 treated with UNC0642, suggesting the involvement of the FOXO activation signaling pathway after G9a inhibition. Overall, with these results, we can suggest that treatment with UNC0642 allows the transcriptional alteration of microRNAs potentially associated with neuronal processes that can promote neuroprotection through the FOXO pathway activation (Fig. 6l).
Figure 6. G9a inhibition treatment induces changes in the miRNome that confirm neuroprotective effects through the antioxidant capacity modulated by FOXO molecular pathway in SAMP8 mice. a. Volcano plot showing the differentially expressed microRNAs. b. Top 10 differentially expressed microRNAs; down-regulated in green and up-regulated in red. c. A representative network between co-regulation of up-miRNAs and down-miRNAs. d. Representative network between co-regulation of down-miRNAs and up-miRNAs. e. Representative microRNA expression of miR-129, f. miR-669, and g. miR-677. h. and i. Representative WB and quantification of FOXO1a protein levels. j. Representative gene expression of Sirt-1, and k. Representative gene expression of OS resistance markers for Sod-1, and Hmox-1. microRNA and gene expression levels were determined by real-time PCR. Values in bar graphs are adjusted to 100% for protein levels of the SAMP8 control. Values in bar graphs are adjusted to 100% for protein levels of the SAMP8 control. Values represented are the mean ± SEM; (n = 12 (SAMP8 control n = 6, SAMP8 UNC0642 (5mg/Kg) n = 6)). Groups were compared by Student’s t-test analysis (significant at *p < 0.05; **p < 0.01). l. Representative scheme of the FOXO1a molecular pathway modulated after UNC0642 treatment in SAMP8. Source data are provided as a Source Data file.
H3K9me2 enrichment regulates pathways associated with the neuronal system after G9a inhibition. We next sought to further characterize the dynamics of the histone mark H3K9me2 after treatment with a G9a inhibitor. We used public H3K9me2 ChIP-seq data corresponding to AML12 cells (murine hepatocyte cell line) treated with UNC0638, a selective inhibitor of G9a with the same specificity as UNC0642. Analysis of H3K9me2 enrichment revealed the presence of this histone mark in distal regions (60.78%) and promoters (13.65%) in cells treated with the G9a inhibitor (Fig. 7a). Furthermore, H3K9me2 has an enrichment around TSS (Fig. 7b), suggesting a role in transcriptional regulation. Interestingly, functional analysis showed that H3K9me2 is enriched at promoters of genes associated with nervous tissue such as neuronal system, transmission across chemical synapses, neuroactive ligand–receptor interaction, positive regulation of neural precursor cell proliferation, gamma-aminobutyric acid transport, among others (Figs. 7c and d). Figure 7e shows the H3K9me2 enrichment in cells treated with G9a inhibitor compared to the control group treated with dimethyl sulfoxide (DMSO) in the genes C-X-C motif chemokine ligand 10 (Cxcl10), tumor necrosis factor-alpha (Tnf-α), heme oxygenase-1 (Hmox-1), and hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (Hcn1).

The distribution of the transcription factor motifs relative to TSS revealed enrichment of binding motifs around TSS (Supplementary Fig. 6a, and Table 3).

To evaluate which transcription factors are associated with H3K9me2, we performed a motif discovery analysis identifying motifs associated with PR/SET Domain 6 (Prdm6), cytoplasmic polyadenylation element binding protein 1 (Cpeb1), TATA-Box binding protein associated factor 1 (Taf1), among others.
(Supplementary Fig. 6b). Additionally, an ENCODE, and ChEA Consensus TF analysis identified an association with **SUZ12 polycomb repressive complex 2 subunit** (**Suz12**), **enhancer of zeste 2 polycomb repressive complex 2 subunit** (**Ezh2**), **RE1 silencing transcription factor (Rest)** and **SMAD family member 4** (**Smad4**) (Supplementary Fig. 6c), suggesting a regulatory network associated with transcriptional repression where G9a could be a central regulator (Supplementary Fig. 6d). Together, these results demonstrate an H3K9me2 enrichment in genes associated with neural function, suggesting that after treatment with a G9a inhibitor, the H3K9me2 reduction could allow the expression of genes associated with the correct function of the CNS.
Figure 7. H3K9me2 enrichment in genes associated with the neuronal system after G9a inhibition. a. Genome region annotation of H3K9me2 peaks in AML12 cells treated with the G9a inhibitor UNC0638. b. Heatmap of H3K9me2 enrichment around the TSS regions of the mouse genome (mm10). c. Pathways regulated by the H3K9me2 enriched genes in AML12 cells treated with UNC0638. d. Reviso visualization of biological processes analyzed by Gene Ontology (GO). e. IGV tracks of H3K9me2 enrichment at the promoters of Cxcl10, Hmox-1, Tnf-α, and Hcn1 in AML12 cells treated with UNC0638 and DMSO as a control. The blue area defines the promoters.

C. elegans AD transgenic strains presented similar epigenetic alterations and modulated pathways altered with increased Aβ levels, reverted by the set-25 knockout in C. elegans. Because our previous in vitro and in vivo results suggested that pharmacological G9a inhibition might act on the β-amyloid pathology, neuronal plasticity, neuroinflammation, and OS, among others, and thus we studied whether set-25 knockout by RNAi also promotes neuroprotective effects in C. elegans due to similar mechanisms. As previously mentioned, SET-25 in C. elegans is similar to the mammalian G9a protein. Firstly, we validated the gene expression levels of set-25 in our generated C. elegans strains. Then, to evaluate the role of SET-25 in aging-related diseases, and more specifically in AD, we used the CL2006 strain, which expresses human Aβ1-42 under the control of a muscle-specific promoter, and also presents paralysis with age-worsening. First, we confirm that the gene expression of set-25 was higher in the transgenic AD strain, CL2006, than in N2 (WT) (Fig. 8a). As expected, due to RNAi suppression, set-25 gene expression was almost completely reduced in our CL2006 strain (set-25 (RNAi)) compared to the CL2006 control group, and this finding is consistent with the observed reduction in H3K9me2 levels (Figs. 8a-c). Then, we examined the effect of set-25 knockout on cognition, using the CL2355 C. elegans strain. This strain expresses neuronal Aβ and significantly reduces the chemotaxis index (CI).
compared to the control strain CL2122. Of note, our results showed that set-25 knockdown fostered the restoration of impaired learning and memory of CL2355 strain by reaching a similar CI as control worms (Fig. 8d, Supplementary Fig. 7). Besides, we showed a reduction in Aβ aggregation (Figs. 8e and f) in CL2006 (set-25 (RNAi)) when compared to the CL2006 control group, suggesting that SET-25 specially modulates memory formation by reducing AD pathology, including in *C. elegans* model. We finally tested if neuroprotective effects found in *C. elegans* AD strains after set-25 knockdown is due the same modulatory pathways. To confirm the transcriptome analysis in *C. elegans*, we assessed some of the genes previously validated in SAMP8 mice. We found a reduction of transcript levels of ric-4 (ortholog of Snap25) and unc-43 (ortholog of Camk2) in CL2006 (set-25 (RNAi)) when compared to the CL2006 control group (Figs. 8g and h), going in line with our previously validated transcriptome analysis in SAMP8 mice. Moreover, we found a significant increase in crh-1c (ortholog of Creb) gene expression in CL2006 (set-25 (RNAi)) (Fig. 8i), which are associated with GMFB pathway. Regarding FOXO signaling activation, we observed an increased daf-16 (ortholog of Foxo) gene expression in CL2006 (set-25 (RNAi)) in comparison with the group control (Fig. 8j). Taken together, these results indicate that set-25 knockdown promotes similar modulatory pathway modifications to confer better cognitive performance in *C. elegans* (Fig. 8k).
Figure 8. *C. elegans* AD transgenic strains presented similar epigenetic alterations and modulated pathways altered with increased Aβ levels, reverted by the set-25 knockout in *C. elegans*. a. Representative gene expression of set-25 in *C. elegans*. Gene expression levels were determined by real-time PCR. Values represented are the mean ± SEM; n = 3-5. Each replicate with at least 350 worms in each group from whole petri dish. Groups were compared by One-Way ANOVA and post-hoc Tukey’s test (significant at ****p < 0.0001 vs CL2006 Control). b. and c. Representative WB, and quantification for histone H3K9me2 of N2 (WT), *C. elegans* AD transgenic strain (CL2006), and the knockout of G9a strain (CL2006 (set-25 (RNAi))). Groups were compared by One-Way ANOVA and post-hoc Tukey’s test (significant at *p < 0.05 vs CL2006 Control)). d. Chemotaxis index (CI) calculated for CL2122 (non Aβ strain), CL2355 (neuronal Aβ strain), and the knockout of G9a strain (CL2355 (set-25 (RNAi))). Values represented are CI mean ± SEM; n=3-6 replicates with at least 200 worms for each analysis. Groups were compared by One-Way ANOVA and post-hoc Tukey’s test (significant at *p < 0.05; **p < 0.01 vs CL2355 Control). e. and f. Representative images from each group and quantification of Thioflavin S-positive particles in the head region of CL2006.
strain and (CL2006 (set-25 (RNAi)) strain. Values represented are mean ± SEM; n = 3-4 with at least 40 worms in each group. Groups were compared by Student's t-test analysis (significant at ****p < 0.0001). g. Representative gene expression of ric-4, h. unc-43, i. crh-1c, and j. daf-16 in C. elegans. Gene expression levels were determined by real-time PCR. Values represented are the mean ± SEM; n = 3-5. Each replicate with at least 350 worms in each group from whole petri dish. Groups were compared by One-Way ANOVA and post-hoc Tukey’s test (significant at **p < 0.01; ****p < 0.0001 vs CL2006 Control). k. Representative scheme of the results found in the set-25 knockout strains. Source data are provided as a Source Data file.

Discussion

The present study demonstrated the relevance of the G9a and its repressive histone mark H3K9me2 in AD patients, which correlates with increased levels of the Aβ42/Aβ40 ratio, an important hallmark of the disease. We also have observed in SAMP8 similar specific epigenetic modifications in G9a and H3K9me2 to those observed in AD patients. More importantly, pharmacological inhibition with UNC0642 restored cognitive status in SAMP8 in the same way as previously published studies in 5XFAD mice. Besides, aggressive behavior displays a direct influence on social interaction, and in the same line, the inhibition of G9a has been associated with a decrease in anxiety-like behaviors in adult male mice. Nevertheless, the beneficial effect of G9a inhibitor treatment on social performance has not been previously described. Thus, for the first time, we demonstrate that G9a inhibition by UNC0642 treatment improved social behavior in SAMP8 mice. Consistently, it has been described that inhibitors of KTMs lead to the remarkable restoration of cognitive behaviors, such as recognition memory, spatial memory, and working memory.

As aforementioned, it is thought that aberrant gene expression is associated with epigenetic alterations such as DNA methylation and histone modifications. Although some relevant in vivo studies are being performed, few describe new modulated pathways after G9a inhibition in detail. Therefore, one of the main...
interests of this study was to determine uncovered pathways by which G9a might act, contributing to transcriptomic changes leading to neuroprotection. Due to these analyses, increased levels of Gmfb expression and decreased levels of Snap25, Tbr1, and Camk2g were found in the hippocampus of SAMP8 mice after UNC0642 treatment. Of note, we validated the activation of the GMFB because its activation leads to the synthesis induction of several important proteins in brain function, promoting neurotrophins production, suggesting a role in neuronal growth and regeneration and were correlated with learning and memory formation. Strikingly, GMFB appears to trigger activation of p38 MAPK, followed by the activation of the transcription factor CREB, up-regulating BDNF expression levels, and thus confirming the pathway activation in SAMP8. Consistent with our results, the generation of GMFB knockout mice revealed its importance in normal cognitive functions since its loss promoted pathological neuroinflammatory disease progression. However, no increase in neurotrophins, such as BDNF and NGF, expression was observed in the cerebellum of GMFB-knockout mice in an in vivo study, displaying an impairment of motor and learning skills. Importantly, Golgi staining analysis revealed an increase in dendritic arborization of cortex with an increase in spine density. These results demonstrated an increase in neuronal complexity, which has been correlated with better cognitive performance. Importantly, therapeutic strategies that can reduce the neurodegeneration and also actively promotes neuro-regeneration leading to better functional phenotype, are of high interest. In fact, G9a has been described as a player in axon formation, controlling the expression of genes associated with cAMP and Ca^{2+}-dependent signaling. Although results on the GMFB-related signaling...
pathway appear beneficial in SAMP8 mice, there are conflicting findings on how GMFB expression is triggered during neuroinflammation and neurodegeneration\textsuperscript{48}. The activation of GMFB is associated with one of the most pivotal neuroinflammatory mediators, the NF-κB, a transcription factor in the positive inflammatory response and a regulator of pro-inflammatory gene expression\textsuperscript{49}. Hence, the precise molecular mechanism of GMFB involved in neurodegeneration remains unclear\textsuperscript{50,51}. For this reason, the assessment of pro-inflammatory markers was of great importance in this work.

A wealth of evidence confirms the link between increased levels of inflammatory markers in the pathogenesis of AD, suggesting that neuroinflammation plays a relevant role in neurodegeneration. Although the interaction between NF-κB and G9a has already been described during an immune response\textsuperscript{52}, it is still unknown in the context of neuroinflammation\textsuperscript{12,53}. As expected, it was found that NF-κB protein levels were decreased after UNC0642 treatment in SAMP8 mice. Accordingly, we observed significantly reduced \textit{Cxcl10}, \textit{Il-6}, and \textit{Tnf-α} gene expression levels in the treated group compared to the control group.

Following our results, clinical research has shown a positive correlation between CXCL10 and cognitive impairment in AD patients\textsuperscript{54}. Furthermore, IL-6 is a pleiotropic inflammatory cytokine secreted by activated glia in the CNS involved in the aging process and the pathogenesis of neurodegeneration\textsuperscript{55}. Besides, the pro-inflammatory cytokine TNF-α exacerbates both Aβ and tau pathology, and thus anti-inflammatory strategies demonstrated amelioration of cognitive function in rodent models of AD\textsuperscript{56}. At first glance, up-regulation of GMFB might compromise the neuroinflammatory state; however, considering our validated \textit{in vivo} results, we report that NF-κB orchestrates a decrease of
pro-inflammatory markers in AD progression in response to G9a inhibition.

Interestingly, clinical imaging studies reported that neuroinflammation in AD patients was characterized by negative correlations between microglial activation and structural integrity or functional activity in the hippocampus of AD patients. As shown by the *in vitro* model of inflammation induced by LPS treatment, the inhibition of G9a reduced microglial specific marker expression, such as Iba-1, iNOS, and Arginase. In line with our results, several studies identified increases in Iba-1 expression in AD patients compared with control groups, considering this protein crucial for activating microglial processes\(^{57}\). Moreover, iNOS is mainly expressed by microglia that become activated in different pathological situations, and its overexpression was associated with the induction of neuronal death\(^{58}\). Then, these results of lower levels of reactive microglial markers reaffirmed the *in vivo* results of a reduction in neuroinflammation after the pharmacological inhibition of G9a, as our group previously reported in an early-onset AD mouse model\(^{12}\).

By overlaying transcriptomic analyses, we identified several microRNAs (*miR-34b-5p, miR-34c-3p*, and *miR-34c-3p*) that were modified. In addition, the KEGG enrichment analysis revealed the association of G9a with cholinergic synapse, axon guidance, PI3K-Akt-signaling pathway, among others. One of the most important up-regulated signaling pathways in the SAMP8 treated mice was FOXO. FOXO is a multifunctional transcription factor, which regulates several molecular events such as OS resistance, cell survival, apoptosis and proliferation, among others, involved in the pathogenesis of AD\(^{35}\). In accordance with our results, it has been reported that G9a protein levels were elevated, and FOXO1 protein levels were decreased in human colon cancer
patients. Hence, to our knowledge, herein we described for the first time the up-regulation of FOXO signaling pathway in SAMP8 brain after a treatment with UNC0642. Accordingly, we evaluated the gene expression of \textit{Sirt-1}, which deacetylates FOXO and regulates gene expression, participating in the process of neuroprotection through OS-involved pathways, and found that it was elevated in the brain of SAMP8 treated with UNC0642. Furthermore, the gene expression evaluation of antioxidant markers \textit{Sod-1} and \textit{Hmox-1} after G9a inhibition revealed that our results align with previously published studies. In supporting our idea that the reduction of H3K9me2 is a promising therapy for AD, we performed a ChIP-seq reanalysis, which revealed an H3K9me2 enrichment in distal and promoter regions, indicating a transcriptional regulation by G9a. Furthermore, in line with our findings, it has been reported that TG2576 mice presented high levels of Aβ$_{40}$ and Aβ$_{42}$ levels with increased H3K9me2 levels in the cortex and hippocampus. This suggests that treatment with G9a inhibitors could reduce the H3K9me2 levels, restoring neural alterations associated with amyloid-β oligomers. In fact, in the RNA-seq data, we found alterations in molecular processes associated with chromatin methylation and H3K9me2 methylation, which suggests evident participation of the proteins associated with the histone mark H3K9me2. This would support the exploratory analysis of H3K9me2 ChIP-seq in AML12 cells. Thus, based on our findings and previous reports, we suggest that G9a inhibition could improve cognition in AD patients. Finally, we used the RNAi method in \textit{C. elegans} as a much cleaner approach than pharmacological inhibition to reduce the off-target effect, and elucidate how SET-25 (similar to the mammalian G9a protein) contributes to the AD.
pathogenesis. Firstly, we showed in *C. elegans* AD strains, similar epigenetic alterations to those observed in AD patients and SAMP8 mice, which were reverted after set-25 knockout. Also, reduced levels of H3K9me2 and Aβ aggregation were determined, suggesting the contribution of SET-25 in AD-like pathology. Thus, foster improved cognitive performance (CL2355), and molecular modifications such as Aβ pathology and transcriptomic changes (CL2006) in the *C. elegans* model after SET-25 suppression. Besides, those changes were associated with the same found in SAMP8 mice, allowing us to confirm our findings attributed to the pharmacological G9a inhibition in SAMP8 mice.

In summary, we first validated the G9a and its repressive mark (H3K9me2) as a potential target candidate for AD treatment. We further demonstrated that G9a inhibition treatment has neuroprotective effects in a mouse model of LOAD, ameliorating behavior and cognitive deficits, correlating with the reduction of H3K9me2. Furthermore, after an integrative data analysis, we proposed new modulated pathways whereby G9a inhibition promotes neuroprotection, elucidating important changes in the transcriptome, which confirms the multifaceted molecular function of the G9a. Finally, those interrelated changes described were validated in *C. elegans* AD strains after set-25 knockdown (Fig. 9).
Figure 9. Scheme of the molecular changes promoted after knockout of set-25 in *C. elegans* or pharmacological inhibition of G9a by UNC0642 in SAMP8 mice model.

Material and methods

Animals and treatment

Female and male SAMP8 and SAMR1\(^{22,23}\) 24-week-old (n = 51) were used to perform cognitive and molecular studies. We divided these mice randomly into three groups: SAMR1 (n = 17), SAMP8 control (n = 17), and SAMP8 treated with G9a histone methyltransferase inhibitor, the UNC0642 (SAMP8 UNC0642 (5 mg/Kg); n = 17). The sample size for the intervention was chosen following previous studies in our laboratory and using one of the available interactive tools (http://www.biomath.info/ power/index.html). Experimental groups either received a daily dose of vehicle (2% w/v, (2-hydroxypropyl)-β-cyclodextrin) or a dose of 5 mg/Kg/day of UNC0642 dissolved in 2% 2-hydroxypropyl-β-
cycloextrin via oral gavage for 4 weeks. Animals had free access to food and water and were kept under standard temperature conditions (22 ± 2°C) and 12h:12h light-dark cycles (300 lux/0 lux). After the treatment period, cognitive tests were performed on the animals.

C. elegans were cultured according to standard procedures, unless otherwise noted. N2 (WT) (Bristol) strain, and the transgenic AD strains (CL2006, CL2355, and its control, CL2122) were used for this study. They were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota, Minneapolis, MN, USA. Moreover, knockout of set-25 strains was generated in this work using the RNAi method in CL2355 and CL2006 strains. WT nematodes were propagated at 20°C, while transgenic AD strains were maintained at 16°C in a temperature-controlled incubator on solid nematode growth medium (NGM) seeded with *Escherichia coli* (*E. coli*) OP50. To obtain synchronized animals, young adults laid embryos for 24 hours before being removed from plates.

**RNAi**

RNAi was performed by feeding method as previously described. Bacteria HT115(DE3) carrying IPTG-inducible were incubated at 37°C for 7-8 hours with 100 mg/mL ampicillin. NGM plates were then seeded with 25 mg/mL carbenicillin and 1 mM IPTG with the incubated cultures and let grow overnight at room temperature. Young adults were plated onto RNAi bacteria at 16°C. Silenced adults were transferred to fresh OP50 plates to produce subsequent generations seven days later.

**Behavioral and cognitive tests**

**Chemotaxis assay**
Fifteen adult hermaphrodites were left to lay eggs for 24h and then removed from the plates. Eggs were incubated at 16°C for 36h, and then at 23°C for another 48h. Briefly, the assay was performed in 100 mm NGM plates, 10 μL of control odorant (96% ethanol) was added to the “control” spot. On the opposite side, 10 μL of odorant (0.025% benzaldehyde in 96% ethanol) was added to the “attractant” spot. Adult worms were washed three times in M9 buffer, and at least 120 worms were placed towards the center of the plate. The test plates were incubated at 23°C for 1.5h, and worms were scored according to the chemotaxis index (CI) as follows: CI = (number of worms at attractant−number of worms at control)/total number of worms.

**Three-Chamber Test**

The social behavior of the mice was evaluated by the TCT following a previously described protocol. A box of transparent polyvinyl chloride (15x15x20 cm) divided into three equally dimensioned rooms with openings among them was used. First, each mouse was placed in the center of the box and allowed to explore the three chambers for 5 min (habituation). Afterward, an intruder (same-sex and age) was placed in a metal cage in one of the rooms, and the behavior was recorded for 10 min. The time spent in each room and interacting with the intruder (sniffing time) were measured manually. The TCT apparatus was cleaned with 70% ethanol between the trials to eliminate olfactory cues.

**Novel object recognition test**

Short- and long-term recognition memory involving cortical areas and the hippocampus was evaluated by NORT. The experimental apparatus used for this test was a 90-degree, two-arm, 25-cm-long, 20-cm-high maze of black
polyvinyl chloride. Light intensity in the middle of the field was 30 lux. First, mice were individually habituated to the apparatus for 10 min per day for 3 days. On day 4, the animals were allowed to freely explore two identical objects (A and A or B and B) placed at the end of each arm for a 10 min acquisition trial (first trial-familiarization). Then, a 10-min retention trial (second trial) was carried out 2 hours (short-term memory) or 24 hours (long-term memory) later. During the Short-term memory retention, objects A and B were placed in the maze replacing one of them (A and B or B and A), and the times that the animal took to explore the new object (TN) and the old object (TO) were recorded. 24 hours after the acquisition trial, the mice were tested again, with a new object and an object identical to the new one in the previous trial (A and C, or B and C). The time that mice explored TN and the time that mice explored TO were measured from the video recordings from each trial session. A Discrimination index (DI) was defined as (TN-TO)/(TN+TO). The maze, the surface, and the objects were cleaned with 70% ethanol between the animals’ trials to eliminate olfactory cues.

Object Location Test

The OLT evaluated the spontaneous tendency of rodents to spend more time exploring a novel object location than a familiar object location and recognizing when an object has been relocated. OLT was performed using a white plywood apparatus (50 × 50 × 25 cm), in which three walls were white and one was black. On the first day, animals just habituated to the empty open field arena for 10 minutes. On the second day, two objects were placed in front of the black wall, equidistant from each other and the wall. The objects were 10 cm high and identical. The animals were placed into the open field arena and allowed to
explore both objects and surroundings for 10 minutes. Afterward, animals were returned to their home cages, and the OLT apparatus was cleaned with 70% ethanol. On the third day, one object was moved in front of the opposite white wall to test the spatial memory. Trials were recorded using a camera mounted above the open field area, and the total exploration time was determined by scoring the amount of time (seconds) spent sniffing the object in the new location (TN) and the object in the old location (TO). DI was calculated, which is defined as (TN-TO)/(TN+TO).

Biochemical experiments

Brain processing

SAMP8 and SAMR1 mice were euthanized 3 days after the behavioral test completion by cervical dislocation. Brains were immediately removed from the skull. Cortex and hippocampus were then isolated and frozen on powdered dry ice. They were maintained at -80 °C for biochemical experiments. For Golgi stain protocol, see the procedure in the section “Spine density and Golgi stain protocol”.

Human cases

Tissue samples were obtained from the Institute of Neuropathology-IDIBELL Brain Bank, Hospitalet de Llobregat, following the guidelines of Spanish legislation on this matter and the approval of the local ethics committee (Fig. S1). The postmortem interval between death and tissue processing was 1 to 10 hours and was processed to minimize postmortem delay artifacts. The brain tissue was immediately frozen on metal plates over dry ice, placed in individual air-tight plastic bags, and maintained at -80 °C for biochemical experiments.
The neuropathologic diagnosis of AD was based on the classification of Braak and Braak.

**Reagents**

All the reagents and kits used for the protocols described below are listed in Supplementary Table 4a.

**Protein levels determination by Western blotting**

Protein, histone extraction, and immunoblot analysis were performed as in Vasilopoulou et al. (n = 6 hippocampal samples from mice, or at least 6 samples from human brain per experimental group). Proteins were separated by SDS-PAGE (8-20%) and transferred onto PVDF membranes. Afterward, membranes were blocked in 5% BSA in 0.1% Tween20-TBS (TBS-T) for 1 hour at room temperature (RT), followed by overnight incubation at 4 ºC with the primary antibodies presented in Supplementary Table 4b. Immunoreactive proteins were viewed with a chemiluminescence-based detection kit, following the manufacturer’s protocol and digital images were acquired using a ChemiDoc XRS+System. Semi-quantitative analyses were carried out using ImageLab Software and results were expressed in Arbitrary Units (AU), considering the control mice group as 100%. Protein loading was routinely monitored by immunodetection of GAPDH.

**Human amyloid-β levels quantification by ELISA**

Elisa measured amyloid-β40 and amyloid-β42 protein levels with the human amyloid-β40 ELISA Kit and human amyloid-β42 Ultrasensitive ELISA Kit, respectively. The samples were diluted by standard dilution buffer at a percentage of 50% and all procedures followed the manufacturer's instructions.
A minimum of 6 human brain samples per experimental group were used for the quantification.

**Mouse amyloid-β levels quantification by ELISA**

Mice brains (n= 6 per experimental group) were homogenized in cold 5 M guanidine-HCl/50 mM Tris buffer containing protease inhibitor cocktail. The quantification of amyloid-β<sub>40</sub> and amyloid-β<sub>42</sub> was performed with the mouse Aβ<sub>40</sub> and Aβ<sub>42</sub> ELISA Kits, following the manufacturer’s instructions.

**RNA extraction and gene expression determination**

Total RNA isolation from cortical samples (n = 6 per experimental group) was carried out using TRIsure™ reagent following the manufacturer’s instructions. The yield, purity, and quality of the RNA were determined spectrophotometrically with a NanoDrop™ ND-1000 apparatus and an Agilent 2100B Bioanalyzer. RNAs with 260/280 ratios and RIN higher than 7.5, respectively, were selected. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the high-capacity cDNA Reverse Transcription kit. SYBR® Green real-time PCR was performed to quantify the mRNA expression of a set of genes listed in Supplementary Table 4c on a Step One Plus Detection System (Applied-Biosystems, Foster City, CA, USA).

Data were analyzed utilizing the comparative cycle threshold (Ct) method (ΔΔCt), where the housekeeping gene level was used to normalize differences in sample loading and preparation. Normalization of expression levels was performed with β-actin for SYBR® Green-based real-time PCR results. Each sample was analyzed in triplicate, and the results represented the n-fold difference of the transcript levels among different groups.
**miRNA extraction and quantitative real-time PCR**

miRNA from cortical sample (n=6 per experimental group) was isolated from 100-125 mg of cortex tissue using mirVana miRNA Isolation kit with phenol. cDNA was obtained using the TaqMan miRNA Reverse Transcription kit, following manufacturer indications. Then, amplification was performed from 1.33 µL of cDNA mix with TaqMan™ Universal PCR Master Mix, and 20X RT primer (Supplementary Table 4c) using the Step One Plus Detection System (Applied-Biosystems, Foster City, CA, USA). Data were analyzed utilizing the ΔΔCt method, where the reference gene for miRNA (U6 snRNA) level was used to normalize differences in sample loading and preparation.

**RNA-sequencing and microRNA-sequencing**

Pooled of 4 hippocampal tissue samples of SAMP8 control and SAMP8 UNC0642 (5 mg/Kg) were aligned with reference genome using Bowtie2, and the gene expression level was estimated using RSEM. Differentially expressed genes were identified using the edgeR program. Genes showing altered expression with p<0.05 and more than 1.3-fold changes were considered differentially expressed.

To determine the altered microRNAs after treatment with UNC0642 we used the QIAseqmiRNA platform. TMM normalization and differential expression analysis were carried out using the edgeR program. The cutoff of differential miRNA was a fold-change threshold of 1.9 and a maximum p-value <0.05. The miRTarVis tool was used to predict miRNA target.

KEGG, Gene Ontology, and GSEA were used to perform the enrichment and pathway analysis. Raw data were deposited at the Gene Expression Omnibus (accession GSE189250), raw fastq files for RNA-seq on mouse hippocampus
SAMP8 (accession GSE189249), and raw fastq files for miRNA-seq on mouse hippocampus SAMP8 (accession GSE 189248).

**ChiP-seq analysis**

Publicly available H3K9me2 ChIP-seq data corresponding to AML12 cells (murine hepatocyte cell line) treated with the selective G9a inhibitor, UNC0638 were obtained from the Genome Sequence Archive (GSA: CRA002762). ChIP-seq analysis was performed as previously described. Briefly, quality control was analyzed in FastQC v0.11.8. The reads were mapped to the reference genome (*Mus musculus* mm10) with Bowtie2 v2.4.2 with default parameters. The unmapped and duplicate reads were filtered with SAMtools v1.11. The H3K9me2 peaks were determined with MACS2 v2.1.1.2. ChIP-seq signal was visualized with deepTools2 v3.3.2 and the bigwig files were viewed in the IGV genome browser v2.8.12.

To determine the annotated genomic region of H3K9me2 peaks and distribution of transcription factors, we used ChIPseeker package. To determine the TF binding at promoters of the genes regulated by H3K9me2 in AML12 cells treated with UNC0638, we used the ENCODE and ChEA Consensus TFs from ChIP-X tool. The detection of transcription factor binding motifs at H3K9me2 peaks was performed with the MEME-ChIP database. Motifs with an E-value<0.05 were considered statistically significant. Protein-protein interaction network between G9a (EHMT2) and transcription factors was performed in STRING database.

**Dendritic length, spine density and Golgi stain protocol**

Mice were sacrificed by cervical dislocation and brains were removed from the skull (n=6 whole brain per experimental group). Then, Golgi stain protocol was
developed using FD Rapid GolgiStain kit according to manufactures instructions. For dendritic branching analysis, images of neurons were collected at 20x magnification in an Olympus BX61 microscope coupled to an Olympus DP70 camera. Measurement of neurite length and complexity was performed using NeuronJ macros and Advanced Sholl Analysis. The number of intersections (branch points) within concentric circles of 10 µm radius was measured and compared between groups. Images for analyzing the spine density were acquired using brightfield microscopy with a 50x oil-objective. All neurites analyzed were around 18 µm and they were at a maximum distance of 150 µm from the soma.

**Primary cultures**

Primary mixed culture of neurons and microglia was prepared from cortex and striatum of fetuses from 18-19 days C57/BL6 pregnant mice. In brief, samples were dissected, carefully stripped of their meninges, and digested with 0.25% trypsin for 30 min at 37°C. Cells were brought to a single cell suspension by repeated pipetting followed by passage through a 100 µm-pore mesh and pelleted (7 min, 200g). Neurons and glial cells were resuspended in medium and seeded at a density of 400,000 cells/ml in 6-well plates. Cultures were maintained at 37°C in humidified 5% CO₂ atmosphere and neurobasal medium supplemented with 2 mM L-glutamine, 5% (v/v) FBS, 100 U/ml penicillin/streptomycin, and 2% (v/v) B27 supplement (Gibco) in a 6-well plate for 12 days.

**Cell viability**

Cell viability assay is based on the principle that living cells maintain intact cell membranes that exclude certain dyes, like trypan blue 0.4%. For quantification
of live cells, cortical and striatal neurons were gently detached and mixed with an equal volume of trypan blue (0.4%). Neurons (%) were counted with a TC20™ Automated Cell Counter (Biorad, 1450102).

**Immunocytochemistry**

Primary microglial culture cells seeded in coverslips were fixed in 4% paraformaldehyde for 15 min and washed twice with PBS containing 20mM glycine, before permeabilization with PBS-glycine containing 0.2% Triton X-100. Microglial cells were treated for 1 h with PBS containing 1% BSA and labeled with the antibodies listed in Supplementary Table 4d. Samples were washed several times and mounted with 30% Mowiol. Samples were observed in a Leica SP2 confocal microscope (Leica Microsystems). Scale bar: 30 µm.

Fluorescence was quantified by Fiji-Image J software.

**Data analysis**

The statistical analysis was conducted using GraphPad Prism ver.9.2 statistical software. Group size may differ depending on power analysis and expertise of the authors and statistical analysis was conducted only for studies where the size of each group was at least n = 5. Data were expressed as the mean ± Standard Error of the Mean (SEM) from at least 5-6 samples per group. Means were compared to One-Way or Two-Way ANOVA analysis of variance, followed by Tukey’s post-hoc analysis. Comparison between groups was also performed by two-tail Student’s t-test for independent samples. Statistical significance was considered when p-values were <0.05. The statistical outliers were carried out with Grubss’ test and subsequently removed from the analysis. For behavioral tests, a blinded analysis was performed.

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Author contributions

M.P and C.G.-F. conceived the idea. A.B.-S., C.G.-F., PA.A.-L., G.N., J.C.-A., R.L.-A., L.M-G. and LL.M. performed research. A.B.-S., C.G.-F., PA.A.-L., G.N., J.C.-A., R.L.-A., B.S.C., A.P.-B. designed the experiments and analyzed the data. A.B.-S., G.N., FX. S., D.O.-S., M.P. and C.G.-F. wrote the manuscript.

Competing interest

None of the authors have any disclosures to declare.
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