

# HDAC6 Inhibitor Blocks Amyloid Beta-Induced Impairment of Mitochondrial Transport in Hippocampal Neurons

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## Abstract

Even though the disruption of axonal transport is an important pathophysiological factor in neurodegenerative diseases including Alzheimer's disease (AD), the relationship between disruption of axonal transport and pathogenesis of AD is poorly understood. Considering that  $\alpha$ -tubulin acetylation is an important factor in axonal transport and that A $\beta$  impairs mitochondrial axonal transport, we manipulated the level of  $\alpha$ -tubulin acetylation in hippocampal neurons with A $\beta$  cultured in a microfluidic system and examined its effect on mitochondrial axonal transport. We found that inhibiting histone deacetylase 6 (HDAC6), which deacetylates  $\alpha$ -tubulin, significantly restored the velocity and motility of the mitochondria in both anterograde and retrograde axonal transports, which would be otherwise compromised by A $\beta$ . The inhibition of HDAC6 also recovered the length of the mitochondria that had been shortened by A $\beta$  to a normal level. These results suggest that the inhibition of HDAC6 significantly rescues hippocampal neurons from A $\beta$ -induced impairment of mitochondrial axonal transport as well as mitochondrial length. The results presented in this paper identify HDAC6 as an important regulator of mitochondrial transport as well as elongation and, thus, a potential target whose pharmacological inhibition contributes to improving mitochondrial dynamics in A $\beta$  treated neurons.

**Citation:** Kim C, Choi H, Jung ES, Lee W, Oh S, et al. (2012) HDAC6 Inhibitor Blocks Amyloid Beta-Induced Impairment of Mitochondrial Transport in Hippocampal Neurons. PLoS ONE 7(8): e42983. doi:10.1371/journal.pone.0042983

**Editor:** Sergio T. Ferreira, Federal University of Rio de Janeiro, Brazil

**Received:** February 13, 2012; **Accepted:** July 16, 2012; **Published:** August 22, 2012

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**Funding:** This work was supported by grants from the 21C Frontier Functional Proteomics Project (FPR08K1301-02210), National Research Foundation 2009-0081673, World Class University-Neurocytomics, and the Korean National Institute of Health R&D Program Project (2009-0443) and supported in part by the Basic Research Program (2008-05943) and MRC at SNU (2011-0030738). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Neurons have extensive processes and asymmetrical organization. The communication between the cell body and the nerve terminal is critical for neuronal functions, which involves microtubule (MT) based axonal transport. MTs are dynamically assembled polymers of  $\alpha$ - and  $\beta$ -tubulin [1]. Tubulin undergoes various post-translational modifications (PTMs), including acetylation, tyrosination, and phosphorylation [2,3]. PTMs of tubulin regulate not only the interaction between MTs and MT-associated proteins (MAP), but also the stability of the microtubule, contributing to controlling axon guidance, synapse formation, and neuronal transport [2,3,4]. Acetylation of  $\alpha$ -tubulin plays a positive role in axonal transport in mammals by increasing microtubule stability [3,5,6]. Histone deacetylase 6 (HDAC6) is a unique cytosolic enzyme that mediates the deacetylation of  $\alpha$ -tubulin, which involves two functional deacetylase domains and a zinc finger motif [7]. The level of acetylated  $\alpha$ -tubulin is decreased [9,10] as the level of HDAC6 is increased in the AD patients' brains [11]. Since impaired axonal transport is an important pathophysiological factor in AD [8], HDAC6 may play a role in

the disruption of axonal transport in AD pathogenesis. Amyloid beta (A $\beta$ ), the cleavage product from amyloid precursor protein (APP), is one of the causative factors of AD pathogenesis. A $\beta$  interrupts vesicular and axonal transport by inducing alteration in microtubule stability and intracellular signaling pathways [12,13,14]. A $\beta$  also causes synaptic degeneration and loss through the disruption of axonal transport [12,15], which leads to impaired trafficking of the mitochondria and neurotransmitters necessary for synaptic function and neuronal viability [8,10,16].

Mitochondria can be delivered along the axon in association with microtubules, which is important for supplying energy required to maintain neuronal functions. During axonal transport, mitochondria are associated with several motor proteins, such as kinesin for anterograde transport and dynein for retrograde transport [1,2]. Adaptor proteins, such as Miro (Miro1 and Miro2 in mammals) and Milton (OIP106 and GRIF1 in mammals), are connected to mitochondria through kinesin [17,18]. Although impaired axonal transport of mitochondria has been reported in the presence of A $\beta$  [12], the precise mechanism for this A $\beta$ -induced impairment remains unclear. In the present study, we

attempted to elucidate the mechanism that links the acetylation of  $\alpha$ -tubulin and A $\beta$ -induced impairment of mitochondrial transport in hippocampal neurons cultured in a microfluidic system. To increase  $\alpha$ -tubulin acetylation, we used the Tubastatin A (TBA) as the HDAC6 inhibitor. Mitochondrial axonal transport was analyzed by measuring the velocity, motility and length of mitochondria. We found that pharmacological inhibition of HDAC6 significantly restored the compromised velocity and motility of the mitochondria of A $\beta$  hippocampal neurons to a normal level in both anterograde and retrograde axonal transports. The inhibition of HDAC6 also recovered the length of mitochondria that had been shortened by A $\beta$ . These results show that the inhibition of HDAC6 rescued neuronal cells from A $\beta$ -induced impairment of mitochondrial axonal transport as well as mitochondrial length, identifying HDAC6 as a potential therapeutic target to modulate AD pathogenesis.

## Results

### Decrease in the acetylation of $\alpha$ -tubulin in the brains of 5XFAD mice

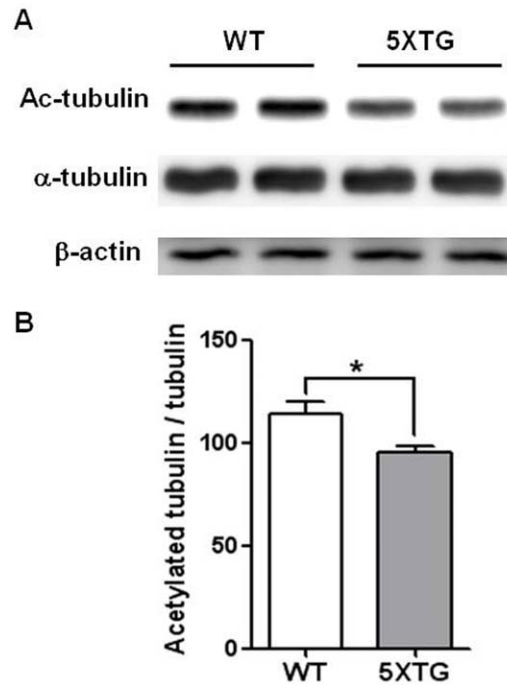
The reduced level of acetylated  $\alpha$ -tubulin [9,10] correlates to the increased level of HDAC6 in the AD patients' brains [11]. To determine the role of acetylation of  $\alpha$ -tubulin in AD, we examined the level of acetylated  $\alpha$ -tubulin in the brains of 5XFAD mice, an AD animal model. Western blot analysis showed that the level of acetylated  $\alpha$ -tubulin in the 5XFAD mice was significantly lower than that of wild type mice (WT:  $1.145 \pm 0.059$ , 5XFAD:  $0.96 \pm 0.026$ , Fig. 1A and 1B,  $*p < 0.05$ ), whereas no difference was observed in the amount of total  $\alpha$ -tubulin. We, therefore, conclude that acetylated  $\alpha$ -tubulin level is decreased in the brains of 5XFAD mice.

### Regulation of the acetylation of $\alpha$ -tubulin by A $\beta$ and HDAC6 inhibitor

It has been reported that A $\beta$  alters the level of acetylated  $\alpha$ -tubulin in primary neuronal cultures and cell lines [10] whereas the HDAC6 inhibitor, TBA, promotes the acetylation of  $\alpha$ -tubulin [19,20,21]. To examine the role of A $\beta$  in reducing acetylated  $\alpha$ -tubulin, primary hippocampal neurons were characterized. Consistent with previous studies [10,22], the level of acetylated  $\alpha$ -tubulin was significantly decreased by the A $\beta$  treatment (Fig. 2), as shown by Western blot analysis (Fig. 2A and 2B,  $*p < 0.05$ ,  $***p < 0.001$ ) as well as immunocytochemistry (Fig. 2C). Western blot analysis (Fig. 2A and 2B,  $*p < 0.05$ ,  $***p < 0.001$ ), together with immunocytochemistry (Fig. 2C), revealed that TBA treatment in the presence of A $\beta$  restored A $\beta$ -induced reduction in the level of acetylated  $\alpha$ -tubulin level, compared to A $\beta$  treatment alone. This indicates that TBA increased  $\alpha$ -tubulin acetylation even in the presence of A $\beta$ .

### The effect of A $\beta$ and TBA on the velocity and motility in mitochondrial axonal transport

Because it has been reported that mitochondrial transport along the microtubule was disrupted in the presence of A $\beta$  [12], the effect of TBA in the presence of A $\beta$  on mitochondrial transport was examined with primary hippocampal neuron cultures in microfluidic system, which could isolate neuronal compartments between the soma and axon. To visualize mitochondrial movement, pDsRed2-Mito construct was transfected into the DIV 7 neurons, and live cell imaging was performed after the neurons were treated with A $\beta$  and/or TBA. 24 hrs after transfection, the neurons were pretreated with 2  $\mu$ M A $\beta$  for 24 hrs and then

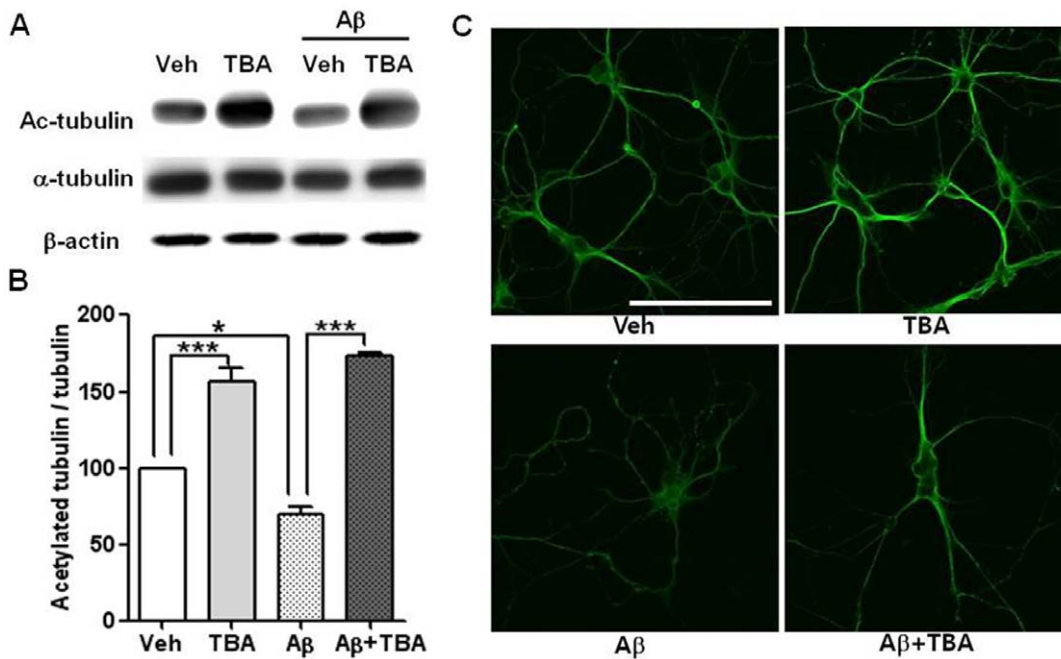


**Figure 1. Reduction of acetylated  $\alpha$ -tubulin in 5XFAD.** (A) Western blot of acetylated  $\alpha$ -tubulin in the brains of both wild type (WT) and 5XFAD mice. Brain extracts were prepared from frontal cortex of 13-month-old mice. Actin is a loading control. (B) Quantitation of the acetylated  $\alpha$ -tubulin normalized by total  $\alpha$ -tubulin is shown as means  $\pm$  SEM (WT  $n = 4$ , 5XFAD  $n = 3$ ,  $*P < 0.05$ ). doi:10.1371/journal.pone.0042983.g001

treated with 5  $\mu$ M TBA for 3 hrs in both somal and axonal sides. Images were taken every sec for 2 mins. To analyze the direction of velocity and motility of mitochondria, we used z-projection and multiple Kymograph plugins functions of the ImageJ program. We found that A $\beta$  greatly reduced mitochondrial movement, and TBA recovered the reduced mitochondrial movement in the presence of A $\beta$ , as shown by the kymographs (Fig. 3A, B, and Movie S1, S2, S3, S4). In A $\beta$  treated neurons, we observed the decreased velocity in both anterograde and retrograde movements of mitochondria. TBA treatment with A $\beta$  induced a recovery of the reduced velocity of mitochondria by A $\beta$  (Fig. 3C,  $***p < 0.001$ ). The results for motility were consistent with those for velocity (Fig. 3D,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ). In this experiment, the ration of motile mitochondria over total mitochondria was calculated to present the motility of mitochondria. Consistent with the data for mitochondrial velocity (Fig. 3C), mitochondrial motility was also decreased by A $\beta$  and recovered by TBA treatment in both directions (Fig. 3D). In the group treated with both A $\beta$  with TBA, the defects caused by A $\beta$  were all rescued by TBA treatment (Fig. 3), suggesting that acetylated  $\alpha$ -tubulin regulates mitochondrial transport even in the presence of A $\beta$ .

### The effect of A $\beta$ and TBA on mitochondrial length in axon

It has been shown that the motor protein dynein is involved in mitochondrial morphology [23,24] and that pharmacological inhibition of HDAC affects mitochondrial elongation [25]. To test the hypothesis that the acetylation of  $\alpha$ -tubulin affects mitochondrial length in hippocampal neurons, we determined the effect of HDAC6 inhibition on mitochondrial length by treating the cells with TBA in the absence or presence of A $\beta$  and



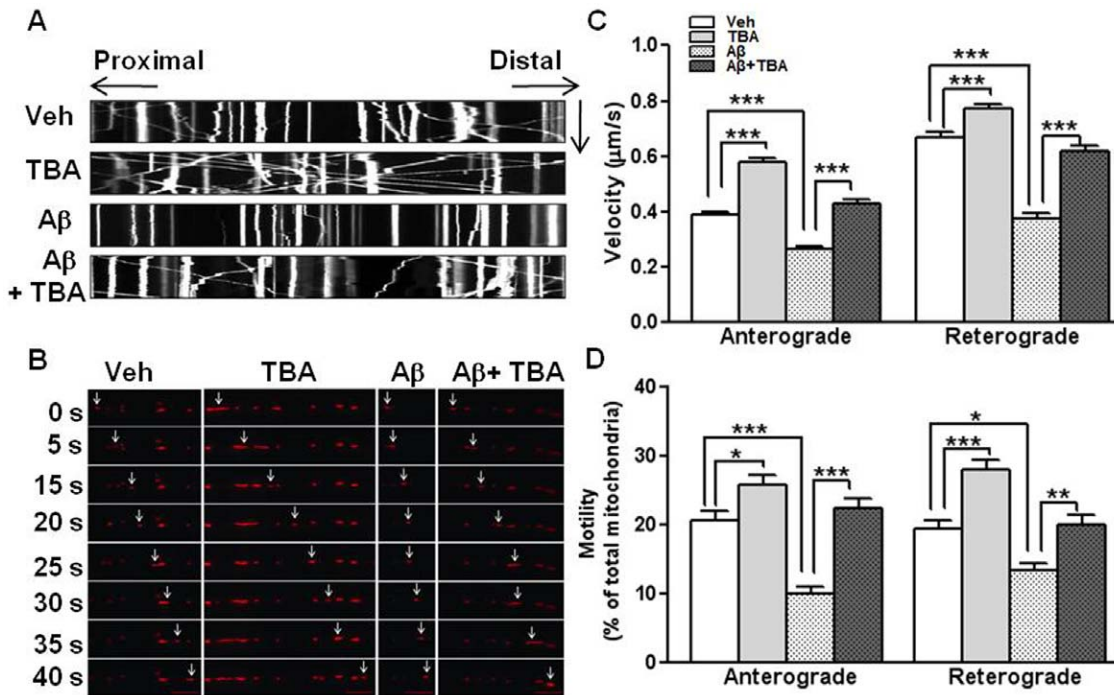
**Figure 2. Modulation of acetylated  $\alpha$ -tubulin by A $\beta$  and TBA.** (A) Western blot of acetylated  $\alpha$ -tubulin in the rat hippocampal neurons. After being pretreated with A $\beta$  (2  $\mu$ M) for 24 hrs, cells were treated with TBA (5  $\mu$ M) for 3 hrs and lysed with RIPA buffer. Actin is a loading control. (B) Quantitation of the acetylated  $\alpha$ -tubulin normalized by total  $\alpha$ -tubulin is shown as means  $\pm$  SEM. Data were acquired from 4 independent experiments (\* $P$ <0.05, \*\*\* $P$ <0.001). (C) Immunocytochemistry of acetylated  $\alpha$ -tubulin in hippocampal neurons. Anti-acetylated  $\alpha$ -tubulin antibody detects  $\alpha$ -tubulin only when acetylated at Lys 40 (Scale bar = 100  $\mu$ m). doi:10.1371/journal.pone.0042983.g002

measured mitochondrial length. In parallel, to compare the length and motility of mitochondria, we separately determined the length of stationary and motile mitochondria. Overall, mitochondrial length was decreased by A $\beta$  and increased in the group co-treated with A $\beta$  and TBA (Fig. 4A; Veh:  $1.168 \pm 0.02$ , A $\beta$ :  $0.904 \pm 0.02$ , TBA:  $1.228 \pm 0.02$ , A $\beta$  & TBA:  $1.159 \pm 0.02$ , \*\*\* $p$ <0.001). Although both motile and stationary mitochondria showed a similar pattern as shown in Fig. 4B, the length of stationary mitochondria was shorter than that of motile mitochondria (Fig. 4B, \*\* $p$ <0.01, \*\*\* $p$ <0.001), suggesting that longer mitochondria have higher motility than shorter ones. This demonstrates that mitochondrial length is related to mitochondrial transport and that TBA treatment affects both mitochondrial length and motility in neurons. However, there was no significant difference among the four groups in the number of mitochondria per 100  $\mu$ m of axon (Fig. 4C). These findings suggest that A $\beta$  and HDAC6 are important in mitochondrial length and axonal transport.

## Discussion

Impaired axonal transport and mitochondrial dysfunctions occur in the early stages of AD [8,15]. These changes are induced by A $\beta$  which forms amyloid plaques, one of the major hallmarks of AD [26,27,28,29]. Increased GSK3 $\beta$  activity induces hyperphosphorylation of tau, resulting in the formation of neurofibrillary tangles (NFT) and/or an increase in A $\beta$  generation [30]. HDAC6 activity, as a substrate of GSK3 $\beta$ , is increased by GSK3 $\beta$  activation [31], suggesting a decrease in both the level of  $\alpha$ -tubulin acetylation and axonal transport. To modulate HDAC6 activity, we used TBA as a potent HDAC6 inhibitor, which is more selective than other HDAC6 inhibitors, such as TSA and tubacin [21,32]. The specificity of TBA to HDAC6 was confirmed

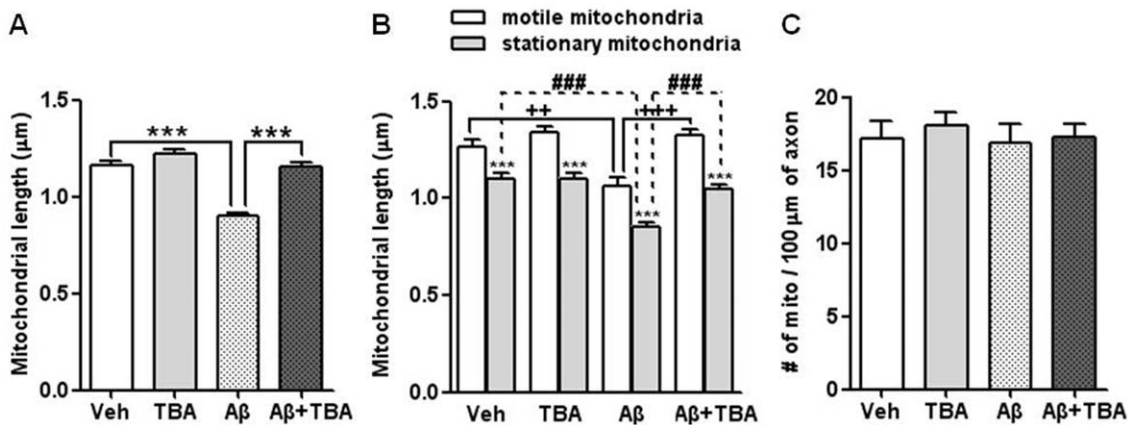
in previous studies by homology modeling and enzyme inhibition experiments using 11 HDAC isozymes [21,32]. TBA affects the acetylation of cytosolic proteins like  $\alpha$ -tubulin but not histones [21]. The knockdown of HDAC6 by siRNA resulted in an increased level of acetylated  $\alpha$ -tubulin, consistent with the results with the HDAC6 inhibitor [33]. It has been reported that TBA treatment increases not only axonal transport and the number of moving mitochondria but also the level of acetylated  $\alpha$ -tubulin in neuropathies of a Charcot-Marie-Tooth model that have been induced by mutant heat-shock protein gene (HSPB1) [32]. Given the results from the current and previous studies, it is increasingly clear that TBA specifically inhibits HDAC6 and, thus, affects the acetylation of  $\alpha$ -tubulin and axonal transport of cellular cargoes, such as mitochondria. Motor proteins, such as kinesin for anterograde transport and dynein for retrograde transport, are involved in mitochondrial transport [34]. Adaptor proteins, such as Milton, syntabulin, miro (for kinesin), and dynactin (for dynein), are also required [17,18]. Axonal transport is regulated by the interaction between adaptor and motor proteins as well as their stability is important in axonal transport [17,18,34]. It has been also known that  $\alpha$ -tubulin acetylation at Lys 40 increases the interaction between kinesin and the microtubule, resulting in improved transport of cargo proteins [3]. Dompierre et al. have reported that MT acetylation increases both anterograde and retrograde transport, which was attributed to the increased interaction of both kinesin family motor protein 5 (KIF5) and dynein with MTs [35]. However, the mechanism by which acetylation of  $\alpha$ -tubulin regulates mitochondrial transport under pathological conditions, such as the accumulation of A $\beta$ , is unclear. Du et al. [15] have reported that mitochondrial motility was significantly reduced in both directions (anterograde and retrograde) in neurons treated with 200 nM A $\beta$  oligomer. By contrast, Calkins et al. [12] found that the treatment with 20  $\mu$ M



**Figure 3. Regulation of mitochondrial transport by Aβ and TBA.** (A) Representative kymographs of mitochondrial movement. Hippocampal neurons from rat (E18) were plated with densities of  $6 \times 10^4$  cells in the somal side of the microfluidic chamber. Cells were transfected with pDsRed2-Mito after 7 days of culture. After being pretreated with Aβ (2 μM) for 24 hrs, cells were treated with TBA (5 μM) for 3 hrs. Images were acquired every 1 sec for 2 min at microgrooves. X axis of kymograph is axonal length (152.7 μm). Proximal to distal indicates the soma to axon terminal direction. Mitochondria which move from proximal to distal region show anterograde movement. Y axis is the time that mitochondria have moved (2 min). (B) Pictures of motile mitochondria for each group were shown every 5 sec (Scale bars = 10 μm). Arrows indicate motile mitochondria. (C) Average velocity of motile mitochondria. Anterograde and retrograde velocity were analyzed separately. (D) Motility of mitochondria. Motility stands for percentage of motile mitochondria over total mitochondria. Anterograde and retrograde motility were analyzed separately. Data were acquired from 4 independent experiments (Veh n=41, TBA n=44, Aβ n=42, Aβ+TBA n=43, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001). doi:10.1371/journal.pone.0042983.g003

Aβ<sub>25-35</sub> oligomer significantly reduced the motility of anterograde mitochondrial transport but not the velocity in mitochondrial transport in both directions. Moreover, Wang and colleagues [36] have shown that the motility of mitochondria was decreased in both directions. In the current study, we showed that the treatment

of neurons with 2 μM Aβ significantly reduced the motility and velocity in mitochondrial transport in both directions. The difference in the level and direction of mitochondrial motility and velocity between this and other studies may be relevant to the form or concentration of Aβ. Our AFM assay showed that Aβ



**Figure 4. Alteration of mitochondrial length by Aβ and TBA.** (A) Average length of total mitochondria, including both motile (anterograde, retrograde transported) and stationary mitochondria. (B) Average length of motile and stationary mitochondria. \*\*\*P<0.001 significance of stationary mitochondria vs. motile mitochondria; ++P<0.01, +++P<0.001 among motile mitochondria; ###P<0.001 among stationary mitochondria. (C) The average number of mitochondria per 100 μm of axon. Data were obtained from 4 independent experiments (Veh n=35, TBA n=42, Aβ n=38, Aβ & TBA n=38, \*P<0.05, \*\*P<0.01, \*\*\*P<0.001). doi:10.1371/journal.pone.0042983.g004

used in this experiment is mostly in oligomer form even though other types of A $\beta$  are present in the mixture (see the figure in File S1). Although the exact mechanism underlying TBA-mediated enhancement of axonal transport is not fully studied, we suggest that TBA promotes the binding of motor proteins to MTs, contributing to the restoration of mitochondrial transport that has been impaired by A $\beta$ . The size and structure of mitochondria are dynamically regulated by mitochondrial fission and fusion [37,38]. A $\beta$  activates two mitochondrial fission proteins, Fis1 and Drp1, leading to excessive fragmentation of mitochondria that may disrupt mitochondrial transport [12]. Misko et al. (2010) also reported that mitofusin 2, an outer mitochondrial membrane protein involved in regulating mitochondrial dynamics, is directly involved in mitochondrial axonal transport [39]. Our results (Fig. 4B) show that motile mitochondria are longer than stationary ones. Thus, it is likely that the change in mitochondrial length is coupled with mitochondrial transport. Therefore, we tentatively anticipate that HDAC6 is more likely to regulate the mitochondrial dynamics. An outstanding issue that remains to be further investigated is how HDAC6 inhibitors affect the interaction between an adaptor protein and MTs and the functions of the mitochondria. In addition, it remains to be further examined whether mitochondrial elongation directly leads to increased mitochondrial transport. Since A $\beta$  has been shown to reduce ATP production in neurons [40], we examined whether TBA-treated neurons are recovered from A $\beta$ -induced impairment in ATP generation. When ATP production was measured in this system, A $\beta$  treatment reduced ATP production significantly, but co-treatment of A $\beta$  with TBA did not rescue A $\beta$ -induced reduction of ATP production (data not shown), suggesting that ATP production is not associated with axonal transport and morphology of the mitochondria in cells treated with TBA. Regarding the relationship of changes in membrane potential to mitochondria and axonal transport, Miller and Sheetz (2004) reported that mitochondria with high membrane potential moved toward the axon terminus, whereas mitochondria with low membrane potential moved toward the soma [41], suggesting that membrane potential is another factor that influences mitochondrial axonal transport. Overall, this study showed that A $\beta$  impaired bidirectional mitochondrial transport, which was subsequently rescued by the HDAC6 inhibitor. Furthermore, we observed that the changes in both acetylation of  $\alpha$ -tubulin and mitochondrial elongation correlate to the alteration of mitochondrial transport in cells treated with A $\beta$  and/or an HDAC6 inhibitor. Therefore, these results suggest that an HDAC6 inhibitor may be a potential therapeutic target for the treatment of AD.

## Materials and Methods

### Animals

Female Sprague-Dawley (SD) rats whose embryos (E18) were used for primary hippocampal neuronal culture were purchased from KOATECH (Gyeonggi-do, Korea). The brain tissues of 13-month-old 5XFAD mice (Tg6799; B6SJL-Tg (APP<sup>SwFLon</sup>, PSEN\* M146L\*L286V) 6799Vas/J, stock number 006554, Jackson Labs, Bar Harbor, ME) overexpressed three mutations (Swedish, Florida, and London) of human APP 695 and two mutations (M146L and L286V) of human PS1 under transcriptional control of the murine Thy-1-promoter and B6SJL wild type (WT) mice were used for western blot analysis. Animals were treated and maintained as per the Helsinki Treaty, the Principles of Laboratory Animal Care (NIH publication No. 85-23, revised 1985), and the Animal Care and Use Guidelines of Seoul National University, Seoul, Korea. All procedures for animal experiments

were approved by the Ethics Review Committee for Animal Experimentation in Seoul National University (approval number, SNU060519-5, IACUC).

### DNA construct and reagents

pDsRed2-Mito (Clontech, Mountain View, CA) is a mammalian expression vector that encodes a red fluorescent protein and the mitochondrial targeting sequence, resulting in neuronal mitochondria appearing as a red fluorescence. Acetylated  $\alpha$ -tubulin and  $\beta$ -actin antibodies were purchased from Sigma-Aldrich (St. Louis, MO) and  $\alpha$ -tubulin antibodies were from abm (Applied Biological Materials Inc., Richmond, BC). Alexa Fluor 488 donkey anti-mouse IgG was used in immunofluorescence analysis. Tubastatin A (TBA; BioVision, Mountain View, California) and A $\beta$ <sub>1-42</sub> peptides (American peptide, Sunnyvale, CA) were also used.

### Cell cultures and transfection

Primary hippocampal neurons were obtained from the brain tissue of SD rat embryos (E18). Preparation of primary hippocampal neurons was followed in previous reports with minor modifications [15,42]. Briefly, brain tissues were isolated in ice-cold Hank's Balanced Salt Solution (HBSS; WelGENE, Daegu, Korea) and digested with 0.5% trypsin (using 2.5% trypsin; Sigma, St. Louis, MO). Cells were dissociated in Neurobasal/B27 medium (Invitrogen, Carlsbad, California) with 0.1 mg/ml penicillin/streptomycin (Sigma, St. Louis, MO) and plated onto poly-D-lysine (Sigma, St. Louis, MO) coated plates and microfluidic chambers. Half of the culture medium was replaced by fresh medium every 2 days for plates and every day for microfluidic chambers. For labeling mitochondria, pDsRed2-Mito was transfected into neurons at 7 days in vitro (DIV) using Lipofectamine 2000 (Invitrogen, Carlsbad, California). After being pretreated with A $\beta$ <sub>1-42</sub> peptides (2  $\mu$ M) at DIV 8 for 24 hrs, cells were treated with TBA (5  $\mu$ M) for 3 hrs [21].

### Microfluidic chamber system

The microfluidic chambers for separating axons were fabricated as described in the reference [43]. After assembling the chambers, the chambers were coated by poly-D-lysine. Cells were loaded in only one compartment of the chamber, the somal compartment. As neurites were growing up, the other compartment was filled with axon which we called the axonal compartment. All chambers have a microgroove length of 900  $\mu$ m and a width of 8  $\mu$ m.

### Live cell imaging and image analysis

Living cells were imaged using Olympus IX81 microscope (Tokyo, Japan) equipped with a Cool SNAP HQ2 CCD camera (Photometrics, Ltd., Tucson, AZ 85706), controlled by MetaMorph Software (Universal Imaging, PA, USA). During imaging, cells were maintained in an incubating chamber at 37°C and supply atmosphere of 5% CO<sub>2</sub>/95% air (Live cell instrument, Seoul, Korea). Time-lapse image recordings were acquired at an exposure time of 500 ms, 1 sec interval and duration up to 2 min. Live cell images were obtained from before and after TBA treatment and focused on axons in microgroove. Movies were processed using MetaMorph. Quantitative analysis of mitochondrial velocity and motility was performed manually using ImageJ (rsb.info.nih.gov, by W. Rasband) installed multiple kymograph plugins (by J. Rietdorf and A. Seitz) at each axon. In order to analyze mitochondrial velocity, we measured the angle of motile mitochondria on the kymographs using ImageJ and then calculated the velocity according to the following equation:

“Velocity ( $\mu\text{m}/\text{sec}$ ) = Tangent ( $\text{Angle}^\circ + 90^\circ$ ) \* 0.111276”. Because the angles measured by ImageJ were all between  $0^\circ \sim 180^\circ$ , the values of “Tangent ( $\text{Angle}^\circ$ )” were all under 0. Therefore, we added  $90^\circ$  to the measured angles to distinguish the anterograde and retrograde mitochondrial movements as positive and negative values, respectively. In our imaging system, 0.111276 represents the length of one pixel ( $\mu\text{m}$ ). Mitochondrial motility was tracked by hand using kymographs and the cut-off value of  $0.0019 \mu\text{m}/\text{sec}$  (mean velocity) was used to distinguish motile from stationary mitochondria. Quantitative analysis of mitochondrial length was also based on manual tracking using ImageJ on the first picture of the live cell images.

### Western blot analysis

To extract brain tissues, mice were anesthetized and perfused transcardially with phosphate-buffered saline (PBS). Cultured primary hippocampal neurons in 6 well plates were harvested as cell pellets. Brain tissues and harvested cell pellets were resuspended in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS, and 50 mM Tris-HCl, pH 7.4) containing protease inhibitors (Sigma, St. Louis, MO), Phenylmethylsulfonyl fluoride (PMSF; Sigma, St. Louis, MO), phosphatase inhibitors (A.G. Scientific, Inc., San Diego, CA) and TBA and then incubated on ice for 20 min and sonicated for 5 sec. After centrifugation at 18,000 g for 10 min, the supernatant was collected to remove membrane lipids. Protein concentrations were determined by BCA assay (Sigma), and equal amounts of protein were loaded on 10% glycine gels. The separated samples were transferred to a PVDF membrane and incubated with antibodies against the target proteins. Protein bands were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Buckinghamshire, England) using a bioimaging analyzer (LAS-3000; Fuji, Tokyo, Japan). This process has been also described in a previous report [44].

### Immunocytochemistry analysis

Primary hippocampal neurons were plated on 18-mm round coverslips coated with poly-D-lysine which were in a 12-well plate. After A $\beta$  and/or TBA treated, cells were washed with chilled PBS, and fixed with 4% paraformaldehyde (BIOSESANG, Inc., Gyeonggi-do, Korea) for 20 min at room temperature (RT). Cells were permeabilized and blocked with 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and 1% goat serum (Vector Laboratories, Inc., Burlingame, CA) diluted in PBS for 10 min at RT. To detect acetylated  $\alpha$ -tubulin, cells were incubated with primary antibodies

(Sigma-Aldrich, St. Louis, MO) overnight at  $4^\circ\text{C}$  and then with secondary antibodies for 1 hr at RT. Primary and secondary antibodies diluted in permeabilizing and blocking solution. DAPI ( $0.1 \mu\text{g}/\text{ml}$ ; Sigma-Aldrich, St. Louis, MO) staining was performed for 10 min at room temperature. Finally, coverslips were mounted on slides using mounting reagent (Biomedica corp., Foster city, CA). Cells were washed by PBS twice every steps between incubations. Images were acquired using confocal microscope (Olympus FV10i; Olympus, Tokyo, Japan).

### Statistical analysis

Significant differences between groups were examined for statistical analysis using t-test or one-way analysis of variance (ANOVA) and Bonferroni post-tests. All data were expressed as means  $\pm$  SEM (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

### Supporting Information

**Movie S1 Mitochondrial movement of control neuron.** (MP4)

**Movie S2 Mitochondrial movement of TBA treated neuron.** (MP4)

**Movie S3 Mitochondrial movement of A $\beta$  treated neuron.** (MP4)

**Movie S4 Mitochondrial movement of A $\beta$  and TBA treated neuron.** Hippocampal neurons from rat (E18) were plated with densities of  $6 \times 10^4$  cells in the somal side of the microfluidic chamber. Cells were transfected with pDsRed2-Mito after 7 days of culture. Cells were treated with A $\beta$  ( $2 \mu\text{M}$ ) for 24 hrs, and then treated with TBA ( $5 \mu\text{M}$ ) for 3 hrs. Images were acquired every 1 sec for 2 min at microgrooves. (MP4)

**File S1 Supporting Materials and Methods.** (DOC)

### Author Contributions

Conceived and designed the experiments: CK IMJ. Performed the experiments: CK HC WL. Analyzed the data: HC WL. Contributed reagents/materials/analysis tools: ESJ SO NLJ. Wrote the paper: CK HC IMJ.

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