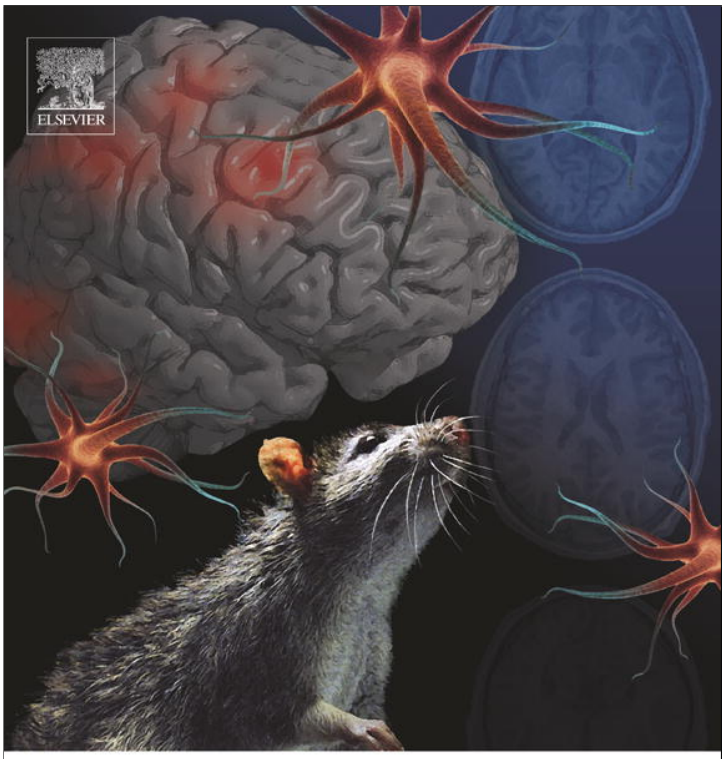

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Research report

Neurorestorative effect of FTY720 in a rat model of Alzheimer's disease: Comparison with Memantine

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HIGHLIGHTS

- Protective effect of FTY720, the S1P analog, is compared to Memantine in AD rats.
- FTY720 as well as Memantine restores memory in male and female AD rats.
- FTY720 as well as Memantine prevents from hippocampal neuron loss in AD rats.
- FTY720 as well as Memantine alters gene transcript profile toward neuroprotection.

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ABSTRACT

Alzheimer's disease (AD) as a neurodegenerative brain disorder is the most common cause of dementia. To date, there is no causative treatment for AD and there are few preventive treatments either. The sphingosine-1-phosphate receptor modulator FTY720 (fingolimod) prevents lymphocytes from contributing to an autoimmune reaction and has been approved for multiple sclerosis treatment. In concert with other studies showing the anti-inflammatory and protective effect of FTY720 in some neurodegenerative disorders like ischemia, we have recently shown that FTY720 chronic administration prevents from impairment of spatial learning and memory in AD rats. Here FTY720 was examined on AD rats in comparison to the only clinically approved NMDA receptor antagonist, Memantine. Passive avoidance task showed significant memory restoration in AD animals received FTY720 comparable to Memantine. Upon gene profiling by QuantiGene Plex, this behavioral outcomes was concurrent with considerable alterations in some genes transcripts like that of mitogen activated protein kinases (MAPKs) and some inflammatory markers that may particularly account for the detected decline in hippocampal neural damage or memory impairment associated with AD. From a therapeutic standpoint, our findings conclude that FTY720 may suggest new opportunities for AD management probably based on several modulatory effects on genes involved in cell death or survival.

1. Introduction

To date, established treatments for Alzheimer's disease (AD) include choline esterase inhibitors [1] and Memantine that is an uncompetitive, moderate-affinity N-methyl-D-aspartate (NMDA) receptors antagonist [2]. Memantine is believed to protect neurons from excitotoxicity, as a major contributing mechanism to escalating dementia [3]. According to systemic clinical studies on AD patients, Memantine improves cognition [4] and may reduce behavioral and psychological symptoms of dementia [5]. In spite of the promising results however, Memantine treatment as other currently available ones for AD (Donepezil, Rivastigmine and Galantamine) is symptomatic and does not halt the disease progression [6], the fact necessitates new drug investigations upon comprehensive knowledge of AD and accompanying dementia.

AD is highly associated with deregulation of lysophospholipids (LPs) of which one of the best known is sphingosine-1-phosphate (S1P) [7]. LPs can play multiple roles in relevance to CNS disorders, especially those associated with CNS injuries and inflammation,

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including memory impairment and neurological disorders like AD [8]. Particularly, FTY720 (Fingolimod) that becomes an analog of S1P upon phosphorylation, has been approved by the FDA in 2010 as a first oral treatment for multiple sclerosis [9,10], even though much is not known about the contributions of S1P to AD. Some scientific data endorse this synthetic sphingosine analog may be promising for development of new therapeutic drugs for AD. For instance, FTY720 may decrease the levels of ceramides [11,12] which have been shown to promote beta amyloid peptide (Aβ) formation and are also linked to neurotoxicity via activation of pro-apoptotic pathways [7]. Upon our previous in vivo experiments FTY720 ameliorates spatial memory impairment in the Morris water maze task in AD rats implying the importance of S1P signaling [13], and suggesting extended studies for elucidation of FTY720 impact on AD outcomes.

FTY720 therapeutic effects are evidently based on a variety of modulatory effects on versatile molecular and cellular mechanisms [14]. Conspicuously, FTY720 treatment down-regulates genes encoding inflammatory mediators [15] and increases BDNF level which earlier has been suggested to be strongly correlated with improved AD out comes [16]. Interestingly upon further experiments FTY720 seems to counteract NMDA-induced neuronal death in a BDNF-dependent manner [17]. Noncompetitive antagonists of NMDA receptor also augment the expression of several genes in limbic cortical [18,19] or hippocampal regions [20,21] which have been suggested partly underlie Memantine's therapeutic effects in AD patients.

Accumulating evidence imply that Aβ aggregation could contribute to memory impairment especially through inducing transcripts of the genes involved in inflammatory or apoptotic pathways [22]. Therefore, to understand the pharmacology of FTY720 in the context of AD animal models it is important to estimate about the responsible alterations occur in the genes transcription within the brain. This prompted us to explore whether FTY720 as well as Memantine could modify the gene expression pattern induced by Aβ in AD animal models. Since comparatively little has been done so far with regard to its possible impact on AD out comes, first of all we provide direct evidence for the experimental therapeutic effects of FTY720 on memory impairment as well as on hippocampal integrity, having a great deal with memory formation. In the same manner all the experiments were performed on AD animals with Memantine, as the standard recognized drug approved for AD patients.

As a valid method for gene transcription assay, QuantiGene Plex system was utilized to detect and analyze the quantity of specific mRNAs. In order to better understand the multiple actions, we selected target genes from a variety of categories approximately may be classified to: (i) pro-apoptotic (Extracellular-signal-Regulated Kinases II, *ERK-II*; c-Jun N-terminal kinases I, *JNK-I*; *p38*mitogen-activated protein kinases and *Caspase 3*), (ii) proinflammatory (*NFkB*, *TNF-α* and *IL-1β*), (iii) protective (*BDNF*, *IRF-3* and *PPAR-γ*) and (iv) pathologically relevant markers (Choline acetyl transferase, *ChAT*; and Calmodulin-Dependent Protein Kinase II, *CaMK-II*) relevant genes. Then we sought to characterize and compare the genes with altered expression in AD animals following FTY720 and Memantine to underlie the concomitant changes.

2. Materials and methods

2.1. Drugs preparation

$A\beta_{1-42}$ fragment (Sigma–Aldrich, USA) was prepared as stock solutions in sterile 0.1 M phosphate-buffered saline (PBS; pH 7.4), diluted to the concentration of 10 ng/μl and then aliquoted, and stored at −80 °C until use. FTY720 (a gift from Pajoohesh Darou Arya Company; Iran) and Memantine (Osvah Pharmaceutical Co.; Iran) were respectively dissolved in normal saline and DMSO, at the concentration of 0.5 mg/ml and 25 mg/ml, and the aliquots were stored at −20 °C. Chloral hydrate (Merck; Germany) was dissolved in sterile saline at the concentration of 400 mg/ml.

2.2. Animals

Adult male and female Sprague Dawley rats weighing 200–250 g were obtained from Monash University (Kuala Lumpur, Malaysia) and housed in groups of five in a controlled environment (temperature 25 + 2 °C and 12 h dark/light cycle) and fed pellet food and water ad libitum. All the experiments were performed according to National Institutes of Health guidelines for the use of experimental animals, approved by the local ethical committee (Faculty of Medicine, University of Malaya). Efforts were also made to diminish the animals used and their suffering.

2.3. Experimental procedure

Animals were accepted to the experiments, at least after 8 days acclimatization to our animal facility. All the rats were anesthetized with intraperitoneal (i.p.) injection of chloral hydrate (400 mg/kg) and placed in a stereotaxic frame. The prepared Aβ solution was bilaterally injected into the animal's frontal cortex (3.2 mm anterior and ±2 mm lateral to the bregma and the depth of 3 mm) [23], using a Hamilton syringe, and at the injection volume of 3 μl per each injection. Sterile 0.1 M PBS was injected instead in control animals. Injections were all made at the rate of 1 μl/min and the needle was left in place for an additional 3 min before it was slowly retracted, to minimize leakage of the injected solution.

After a recovery period of 24 h, rats in appropriate groups received daily i.p. injections of FTY720 (0.5 mg/kg) [13], Memantine (2 mg/kg) [24], or vehicles for five consecutive days. The day after the last i.p. injection, animals were trained and 24 h later tested for passive avoidance test followed by animal sacrifice and tissue preparation for further histological or molecular analysis. The number of animals per group was 10 for behavioral analysis and 4 animals per group were randomly selected for each tissue staining and QuantiGene Plex assay. The experimental procedure is schematically shown in Fig. 1.

2.4. Passive avoidance test

The passive avoidance test was used to measure learning and memory retention abilities in AD model rats as well as control and drug administered animals. On the training day, male and female rats were firstly allowed to habituate to the experimental room for at least 1 h prior to the experiments and then were placed in the behavioral apparatus (shuttle box), consisted of an illuminated and a dark compartment adjoining each other through a guillotine door. Each animal was placed in the light compartment, facing away from the guillotine door, which was in the closed position and was opened after 10s. When a rat entered the dark compartment to the extent that all four paws were on the dark side, the door closed, and a footshock (1 mA, 1.5ʏs) was delivered. Rats were then replaced in the light compartment and received another footshock if reentered again to the dark side within 120ʏs. The number of foot shocks required to retain the animal in the light side for 120ʏs was recorded as a measure of the acquisition of passive avoidance. Memory retrieval was examined 24 h later using a same procedure, except that no footshock was administered. The latency to enter the dark compartment (step-through latency; STL) and the total time spent in the dark compartment (TDC) were recorded. A maximum STL of 180ʏs was allowed per test session. All behavioral experiments were performed between 10 am and 15 pm, during the light period.

After the behavioral assessment, 4 rats of each experimental group of male rats were anesthetized with CO_2 and sacrificed by decapitation. One hemisphere of each brain was used for histological analysis and the other hemisphere was snap frozen and kept at −80 °C to be later used for gene expression analysis using QuantiGene Plex assay, as described below.

2.5. Brain tissue fixation, hematoxylin–eosin (HE) staining and histological examination

Brain hemispheres were fixed in PBS fixation solution containing 4% paraformaldehyde at 4 °C for 5 days, and subsequently embedded in paraffin. Coronal sections (4–5-μm thickness) were prepared serially using a microtome rotary apparatus (Cut5062, Germany). Five sections with at least 40 μm intervals, delimited to the −3.5 to −4.2 mm from the bregma, were selected from each animal and assigned to hematoxylin–eosin (HE) staining.

The dentate gurus region of each brain slice was evaluated under a light microscope and the number of live granular cells was quantified at 400× magnification according to the method described previously [25].

2.6. QuantiGene Plex 2.0 assay

QuantiGene Plex 2.0 assay has been validated as a reliable, reproducible, and sensitive method for measuring mRNA transcript levels as an alternative to RT-PCR, and enables the detection and quantitation of multiple mRNA targets simultaneously [26,27]. To determine the expression level of a set of genes, displayed in Table 1, we used a custom-designed QuantiGene Plex 2.0 Reagent System from Panomics.

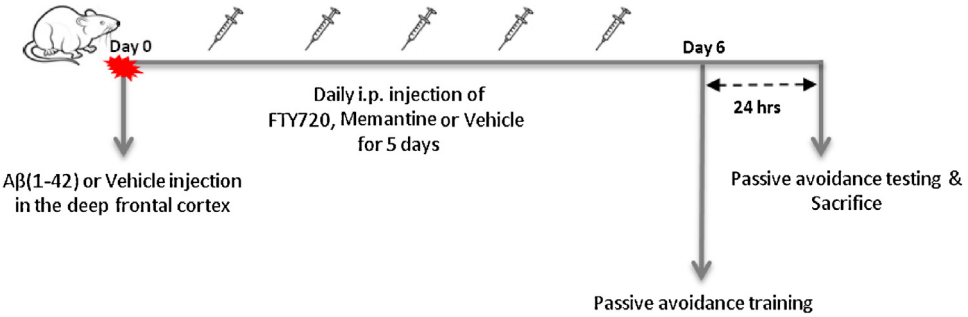

Fig. 1. Schematic representation of the experimental procedure. Aβ (30 ng/side) or its vehicle (sterile PBS) were injected stereotaxically into bilateral cerebral cortex of adult male and female rats. Starting from the day after stereotaxic surgery, both Aβ or vehicle-injected rats were separated into three different groups each receiving daily i.p. injection of FTY720 (0.5 mg/kg), Memantine (2 mg/kg) or DMSO as vehicle for 5 consecutive days. On day 6, passive avoidance training was performed, followed the next day by the probe trial test. Animals were sacrificed within 2 h after the retention test and subjected to histological examination and gene profiling assays.

In brief, frozen hippocampus tissues were homogenated using Panomics' QuantiGene Sample Processing Kit. Tissue homogenates were then subjected to capturing target RNAs, signal amplification and then detection of RNA targets according to the QuantiGene Plex 2.0 Reagent System user manual. Three technical replicates per each sample from each experimental group were run in the assay. Three housekeeping genes representing high (*ACTB*), medium (*HPRT*), and low (*GUSB*) relative expression levels were used to normalize gene expression data across samples. The signal, proportional to the number of captured target RNA molecules, was finally reported as median fluorescence intensity (MFI). The mean of background-subtracted MFI data from technical replicates of each sample, for each mRNA, were divided into the mean of background-subtracted housekeeping RNA signals and then further analyzed for multiple compressions between different experimental groups.

2.7. Statistical analysis

The results shown are presented as means ± SEM. All the data was established with a one-way ANOVA (using SPSS16) followed by the Tukey HSD test for post hoc comparisons between groups. Statistically significant was considered when a p value of <0.05 was determined.

3. Results

3.1. Effects of FTY720 and Memantine on Aβ-induced passive avoidance memory deficit

To determine whether FTY720 (0.5 mg/kg) administration as well as Memantine (2 mg/kg) has a protective effect on AD animals, male and female adult rats were subjected to a passive avoidance task, which is a well established behavioral test for investigating learning and memory processes. It has been shown that beside the aging, gender is also one of the main risk factors for AD development and that the incidence of the disease is higher in women than in men [28–30]. On the other hand, there are lots of evidences showing the different responsiveness of male and female subjects in memory impairment in response to pathological insults [31]. Accordingly, we used both male and female rats in the present study, to compare the efficacy of FTY720 with Memantine in Aβ induced-memory deficit.

In the passive avoidance training trial, all the rats in control, Aβ, Aβ + FTY720, Aβ + Memantine, FTY720 and Memantine groups displayed similarly short baseline STL to enter the dark side. All the animals were also retained for more than 120ʏs in the light side by receiving one footshock, which demonstrate the same acquisition ability (data not shown). In the probe trail, 24 h after training session, male and female AD rats (Aβ groups) exhibited significantly decreased STL to enter the dark compartment and increased TDC ($p < 0.01$and $p < 0.001$ for male and female groups, respectively) in comparison with the controls. However, the FTY720- and Memantine-treated AD animals entered the dark compartment with much higher STL and showed lower TDC than non-treated AD rats ($p < 0.001$). This is while; administration of FTY720 or Memantine for 5 consecutive days in non-AD rats had no significant effect on STL and TDC parameters in passive avoidance probe trail in comparison with the control groups ($p > 0.05$) (Fig. 2A and B).

3.2. Effects of FTY720 and Memantine on Aβ-induced hippocampal neuron loss

Histological analyses via HE staining is an effective and simple way to evaluate cell viability, and it was used in this study to evaluate the hippocampal neuronal survival in our AD animals and those treated with FTY720 or Memantine. In this method, generally the dead neurons represent condensed polygonal nucleus and pink cytosol. Assessment of slices from Aβ-injected rat brains showed an increase in the number of dying neuronal cells and also a remarkable neuronal loss in different hippocampal subregions. Although neuronal death and sites of neuronal loss were partially localized

Table 1
Aβ-induced gene expression changes in rat hippocampus and the effect of FTY720 and Memantine.

Gene	Control	Aβ	Aβ + FTY720	Aβ + Memantin	FTY720	Memantin
ERK2	0.401 ± 0.092	0.902 ± 0.029^{aa}	0.766 ± 0.048^{a}	0.838 ± 0.026^{aa}	0.566 ± 0.031	0.518 ± 0.112
JNK1	0.258 ± 0.02	0.458 ± 0.06^{aa}	0.31 ± 0.025^{b}	0.385 ± 0.031^{a}	0.279 ± 0.043	0.297 ± 0.035
P38	9.333 ± 0.255	12.881 ± 0.504^{a}	9.981 ± 0.399^{b}	9.212 ± 0.649^{b}	8.866 ± 1.035	9.701 ± 0.642
Caspase3	0.076 ± 0.007	0.152 ± 0.011^{aa}	0.098 ± 0.009^{bb}	0.099 ± 0.014^{b}	0.105 ± 0.012	0.096 ± 0.014
NF-κB	0.102 ± 0.014	0.161 ± 0.017^{a}	0.107 ± 0.006^{b}	0.145 ± 0.021	0.109 ± 0.009	0.105 ± 0.016
TNF-α	0.025 ± 0.0005	0.058 ± 0.008^{aa}	0.031 ± 0.004^{bb}	0.032 ± 0.002^{bb}	0.025 ± 0.004	0.02 ± 0.001
IL-1β	0.0008 ± 0.0003	0.0029 ± 0.0006^{aa}	0.0016 ± 0.0003^{b}	0.0018 ± 0.0002^{b}	0.0018 ± 0.0002	0.0014 ± 0.0003
BDNF	0.155 ± 0.025	0.298 ± 0.023^{a}	0.259 ± 0.023	0.263 ± 0.039	0.148 ± 0.065	0.168 ± 0.031
IRF3	0.273 ± 0.013	0.372 ± 0.031^{a}	0.235 ± 0.023^{bb}	0.292 ± 0.012^{b}	0.219 ± 0.019	0.259 ± 0.034
PPAR-γ	0.043 ± 0.006	0.044 ± 0.008	0.045 ± 0.001	0.041 ± 0.003	0.042 ± 0.002	0.051 ± 0.008
ChAT	0.011 ± 0.0005	0.004 ± 0.0004^{aaa}	0.005 ± 0.0007^{aaa}	0.005 ± 0.0001^{aaa}	0.011 ± 0.0009	0.008 ± 0.0004
CaMKII	0.994 ± 0.046	0.651 ± 0.037^{aa}	0.733 ± 0.109^{a}	0.826 ± 0.017	1.138 ± 0.068	1.159 ± 0.117

These genes represent the set of genes analyzed by QuantiGene Plex 2.0 assay in male rat hippocampal homogenates.
Data are represented as mean ± SEM. $^{a}p < 0.05$, $^{aa}p < 0.01$ and $^{aaa}p < 0.001$ vs control group; $^{b}p < 0.05$ and $^{bb}p < 0.01$ vs Aβ group.

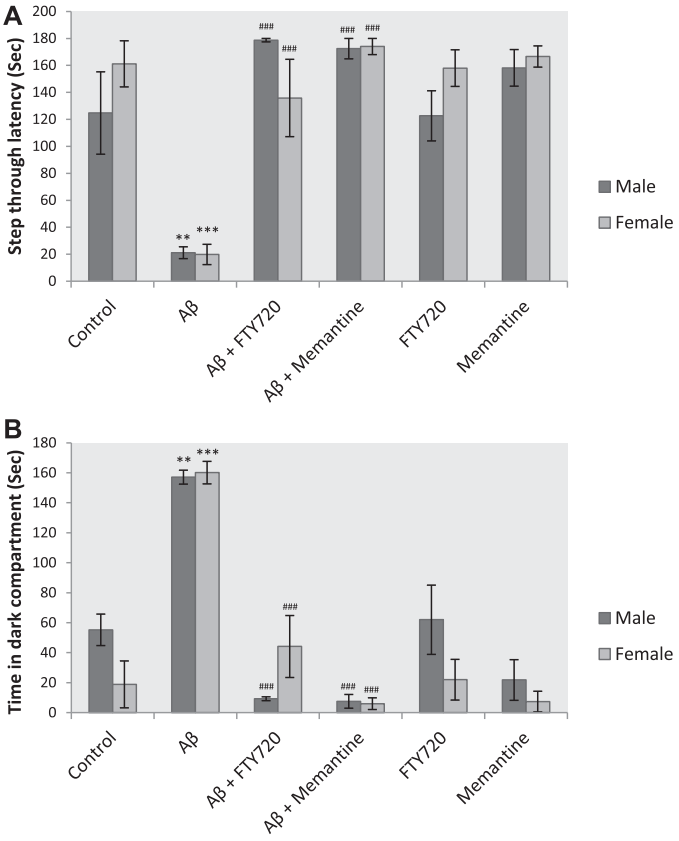

Fig. 2. Effects of FTY720 and Memantine on Aβ-induced passive avoidance memory deficit. (A) Step-through latencies (STL) into the dark compartment, and (B) the total time spent in the dark compartment (TDC) in passive avoidance probe trial in male and female rats ($n = 10$ per group). Data are represented as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs control group; $^{###}p < 0.001$ vs Aβ group.

rather than widespread in CA1, CA2 and CA3 subfields of hippocampus, expected given the short time exposure to Aβ, were more obvious in dentate gyrus granular cell layer. Quantitative analysis of live neurons in DG sub region showed a significant reduction in the number of alive neurons in AD rats ($p < 0.01$). However, treatment with FTY720 ($p < 0.05$) as well as Memantine ($p < 0.01$) significantly attenuated the Aβ-induced neuron loss (Fig. 3).

3.3. Altered gene expression profiles in the hippocampus

Using the QuantiGene Plex system, we measured the transcriptional level of pro-apoptotic genes including *ERK-II*, *JNK-I*, *p38* and *Caspase 3*; proinflammatory genes including *NFkB*, *TNF-α* and *IL-1β*; and protective genes including *BDNF*, *IRF-3* and *PPAR-γ*; as well as those are pathologically relevant to AD pathology including *ChAT* and *CaMK-II*. Of the 12 genes examined for transcriptional changes, we observed significant increases in the mRNA levels of *ERK-II*, *JNK-I* ($p < 0.01$), *p38* ($p < 0.05$), *Caspase 3* ($p < 0.01$), *NFkB* ($p < 0.05$), *TNF-α*, and *IL-1β* ($p < 0.01$) in Aβ-injected animals compared to the control group. Interestingly, the transcription of protective genes including *BDNF* and *IRF-3* ($p < 0.05$) significantly increased in response to Aβ-induced neurotoxicity, which may be referred as a defense mechanism. As expected, the mRNA level of *ChAT* ($p < 0.05$) as well as *CaMK-II* ($p < 0.01$), which are both essential molecules for hippocampus dependent learning and memory processes, were significantly decreased in Aβ-injected AD animals in comparison with the control group. Treatment of AD rats with FTY720 prevented from gene transcriptional changes of *JNK-I*, *p38* ($p < 0.05$), *Caspase 3* ($p < 0.01$), *NFkB* ($p < 0.05$), *TNF-α* ($p < 0.01$), and *IL-1β* ($p < 0.05$), and *IRF-3* ($p < 0.01$), while was not able to restore the changes of *BDNF*, *ERK-II*, *ChAT* and *CaMK-II* mRNA. Consistent with those results obtained from behavioral and histological experiments, FTY720 treatment resulted in the same results as Memantine treatment, since there were no significant differences between FTY720 and Memantine on the gene profile analyzed in AD rats. Among the genes examined herein, *PPAR-γ* did not show any significant

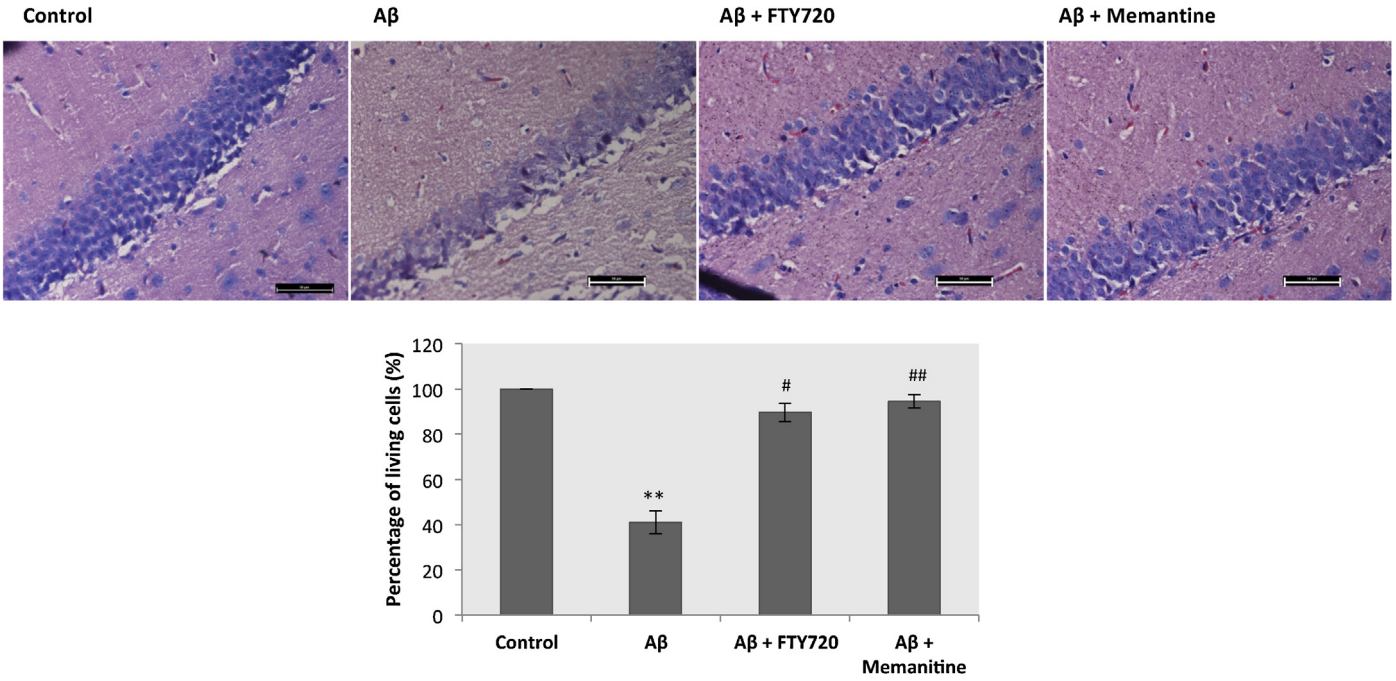

Fig. 3. Histological examination of hippocampus via hematoxylin–eosin (HE) staining and quantitative analysis of live neurons. Although less than half of the DG granular neurons were found to be alive in Aβ group of male rats, treatment with FTY720 as well as Memantine significantly prevented from such neuronal loss. Results shown are the representatives of four animals under each experimental condition. Scale bar, 50 μm. Quantitative data are represented as mean ± SEM. $^{**}p < 0.01$ vs control group; $^{#}p < 0.05$ and $^{##}p < 0.01$ vs Aβ group.

alteration in all of the experimental groups. Furthermore, administration of FTY720 and Memantine for 5 consecutive days in non-AD rats, did not affect the transcription of genes analyzed (Table 1).

4. Discussion

AD has triggered vast numbers of researches to understand its pathogenic mechanisms and to define drug targets and therapeutic approaches. Accordingly AD pathology seems to be a multifactorial process resulting from altered Aβ production, abnormal tau protein procession, oxidative stress and neuro-inflammatory reactions [32,33]. The present study emphasizes that phospholipids also play a key role in AD development and may represent a new therapeutic target for AD, as comparable to Memantine modulating S1P receptors by FTY720 could restore passive avoidance memory in male and female AD rats.

Although so far, not much direct evidences have been reported of S1P signaling involvement in the pathology of AD, a variety of correlative signals have been focused in AD. For instance, abnormal Aβ accumulation decreases in response to S1P exposure [34,35] and enzymes partially responsible for LPA or S1P production have been reported to be up-regulated in AD brain [34,36]. It has been also apparent that S1P levels fall in AD [7], and SPK1 (S1P producing enzyme) overexpression, promotes neuronal survival upon Aβ exposure [37]. Consistently, our in vivo experiments confirm that FTY720 presents neurorestorative effects on AD context. In the present utilized AD model, Aβ injection caused a significant passive avoidance memory impairment in male and female rats that was almost fully restored by FTY720 as well as Memantine, the standard drug for AD. In pursuing our main aim of evaluating the protective and rescue effects of FTY720 in comparison with Memantine, we continued only with male rats in further experiments.

To more clarify the neurorestorative effects of FTY720 in AD, we used histological tests and also analyzed the transcription level of key molecules that have been evidently linked to AD pathogenesis in earlier studies [21,38]. Neural cell death could obviously account for the observed memory deficit at least partly [39,40]. Our HE staining results showed that the dentate gyrus granular neurons in AD animal hippocampus is apparently reduced in comparison with control animals and this while is AD animals received Memantine or FTY720, represents a meaningful reduced neuronal loss.

Concomitant caspase-3 mRNA rise may demonstrate a reasonable underlying mechanism; however, the involvement of MAPKs could not be ruled out [41]. JNK, ERK and P38 are well-characterized subfamilies of MAPKs that control a vast array of physiological processes [42,43] including apoptosis like that occurs in AD brains [44]. According to the previous reports, Memantine attenuates NMDA receptors downstream signaling cascades triggered by P38 [45] and ERK [46] activation. However, whether it raises transcriptional levels of MAPKs has remained controversial [47]. FTY720 corresponding effects on MAPKs also have been investigated [48,49] especially for ERK which the results strictly depend on the cellular and experimental context [50–52].

Here we demonstrated that both Memantine and FTY720 prevented hippocampus neuronal death in parallel with p-38 over transcription. Although by FTY720, JNK mRNA was also inhibited from over expression but it seems not to contribute to more protection against neural death upon the quality of granular neurons layer in DG.

Aβ has been determined to induce apoptosis via several mechanisms including JNK activation which in turn leads to c-JUN phosphorylation and the consequent inflammatory responses [22]. Inflammatory changes in AD brain are particularly observed at the amyloid deposits and potentially contribute to neuronal dysfunction and eventually cell death [53]. In consistent with previous reports [54,55] the present QuantiGene Plex results confirms that Memantine significantly reduces neuroinflammation on AD context as it was determined by the diminished IL-1β and TNF-α mRNA levels compared to AD animals. This indicates that Memantine may prevent Aβ-induced cytokines transcription however NFkB mRNA levels remains unchanged.

Firstly described as an immunosuppressive drug [56], FTY720 was then proved to exert therapeutic benefit in MS [57] and various CNS injuries, such as stroke and trauma [58–60] through potent anti-inflammatory actions. Its impact on inflammatory responses occurs in AD brains, however, has not yet been reported. Upon the QuantiGene Plex results in this work, we found FTY720 could suppress transcription of inflammatory cytokines like IL-1β and TNF-α probably through NFkB downregulation. These data corroborate with earlier studies indicating TNF-α and IL-1β proteins decreased levels after FTY720 [61].

On the other hand some neuroprotectant molecules may markedly attenuate the ultimate neural damage caused by these aberrant signals. According to our QuantiGene Plex results the alterations detected in PPAR-γ, BDNF and IRF-3 transcriptional level could not account for behavioral advantages obtained by the drugs. IRF3 is a crucial transcription factor to regulate immune responses against pathogen- and damage-associated molecules stimulating TLR-4 receptors [62,63] that changes microglial phenotype from proinflammatory to anti-inflammatory [38]. Aβ could significantly elevate IRF-3 mRNA level in AD animal which was prevented by FTY720 as well as Memantine. For the other protective agent, BDNF, there are evidences implying Memantine's therapeutic effect in AD patients relatively depends on increased BDNF expression in the brain [21] and reportedly it is plausible for FTY720 as well [17]. But upon our data none of these treatments could change BDNF mRNA profile induced by Aβ. As for the mentioned neuroprotectants, the further steps in proteins synthesis and activation were not considered in our experiments, here we may not suggest about the link between their exact expression and behavioral out comes in each experimental group. The enhanced neuroprotectants transcriptional level may be a rapid compensatory response to Aβ. Still this probability remains to be clarified that if more prolonged treatments had been allocated, these protective transcripts might have been enhanced by FTY720 and/or Memantine.

Such limitations we had in our experiments may convincingly demonstrate why in some instances the aimed treatments could not change ChAT as an AD pathological associated markers. The decreased ChAT mRNA we detected in AD animals is in agreement with earlier evidence pointing to a decline in ChAT activity as an underling mechanism for cholinergic impairment in AD forebrains [64–66]. However, in Memantine treated rats these results still is not in corroboration with previous controversial reports not detecting any change in ChAT activity [67] or finding marked rise in ChAT positive neurons in AD animals forebrain [68]. For CaMKII, quantitative analysis has indicated that the expression of CaMKII mRNA in the hippocampus correlates with enhancing effect of chronic multiple-stress on learning and memory [69]. In contrary to FTY720, Memantine restored the CAMII transcriptional level to normal in our set of experiments as it was reported earlier [70].

Conclusively our behavioral results taken together with histological and genetic examinations suggest that S1P signaling provides protection in AD brains and FTY720 administration as an approved medication in MS could also be considered as a potential therapeutic for AD patients. Kaneider and her colleagues in vitro experiments [71] have suggested that FTY720 presumably is an effective chemical for AD and linked it to the detected inhibition of monocytes migration in response to Aβ. Here we did not examine a particular cellular or molecular mechanism. But we may conclude that almost to the same extend as Memantine, FTY720 improves passive avoidance memory retrieval in AD animals

probably through mechanisms which alter the overall inflammatory and apoptotic mechanisms toward less brain damage and memory loss. Further works are highly suggested to reveal the S1P analog, FTY720, therapeutic effects in AD animal models with prolonged chronic treatment.

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