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The BET-Bromodomain Inhibitor JQ1 Reduces Inflammation and Tau Phosphorylation at Ser396 in the Brain of the 3xTg Model of Alzheimer's Disease

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Abstract: ***Background:*** Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by well-defined neuropathological brain changes including amyloid plaques, neurofibrillary tangles and the presence of chronic neuroinflammation. ***Objective:*** The brain penetrant BET bromodomain inhibitor JQ1 has been shown to regulate inflammation responses *in vitro* and *in vivo*, but its therapeutic potential in AD is currently unknown. ***Method:*** Three-month-old 3xTg mice were injected once a day with JQ1 (50 mg/kg) or vehicle for 15 weeks. At the end of the treatment learning and memory was assessed using the modified Barnes maze and the Y maze behavioral tests. Tissue from the brain and other organs was collected for molecular evaluation of neuroinflammation tau pathology and amyloid β. ***Results:*** JQ1 treatment reduced splenomegaly and neuroinflammation in the brain of treated mice where we observed a reduction in the expression of the pro-inflammatory modulators *Il-1b*, *Il-6*, *Tnfa*, *Ccl2*, *Nos2* and *Ptgs2*. Additionally, JQ1-treated mice showed a reduction of tau phosphorylation at Ser396 in the hippocampus and frontal cortex while total levels of tau remained unaffected. On the other hand, JQ1 did not ameliorate learning and memory deficits in 7-month-old 3xTg mice. ***Conclusion:*** Taken together, our data suggest that BET bromodomain inhibitors hold the promise to be used for the treatment of neurological disorders characterized by neuroinflammation.

Keywords: Alzheimer's disease, astrocytes, inflammation, microglia, neuroinflammation, bromodomain, JQ1, splenomegaly.

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INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disease characterized by progressive loss of memory and other cognitive functions. AD represents the most common form of dementia with more than 5 million Americans affected and for which there are currently no disease-modifying drugs available [1]. Dr. Alois Alzheimer first diagnosed the pathology in 1901 when he noticed the presence of extracellular (amyloid plaques) and intracellular (neurofibrillary tangles) proteinaceous accumulations in the brain of a patient affected by pre-senile dementia [2]. This early onset form of Alzheimer's disease (EOAD) is caused by autosomal mutations in amyloid precursor protein (APP), presenilin 1 (PS1) and presenilin 2 (PS2) and represents only a small percentage of AD cases (below 5%). APP encodes for a transmembrane protein that is sequentially cleaved by different secretases including PS1 and PS2 (For review see [3]). Mutations in these genes cause aberrant processing of APP and result in the imbalanced production of amyloid β peptides (Aβ), which is believed to be the driving force of AD pathophysiology. Increase of soluble Aβ and its deposition in insoluble amyloid plaques together with neurofibrillary tangle (NFTs) also represent the hallmark of the most common, late onset form of Alzheimer's disease (LOAD). Intracellular accumulation of NFTs, which are composed of hyperphosphorylated form of the microtubule-associated protein tau, represents a later event during disease progression and strongly correlates with neuronal death and cognitive impairments [4]. Evidence in rat and human neuronal culture [5], and in mouse models [6], suggest that accumulation of Aβpeptidesdrives tau phosphorylation but the exact mechanisms remain elusive. Beside known environmental and genetic risk factors, the etiology of LOAD still remains largely unexplained [7]. Recent genetic studies identified mutations in immune receptor genes *TREM2* [8] and *CD33* [9, 10] that confer a significant high risk of developing Alzheimer's disease. These genes, expressed by microglia cells, are important for glia cell function and inflammation regulation and play a major role in the phagocytosis of cellular debris and amyloid beta. Glia cells and in particular microglia represent the major players in orchestrating the innate immune response in the brain. Upon binding cellular debris or soluble and insoluble amyloid via specific receptors, microglia trigger the inflammatory response by assuming an activated state and producing pro-inflammatory cytokines and chemokines. In the brain of AD patients, activated microglia

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exposed to amyloid β increase the production of pro-inflammatory cytokines such as TNF-a, IL-1 and IL-6 [11]. This non-resolved, chronic neuroinflammation caused by amyloid-induced microglia activation and the sustained levels of pro-inflammatory cytokines may promote tau hyperphosphorylation and aggregation [12, 13]. Epigenetic and transcriptional control of pro-inflammatory genes in immune cells play a major role during the inflammatory response. Pro-inflammatory gene expression needs to be tightly modulated since an imbalanced production of pro-inflammatory cytokines and chemokines could lead to detrimental effects to the host organism [14]. Inflammatory gene expression is regulated in part by the bromodomain and extraterminal domain (BET) family of proteins, which in human consists of BRD2, BRD3, BRD4 and the testes specific BRDT. In the nucleus of immune cells, BET proteins bind to acetylated histones and transcriptional co-regulators to activate transcription and elongation of pro-inflammatory genes, among which E-selectin, Tnfa, Il1b, Il1a and Ptgs2 [15, 16]. Recently developed BET-bromodomain inhibitors function as specific and potent regulators of the innate immune response by reducing the expression of pro-inflammatory cytokines and chemokines [17]. Administration of BET inhibitors attenuates mice death induced by endotoxin shock and sepsis [17, 18] and is effective in reducing inflammation in mouse models of autoimmunity [19, 20]. Even though a BET bromodomain inhibitors (RVX-208) is currently in phase I clinical trial for Alzheimer's disease [21], mechanistic data from animal models are missing. The triple transgenic 3xTg mouse model of Alzheimer's disease harbors three human transgenes: the familial AD mutations APPSwe and PS1m146v and the TauP301L mutation which is found in frontotemporal dementia [22]. These mice start developing intracellular amyloid β at 3 months of age, which is followed by hyperphosphorylation of tau, extracellular deposition of amyloid plaques and intracellular formation of neurofibrillary tangles between 18 and 26 month of age [22]. Moreover 3xTg mice develop systemic autoimmunity and inflammation between 6 and 12 month of age [23] and show cognitive deficits similar to those observed in AD patients [22]. In this study we showed that prolonged treatment with the brain-penetrant BET-bromodomain inhibitor JQ1 reduced systemic and brain inflammation and tau phosphorylation at Ser396 in the 3xTg model of Alzheimer's disease.

MATERIAL AND METHODS

Drug Preparation

The BET inhibitor (+)-JQ1 was kindly provided by the Bradner Lab at Dana-Farber Cancer Institute, Harvard Medical School. JQ1 was initially dissolved in DMSO to have a stock solution of 50 mg/ml. For injections, the stock solution of JQ1 was diluted with vehicle solution (saline, 5% Tween 80) and bath-sonicated to properly dissolve the drug. Mice received intraperitoneal injection of JQ1 (50mg/kg) in a volume of 150 ml.

Animals

20 male 3xTg mice were purchased from Jackson Laboratories and the Mutant Mouse Resource and Research Center (MMMRC) repository at two months of age. Upon arrival from the supplier, all mice were group-housed (5 mice/cage) and kept under standard laboratory conditions. All protocols were performed in accordance with the rules and regulations of the Institutional Animal Care and Use Committee at the University of Miami. Mice started to receive intraperitoneal (IP) injections when they were 3 months old. Half of the mice received 1 injection per day for 5 days a week of JQ1 (50mg/kg), while the other half received vehicle control solution. 12 weeks-old mice were injected for 15 weeks (Fig. **1**). To avoid possible acute effects of the drug we suspended injections for two weeks prior to behavioral testing. Behavioral experiments lasted for one week, during which mice did not receive any treatment (Fig. **1**). After completion of behavioral testing mice were injected for 1 week before they were euthanized and their organs collected for downstream molecular analyses. One of the JQ1-treated mice prematurely died during the second month of treatment.

RNA Extraction and RT-qPCR

Mice were first anaesthetized with isoflurane inhalation, then euthanized by cervical dislocation and decapitated to collect their brains, which were separated in the two hemispheres. From each hemisphere the hippocampus and frontal cortex were dissected over ice and then flash frozen in liquid nitrogen for RNA and protein analysis. Brain tissues were homogenized and RNA was extracted and purified using a combination of TRIzol reagent and QIAGEN RNeasy columns as previously described [24]. 1 mg of RNA was re-

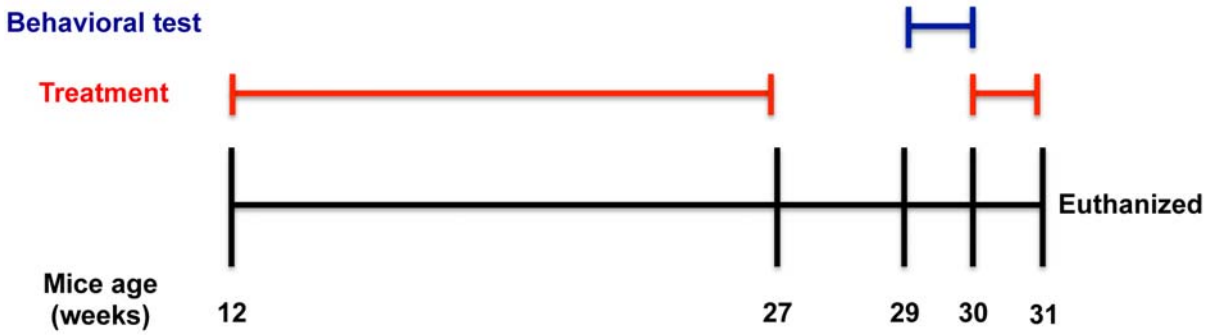

Fig. (1). Treatment scheme.
12 weeks-old mice received 50 mg/kg of JQ1 or control saline injections once a day for 5 days/week for 15 weeks. Treatment was interrupted for two weeks. After this, mice underwent behavioral testing during which time they did not receive any treatment. After completion of behavioral testing, mice received treatment for 1 week before they were euthanized.

verse transcribed using the High-Capacity cDNA Reverse Transcroption Kit (Applied Biosystems). cDNA was diluted 1 to 5 and used as template for TaqMan qPCR on the QuantStudio™ 6 Flex Real-Time PCR System. *Actin*, *Ccl2*, *Il-1b*, *Tnfa*, *Il-6*, *Ptgs2*, *Nos2* and *MAPT* TaqMan assays were purchased from Life Technologies. For all RT-qPCR reactions we included three technical replicates. To compare the expression of genes between JQ1- and vehicle-treated mice we used Student's t–test. A p-value less 0.05 was considered as statistically significant.

Western Blotting and ELISA

Brain samples were homogenized in mammalian protein extraction reagent (M-PER) lysis buffer (Pierce/ThermoFisher) supplemented with complete protease (Roche) and phosphatase inhibitor (Sigma Aldrich) cocktails. Gel electrophoresis and immunoblotting were done as previously described [25]. Immunoblots were developed using primary antibodies directed to anti-Phospho-Tau Ser396 (Cell Signaling, #9632), anti-Tau (Abcam, ab64193) and GAPDH (Santa Cruz Biotechnology, sc-32233) and HRP-conjugated secondary antibodies. For the measurement of soluble Aβ, brain tissues were homogenized as described above. ELISA was performed to quantify Aβ using the human Aβ42 and Aβ40 kit from Thermo Fisher Scientific.

Behavioral Studies

The modified Barnes maze, Y maze and open field test were performed on 10-vehicle treated and 9 JQ1-treated 7-months-old 3xTg mice. The Barnes maze consisted of 12 equally spaced holes around the circumference of a circular table of 100 cm in diameter and elevated 90 cm from the floor. Below one of the hole was allocated an escaping cage that mice could use to escape the maze. The maze was positioned in a room with distinct visual cues with a bright light and a loud buzzer acting as stressors to facilitate escape behavior. At the beginning of each trial, the mouse was confined for 1 min in the center of the maze with a box; time began when the box was lifted and the mice were able to explore the maze. Latency to escape was calculated using EthoVision tracking software. Mice were trained to find the escape hole with two 5 min trials per day for 3 consecutive days (training phase). Each trial ended when the mouse entered the escape hole. Following the training phase, mice were returned to their home cage for 3 days and then tested for their ability to remember the location of the escape hole for 2 days, with two 5 min trials per day (testing phase). Repeated measures two-way ANOVA was used to evaluate the effects of JQ1 over time. The Y maze spontaneous alternation test consisted of a Y-shaped maze with three plastic arms (40 × 9 × 16 cm) at 120 degree angle from each other. The mice were placed at the center of the maze and their activity was recorded using EthoVision tracking software. The number of entries in each arm was scored manually when all four limbs of the mice were within the arm. Alternations were defined as successive entries into each of the three arms as on overlapping triplet sets (i.e., ABC, BCA). The percentage of alternation was calculated as the ratio between the numbers of spontaneous alternation over the total number of arm entries. Locomotor activity (distance traveled and speed) was measured for 10 min in a 27 X 27 cm open-field Plexiglas chamber using EthoVision tracking software.

RESULTS

JQ1 Reduces Expression of Pro-Inflammatory Genes in the Brain

In this study we tested the effects of chronic administration of the brain-penetrant BET-bromodomain inhibitor JQ1 to the 3xTg model of AD. 3-month-old mice received daily intraperitoneal (IP) injections of JQ1 or vehicle over a 15 weeks period (see Methods section and Fig. **1**). Mice cognition was then assessed using several behavioral tests (see below), and tissue from the brain and other organs was collected for molecular examination. We examined the effects of JQ1 on the expression of microglia-secreted pro-inflammatory cytokines and chemokines and genes expressed by activated glia cells in brain of 3xTg mice. A statistically significant reduction in the expression of *Tnfa* and *Il-6* in the hippocampus and in the frontal cortex was found in JQ1 treated mice (Fig. **2**). JQ1 treatment also reduced the expression of *Il-1b* and *Ccl2* in the hippocampus and a similar trend, although not statistically significant, was observed in the frontal cortex (Fig. **2**). We then looked at the levels of inflammation-related proteins that are expressed by activated glia cells. JQ1 treatment reduced the expression of *Ptgs2* and *Nos2* in the hippocampus and frontal cortex of 3xTg mice (Fig. **2**). In line with previous data demonstrating a systemic anti-inflammatory effect of BET-bromodomain inhibitors, our results showed that JQ1 negatively regulates brain expression of pro-inflammatory genes that play a major role in neuroinflammation.

JQ1 Treatment Attenuates Tau Phosphorylation at Ser396

In 3xTg mice, microglia become activated in an age-dependent manner that correlates with tau hyperphosphorylation [26]. Here we wanted to verify whether the anti-inflammatory activity of JQ1 had an effect on the phosphorylation of tau at Ser396, which represents an early event in Alzheimer's disease [27]. Western blot (WB) analysis using phospho-tau (Ser396) antibody showed hyperphosphorylation of tau in the hippocampus (Fig. **3A**) and frontal cortex (Fig. **4A**) of 7-month-old 3xTg mice. Compared to mice that received vehicle control injections, JQ1-treated mice showed a statistically significant reduction of hyperphosphorylated tau in both brain regions (Fig. **3A, 3B** and Fig. **4A, 4B**). Western blot and reverse transcriptase quantitative PCR (RT-qPCR) analyses showed no statistically significant changes in the expression of total tau protein (Fig. **3A** and **4A**) and *MAPT* mRNA (Fig. **3C** and **4C**) in JQ1-treated versus vehicle-treated mice. Our data revealed significant reduction of Tau phosphorylation at Ser396 without alteration of total tau protein in the JQ1 treated mice and suggest a role for immune response in Tau hyperphosphorylation.

JQ1 Does Not Affect the Brain Levels of Soluble Aβ42 and Aβ40

In AD, Aβ production directly induces microglia activation and decreased amount of Aβ results in attenuation of tau

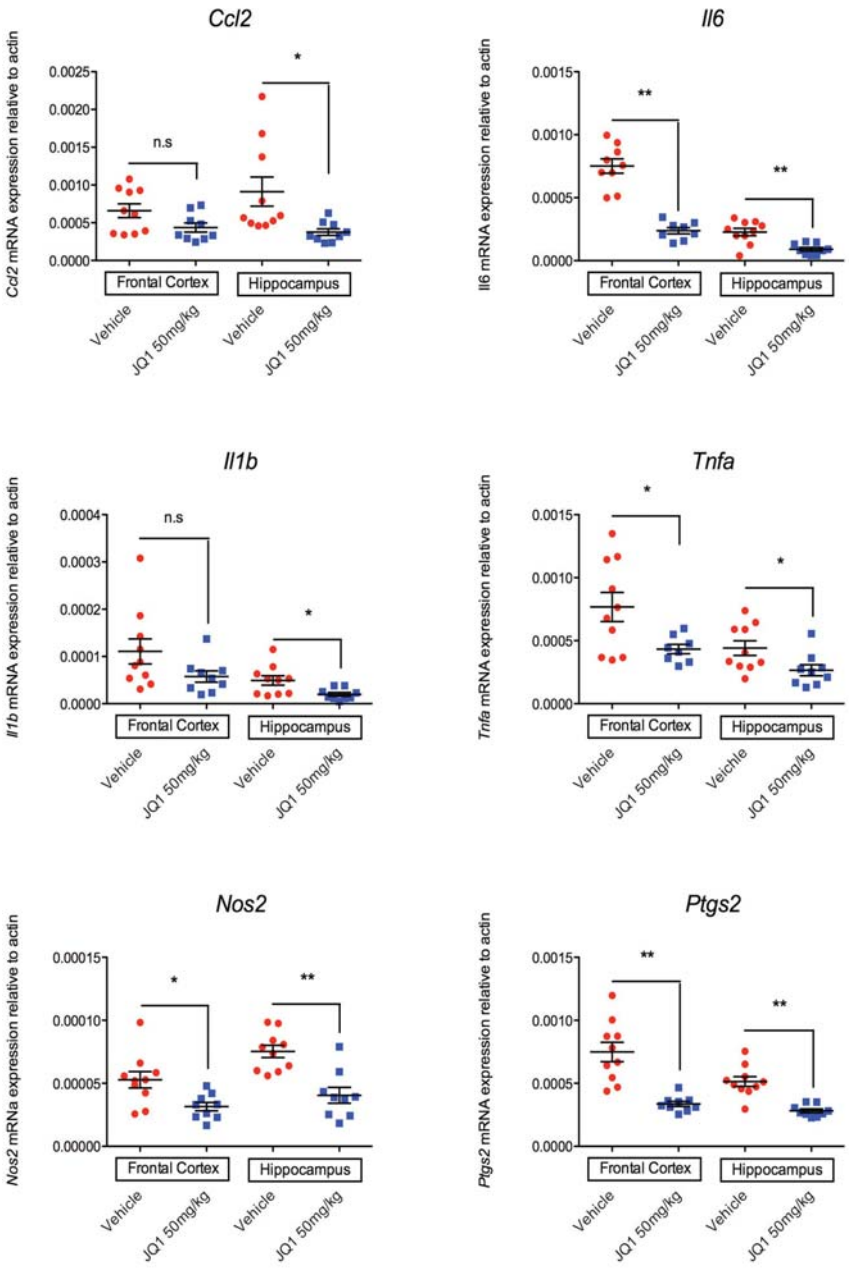

Fig. (2). JQ1 reduces the expression of pro-inflammatory genes in the hippocampus and frontal cortex.
RT-qPCR analysis of pro-inflammatory gene expression changes in the hippocampus of 3xTg mice treated with JQ1. On the Y exe is depicted the expression of the analyzed gene relative to the housekeeping gene β-Actin. Error bars are S.E.M.; * p value < 0.05; ** p value < 0.001.

pathology [5, 6]. Thus, it was possible that the changes in the expression of neuroinflammatory markers and in tau phosphorylation that we observed after JQ1 treatment were due to a reduction in Aβ levels. To verify this hypothesis we used ELISA to measure the amounts of human soluble Aβ42 and Aβ40 in the frontal cortex and hippocampus of JQ1- and vehicle-treated mice. Our data showed no reduction in the levels of Aβ42 and Aβ40 in both brain regions (Fig. **5**).

JQ1 Reduces Splenomegaly in 3xTg Mice

In young adult mice the spleen weights around 150 mg and measures approximately 15 mm in length. During animal dissection we noticed that most of the 3xTg mice that received vehicle-control injections had severe enlargement of the spleen (Fig. **6A**), with some of the spleens reaching 1g in weight (Fig. **6B**). Splenomegaly was not present in mice #6 and #8, which had spleen of normal size (Fig. **5A**). Splenomegaly has been previously observed in 12-month-old 3xTg mice [23] and could be a consequence of the sustained chronic inflammatory response due to amyloidosis. Interestingly, JQ1-treated mice showed a statistically significant reduction of their spleen size compared to vehicle-treated mice (Fig. **6A** and **6B**), possibly due to the systemic anti-inflammatory effects of JQ1. Importantly, we noticed a statistically significant correlation between spleen size and the level of tau phosphorylation in the frontal hippocampus (Fig.

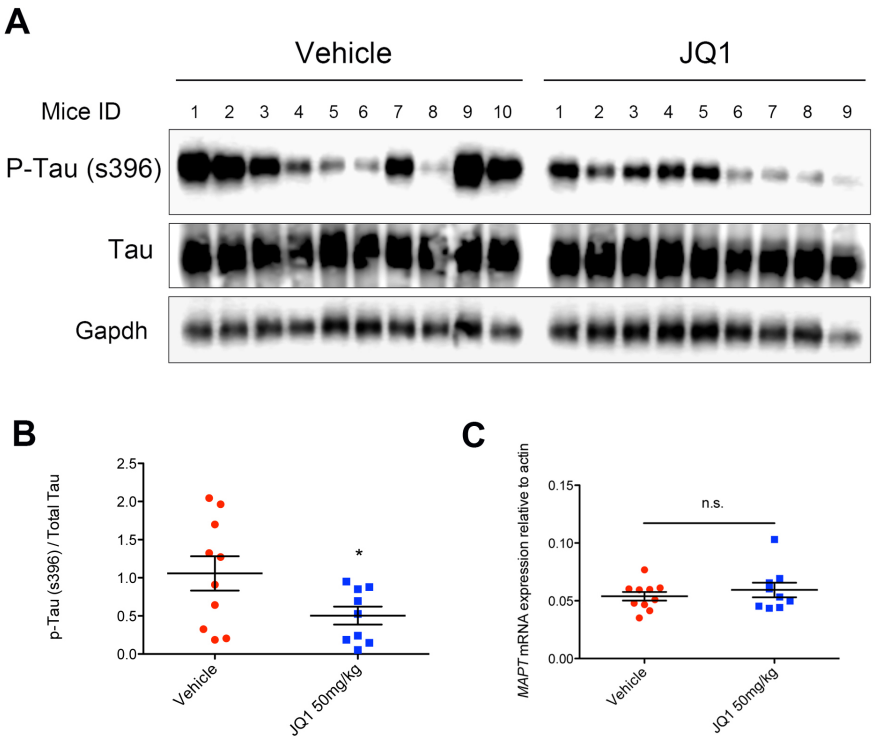

Fig. (3). JQ1 reduces tau phosphorylation in the hippocampus.
(**A**) Western blot analysis of total tau and tau phosphorylation at Ser396 (P-Tau(s396)) in the hippocampus of 3xTg mice treated with JQ1. The expression of the housekeeping gene Gapdh was used as loading control. (**B**) Densitometric analysis of P-tau(Ser396) using ImageJ. On the Y axe is depicted the ratio between the intensity of the gel bands corresponding to P-tau(Ser396) and total Tau. (**C**) RT-qPCR analysis of MAPT expression in the hippocampus of vehicle control- (C) and JQ1-treated (J) mice. Error bars are S.E.M.; * p value < 0.05.

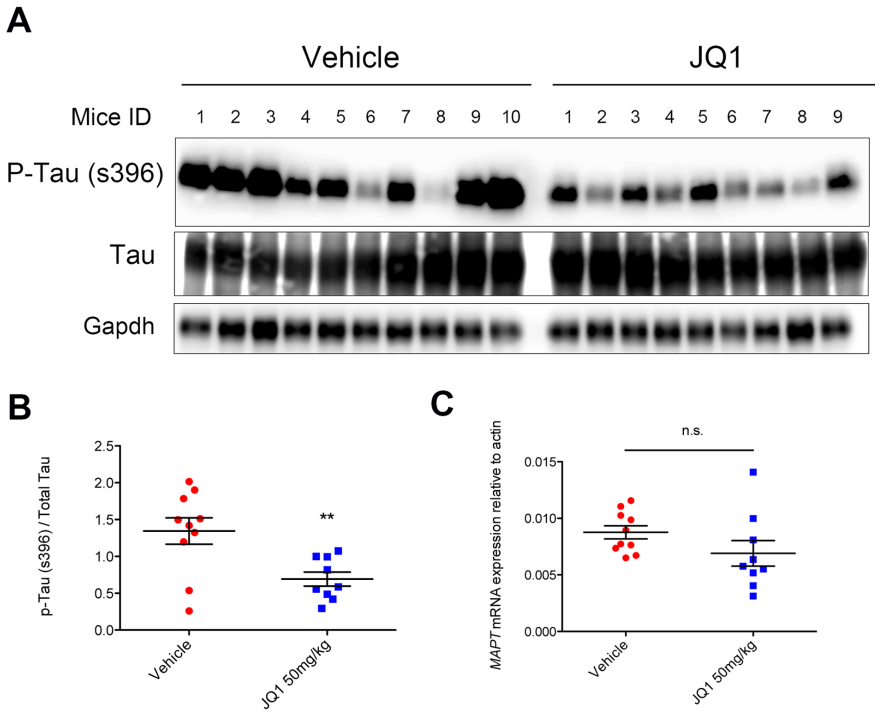

Fig. (4). JQ1 reduces tau phosphorylation in the frontal cortex.
(**A**) Western blot analysis of total tau and tau phosphorylation at Ser396 (P-Tau(s396)) in the frontal cortex of 3xTg mice treated with JQ1. The expression of the housekeeping gene Gapdh was used as loading control. (**B**) Densitometric analysis of P-tau(Ser396) using ImageJ. On the Y axe is depicted the ratio between the intensity of the gel bands corresponding to P-tau(Ser396) and total Tau. (**C**) RT-qPCR analysis of MAPT expression in the frontal cortex of vehicle control- (C) and JQ1-treated (J) mice. Error bars are S.E.M.; * p value < 0.05.

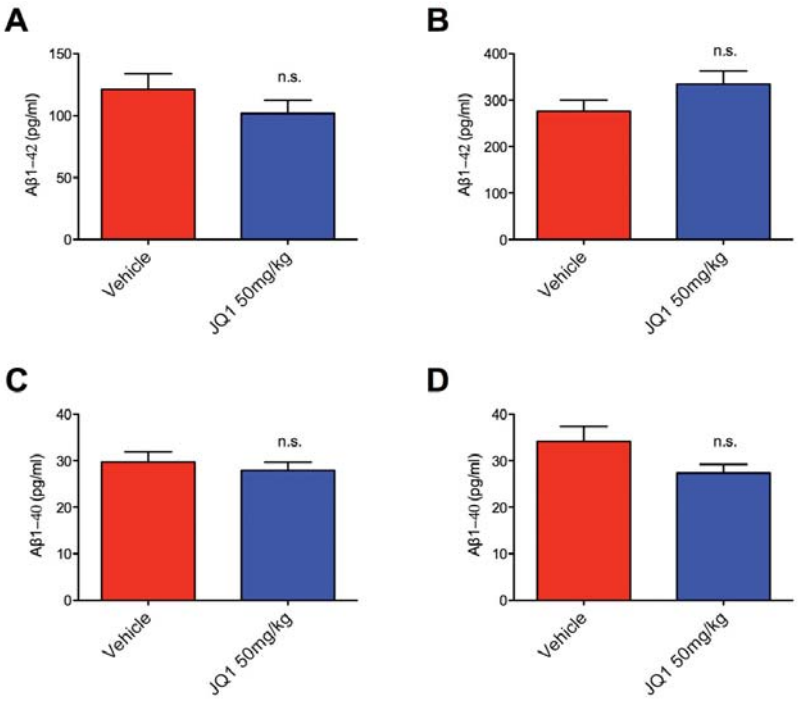

Fig. (5). JQ1 treatment does not affect Aβ levels.
The levels of human Aβ1-42 (upper panels) and Aβ-1-40 (lower panels) were measured by ELISA in the frontal cortex (**A** and **C**) and in the hippocampus (**B** and **D**) of JQ1- and vehicle-treated mice. Error bars are S.E.M. N=9 for JQ1-treated mice. N=10 for vehicle-treated mice.

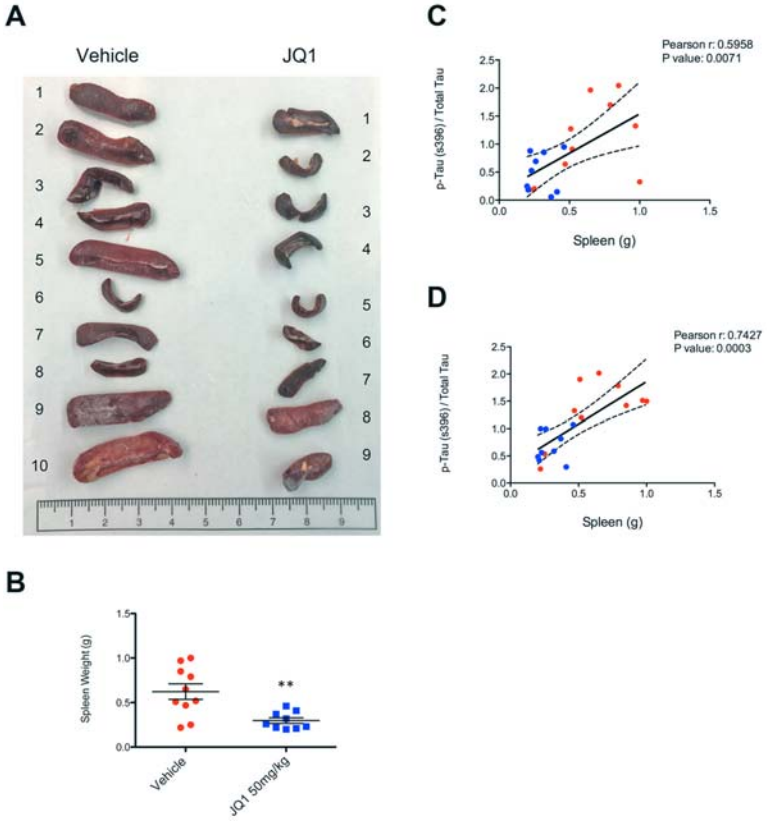

Fig. (6). JQ1 reduces splenomegaly.
7 months old 3xTg mice that received IP injections of JQ1 or vehicle control solution were sacrificed and their spleen collected for examination (**A**). The spleens of JQ1- and vehicle-treated mice were weighted and compared using T test (**B**). Correlation analysis between P-tau(Ser396) and spleen weight in the hippocampus (**C**) and frontal cortex (**D**). Red circles represent vehicle-treated mice; blue circles represent JQ1-treated mice**.** Error bars are S.E.M.; ** p value < 0.001.

6C) and frontal cortex (Fig. **6D**) of 3xTg mice, further highlighting the correlation between inflammation and tau pathology.

Learning and Memory is not Improved by JQ1 Treatment in 7-Months-Old Mice

Open field test was performed to verify whether JQ1 treatment had any consequences on locomotor activity that could affect the interpretation of the results of other behavioral tests aimed at the evaluation of mice cognition. In the open field test, JQ1-treated mice showed no differences in terms of velocity (Fig. **7A**) or distance traveled (Fig. **7B**) compared to vehicle-treated mice, suggesting that JQ1 treatment does not affect locomotor activity.

Hippocampal and frontal cortex dependent memory were assessed using two different behavioral tests: the modified Barnes maze test and the Y maze spontaneous alternation test. The modified Barnes maze is a behavioral test to measure hippocampal-dependent spatial learning and memory in which mice utilize visual cue to find the escape hole in the maze [28]. At the age of 7 months and following 3 months of JQ1 treatment, we noticed no differences between JQ1- and vehicle-treated mice in finding the escape hole during the three days of training phase (Fig. **7C**). During the first day of testing JQ1-treated mice performed worse than control animal, while no differences were observed during the second day of training (Fig. **7C**).

The Y maze is a behavioral test that relies on the predisposition of mice to explore new environments. The test is based on mice ability to remember the most recently explored arm of the arena and to frequently alternate entries in different arms. The hippocampus and the pre-frontal cortex, among other parts of the brain, are involved in this task. We found no statistically significant differences in the percentage of alternations between JQ1- and vehicle-treated mice at the age of behavioral test (Fig. **7D**).

DISCUSSION

In the past two decades the important role of neuroinflammation in the pathophysiology of AD, in both preclinical and late stage of the disease, has been deeply investigated. Prospective longitudinal studies have shown that neuropathological changes in the brain of AD patients, including neuroinflammation, precede neurodegeneration and the appearance of cognitive impairments [29]. Epidemiological studies using anti-inflammatory drugs suggest that inhibition of inflammation may reduce the risk of developing AD [30-32], however results from clinical trials using non-steroidal anti-inflammatory drugs (NSAIDs) have provided inconclusive results. Because neuroinflammation is an early pathological event, therapeutic interventions aimed at blocking inflammation may have beneficial effects if administered during the early asymptomatic phase of the disease. In the current study, we treated young (three-month-old) asymptomatic 3xTg AD mice with the BET bromodomain inhibitor JQ1 for an extended period of time (over four months) and evaluated JQ1's effects on pathological brain changes and cognition. Microglia are the innate immune cells of the central nervous system where they are crucial in protecting the brain from pathogens and in removing cellular debris and toxic protein aggregates, among which Aβ [33]. Under pathophysiological conditions microglia cells become activated, extend their processes and migrate to the site of injury where they adopt two different activated phenotypes: a pro-inflammatory M1 phenotype and an anti-inflammatory M2 phenotype. M1 microglia are characterized by the increased expression of pro-inflammatory proteins including IL-1, IL-6, IL-12, TNFą,NOS2, COX2, while M2 microglia mostly secrete anti-inflammatory cytokines like IL-4, IL-10 and TGFβ [34]. In the brain of AD patients activated M1 microglia localize around amyloid plaques where they bind Aβ through specific receptors and start producing pro-inflammatory cytokines, and proteins and release reactive oxygen and nitrogen species [35]. In AD brain the constant production of Aβ sustains microglia activation transforming the innate immune response in a detrimental non-resolving chronic inflammation state. A recent transcriptomic analysis in BV-2 microglial cells showed that JQ1 treatment inhibits the expression of several LPS-induced pro-inflammatory genes [36], while little changes in gene expression were observed when non-activated BV-2 cells were treated with JQ1. Among the genes activated by LPS stimulation and inhibited by JQ1 there were several important pro-inflammatory modulators like *Il-1, Nos2, Ptgs2* and *Ccl2* [36]. These observation together with the fact that JQ1 has proven to be able to cross the blood brain barrier and target the CNS [36-38], make this bromodomain inhibitor a promising compound for the treatment of neuroinflammation. Indeed, i*n vivo* data presented here support this hypothesis. Similarly to what has been shown in the above-mentioned *in vitro* study, in our *in vivo* experiments intraperitoneal injections of JQ1 reduced the expression of several microglia-secreted pro-inflammatory molecules in the brain of 3xTg mice. JQ1 treatment efficiently reduced brain expression of *Ccl2*, also know as monocyte chemotactic protein 1 (*Mcp1*), an important modulator of immune response that mediates microglia recruitment at inflammatory sites. The role of CCL2 in AD neuroinflammatory process was recently suggested by the initial findings that Aβ exposure drives CCL2 expression in human monocytes and astrocytes [39] as well as in microglial cells isolated from the brain of AD patients [40]. Another important pro-inflammatory cytokine that is reduced by JQ1 treatment is interleukin 1b. High levels of IL-1b have been found in microglia surrounding amyloid plaques in addition to the brain and CSF of AD patients [41, 42]. Moreover, a polymorphisms in IL-1b locus is associated with increased risk of developing AD [43]. In AD brain IL-1þis produced by glia and neurons exposed to Ab where it acts as a central player in modulating the innate immune response. IL-1þfunctions in a positive feedback loop by activating the release of other pro-inflammatory molecules from glia and neurons. For example it has been shown that IL-1b induces the expression of IL-6 and TNFa in microglia and astrocytes [44]. Interestingly these two pro-inflammatory cytokines, that are also directly produced by microglia in response to amyloid beta load in AD brain [45], were significantly reduced by JQ1 treatment in the brain of 3xTg mice. IL-1b also drives COX-2 expression and prostaglandins (PGs) production in astrocytes [46, 47] and neurons [48]. COX2 expression increases during early phases of AD [49] and in-

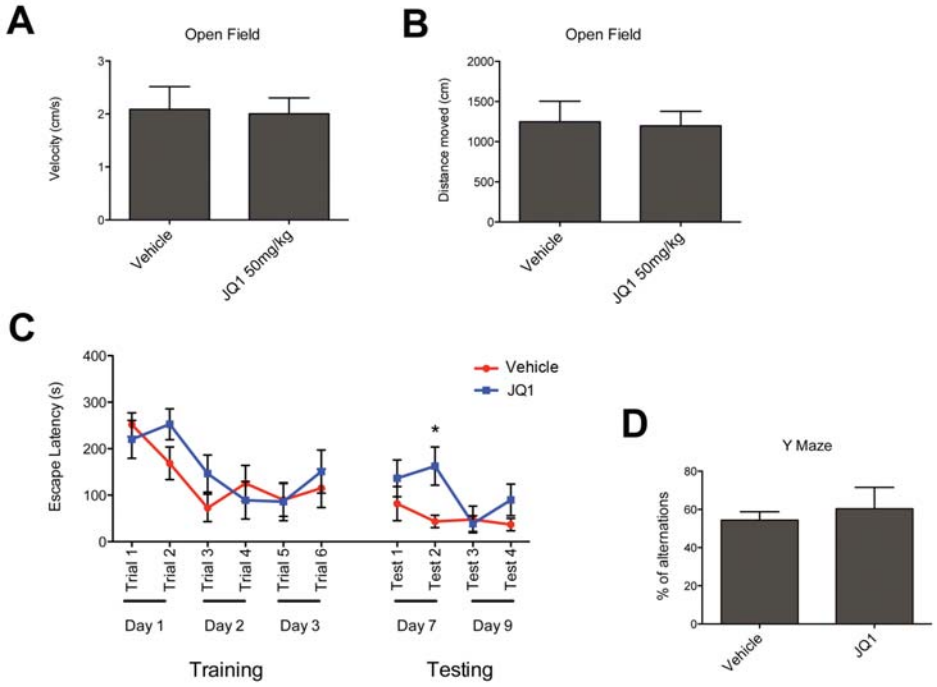

Fig. (7). JQ1 treatment doesn't improve cognition

Open field test measuring the velocity (**A**) and the distance traveled (**B**) by vehicle- and JQ1-treated mice. **(C)** Vehicle control- and JQ1-treated mice were trained and subsequently tested in the modified Barnes maze to evaluate their ability to discover the escape hole. On the Y axe is depicted the mean latency to locate the escape hole per trial. Vehicle control-treated mice are shown in red, JQ1-treated mice are shown in blue. (**D**) Y maze test measuring the % of alternation in the entrance of different arms by vehicle- and JQ1-treated mice. Error bars are S.D.; * p value < 0.05.

creased levels of PGs have been reported in the CSF of AD patients [50]. Inhibition of COX-2 prevents the Aβ-mediated suppression of hippocampal long-term plasticity [51]. Interestingly, our data showed that JQ1 reduces the expression of COX2 by almost 2 fold in the hippocampus (Fig. **2**) and frontal cortex (Fig. **3**) of 3xTg mice. In microglia and astrocytes exposure to different pro-inflammatory cytokines leads to increased production of iNOS, which catalyze the synthesis of nitric oxide, a toxic molecule for neurons. Not surprisingly iNOS expression was found to be upregulated in the brain of AD patients [52], while reduction of iNOS in AD mouse models reduced disease severity [53]. In our study JQ1 treatment of 3xTG mice resulted in a statistically significant reduction of iNOS in the hippocampus and frontal cortex.

The role of inflammation in the development of tau pathology has been extensively studied in the 3xTg [26, 54] and other mouse models of neurodegeneration [12, 13], where it has been shown that microglia activation and the augmented production of pro-inflammatory cytokines increase tau-phosphorylation in the brain via different mechanisms. For instance it has been shown that microglia-secreted IL-1β triggers tau phosphorylation via P38-MAPK pathway [55]. Because of the JQ1-mediated reduction of several pro-inflammatory modulators we decided to look at the level of tau phosphorylation in treated mice. We noticed a positive correlation between systemic inflammation (as a measure of spleen size) and tau phosphorylation. Furthermore, our data showed that JQ1 treatment reduced the levels of tau phosphorylation at Ser396 but not total tau expression in the hippocampus and frontal cortex of treated mice, suggesting that JQ1 anti-inflammatory effects may be effective in reducing tau pathology.

One of the first things that we noticed when we dissected the mice that received vehicle injections was the presence of significantly enlarged spleens. Splenomegaly was previously reported in 3xTg mice where it was associated with a chronic systemic autoimmune/inflammatory disease of the mice [23]. Interestingly, splenomegaly was also reported in different mouse models with reduced dosage of γ -secretase: $PS1^{(+/-)}PS2^{(-/-)}$ [56] and $Nct^{(+/-)}$ and $Nct^{(+/-)}$ $PS1^{(+/-)}$ [57]. In these mice, reduced **γ**-secretase activity leads to the development of an age-dependent myeloproliferative disease, characterized by an overproduction of granulocyte-monocyte progenitors (GMPs) [56]. These observations suggest an intriguing scenario involving a possible link between γ-secretases, monocytes/microglia production, neuroinflammation and AD. Other than the know effects on APP processing, the altered activity of **γ**-secretase may lead to (possibly via NOTCH signaling [58]) an imbalanced production of monocytes which are important factors in chronic neuroinflammatory process associated with AD [59, 60]. The effects of JQ1 on spleen size could also be due to inhibition of myeloid-precursor cells proliferation as suggested by previous studies showing the ability of BET inhibitors to antagonize the growth of myeloid malignancies [61].

Recent failures in clinical trials suggest that therapeutic intervention with disease-modifying drugs during the preclinical phase of LOAD may represent a successful therapeutic strategy. This is confirmed by data from animal models

showing that drugs targeting amyloid pathway have little efficacy in restoring cognition once neuronal degeneration has already occurred. To test whether JQ1 treatment had any beneficial effects on animal cognition we began drug treatment when 3xTg mice were 3 months old and we daily administered the drug for almost 4 months. At this point we tested animals cognition with a series of behavioral tests aimed at measuring hippocampus- and cortex- dependent learning and memory. Despite the encouraging results showing reduction of neuroinflammation and tau phosphorylation, behavioral tests revealed no improvement in cognition in JQ1-treated 7-month-old mice. This could be due to the relatively young age of treated mice cohort and the late start of behavioral impairment in 3xTg mice. As reported by previous studies, 3xTg mice at this age start to develop impairments in spatial working memory [62, 63]; however, more severe deficits in recognition memory and reference memory only appear between 9 and 12 months of age [64, 65]. More experiments using JQ1 for a prolonged period of time and in different age windows should be performed to test the effects of the drug on mice cognition. To further complicate this picture is a recent study showing that the BET protein Brd4 mediates the transcriptional response that occurs during memory formation and that Brd4 inhibition by JQ1 affects memory formation [66].

CONCLUSION

BET bromodomain inhibitors are potent modulators of inflammation and hold the promise to be utilized in the future for the prevention and treatment of neurological disorders characterized by chronic neuroinflammation. The significant decrease in tau phosphorylation we observed also suggests that BET bromodomain inhibitors hold potential therapeutic effects for diseases characterized by tau hyperphosphorylation. Nevertheless, the pharmacodynamics and pharmacokinetics of currently available BET inhibitors need to be further improved. New drugs with better brain accessibility and improved selectivity for the individual BET proteins will be more potent and specific modulators of CNS inflammation.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Marco Magistri: conceived and designed the study, collected, assembled and interpreted data and wrote the manuscript.

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