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ORIGINAL ARTICLE

3,6′-Dithiothalidomide, a new TNF-α synthesis inhibitor, attenuates the effect of $A\beta_{1-42}$ intracerebroventricular injection on hippocampal neurogenesis and memory deficit

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Abstract

Evidence indicates altered neurogenesis in neurodegenerative diseases associated with inflammation, including Alzheimer's disease (AD). Neuroinflammation and its propagation have a critical role in the degeneration of hippocampal neurons, cognitive impairment, and altered neurogenesis. Particularly, tumor necrosis factor (TNF)-α plays a central role in initiating and regulating the cytokine cascade during an inflammatory response and is up-regulated in brain of AD patients. In this study, we investigated the effects of a novel thalidomide-based TNF-α lowering drug, 3,6′-dithiothalidomide, on hippocampal progenitor cell proliferation, neurogenesis and, memory tasks after intracerebroventricular injection of β-amyloid $(A\beta)_{1-42}$ peptide. Seven days after $A\beta_{1-42}$ injection, a significant proliferation of hippocampal progenitor cells and memory impairment were evident. Four weeks after $A\beta_{1-42}$ peptide injection, elevated numbers of surviving 5-bromo-2′-deoxyuridine cells and newly formed neurons were detected. Treatment with 3,6′-dithiothalidomide attenuated these $A\beta_{1-42}$ provoked effects. Our data indicate that although treatment with 3,6′-dithiothalidomide in part attenuated the increase in hippocampal neurogenesis caused by $A\beta_{1-42}$-induced neuroinflammation, the drug prevented memory deficits associated with increased numbers of activated microglial cells and inflammatory response. Therefore, 3,6′-dithiothalidomide treatment likely reduced neuronal tissue damage induced by neuroinflammation following $A\beta_{1-42}$ injection. Understanding the modulation of neurogenesis, and its relationship with memory function could open new therapeutic interventions for AD and other neurodegenerative disorders with an inflammatory component.

Keywords: 3,6′-dithiothalidomide, $A\beta_{1-42}$ peptide, Alzheimer's disease, neurogenesis, neuroinflammation, TNF-α.

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Within the adult brain, neurogenesis constitutively occurs in the subventricular zone of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus (Doetsch 2003; Russo *et al.* 2011a). After proliferation, hippocampal progenitor cells migrate from the SGZ into the granule cell layer

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Abbreviations used: 3′-UTR, 3′-untranslated region; AD, Alzheimer's disease; Aß, β-amyloid; BrdU, 5-bromo-2′-deoxyuridine; DCX, doublecortin; DG, dentate gyrus; i.c.v., intracerebroventricular injection; SGZ, subgranular zone; SYN, synaptophysin; TNFR, tumor necrosis factor receptor; TNF-α, tumor necrosis factor- α.

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of the dentate gyrus (DG) where they differentiate into granule cells (Kempermann *et al.* 2003; Russo *et al.* 2011a,b) and, thereafter, morphologically and functionally integrate into the existing hippocampal circuitry (van Praag *et al.* 2002). Hippocampal neurogenesis thereby plays an important role in learning, memory, and repair processes, whereas abnormalities in neural progenitor cell capacity may well contribute to the pathogenesis of diseases such as Alzheimer's disease (AD) (Haughey *et al.* 2002).

A primary neuropathological hallmark of AD is the accumulation of β-amyloid (Aß) peptide in brain (Glenner and Wong 1984), which is derived from the proteolytic processing of β-amyloid precursor protein. Soluble oligomeric assemblies of Aß (Shankar *et al.* 2008; Ashe and Zahs 2010) and Aß-derived diffusible ligands (De Felice *et al.* 2007) have been reported to target synapses, induce neuronal dysfunction, and impair memory. Furthermore, the administration of $Aß_{1–42}$, the most toxic and pro-aggregation form of Aß (Li *et al.* 1999; Takeda *et al.* 2009), into the lateral ventricle of mice has been used to model neuroinflammation, synaptic dysfunction, neuronal death, and memory impairment (Yankner 2000; Hardy and Selkoe 2002; Yamada *et al.* 2005; Choi and Bosetti 2009), all of which are observed during the progression of AD (Yamada and Nabeshima 2000; Van Dam and De Deyn 2006). It has been suggested that amyloid deposition may also impact processes that regulate neurogenesis (Hardy and Selkoe 2002). In this regard, numerous growth factors that are potent modulators of neural stem cell activity (Neeper *et al.* 1996; Gomez-Pinilla *et al.* 1997; Fabel *et al.* 2003) have been found up-regulated in proximity of amyloid plaques (Tarkowski *et al.* 2002; Burbach *et al.* 2004). Furthermore, inflammation accompanying amyloid abnormal production and deposition (Akiyama *et al.* 2000) may negatively affect neurogenesis (Monje *et al.* 2003; Russo *et al.* 2011a,b). The inflammatory response and its propagation have a critical role in the degeneration of hippocampal neurons and the progression of AD (Hoozemans *et al.* 2008; Choi and Bosetti 2009), and ultimately lead to massive degeneration of pyramidal neurons and cognitive impairment (Haughey *et al.* 2002).

The pro-inflammatory cytokine tumor necrosis factor (TNF)-α, which plays a central role in initiating and regulating the cytokine cascade during an inflammatory response (Makhatadze 1998; Sutton *et al.* 1999), has been found up-regulated in postmortem brain from AD patients (Perry *et al.* 2001). However, its role in the modulation of the neurogenic niche remains unclear (McAlpine *et al.* 2009). In this study, we investigated the activity of a novel thalidomide-based TNF-α lowering agent, 3,6′-dithiothalidomide (Scheme 1a). This compound, similar to but more potent than thalidomide (Baratz *et al.* 2011), readily enters the brain and lowers the rate of synthesis of TNF-α post-transcriptionally *via* the 3′-untranslated region (3′-UTR) of TNF-α mRNA (Zhu *et al.* 2003; Greig *et al.* 2004; Tweedie *et al.* 2009). Recently, 3,6′-dithiothalidomide was shown to reverse hippocampus-dependent cognitive deficits in a model of neuroinflammation induced by chronic lipopolysaccharide infusion (Belarbi *et al.* 2012), and attenuated neuroinflammation, AD pathology, and behavioral deficits in animal models of neuroinflammation and AD (Tweedie *et al.* 2012, in press). The use of 3,6′-dithiothalidomide allowed us to evaluate the role of TNF-α on hippocampal neurogenesis during $Aβ_{1–42}$-induced neuroinflammation, in relation to hippocampal progenitor cell proliferation, survival, and phenotypic differentiation, as well as actions on memory, and additionally to assess the agent as a potential therapeutic for specific aspects of AD neuropathology.

Materials and methods

Animals: stereotaxic intracerebroventricular $Aβ_{1–42}$ injection and 3,6′-dithiothalidomide administration

All animal procedures were approved by the National Institutes of Health (NIH) Animal Care and Use Committee in accordance with NIH guidelines on the care and use of laboratory animals. Three-month-old male C57BL/6 mice (purchased from Taconic) were housed at 25°C in our facility with a 12-h light/dark cycle with free access to food and water. Studies were aimed at examining the effects of Aβ peptide administration on (i) hippocampal progenitor cell proliferation and (ii) progenitor cell survival and generation of newly derived neurons. To assess these features, two different experimental time courses were followed.

For the former (i), the experimental duration was for 14 days to allow quantification of $Aβ_{1–42}$ -induced hippocampal progenitor cell proliferation and accompanying memory deficits (Scheme 1b). Mice received drug or vehicle daily for 14 consecutive days. Seven days after the initiation of drug treatment, Aβ peptide (1–42 or 42–1) and a marker for cell proliferation were administered. After additional 7 days (at 14 days after initiation of the study), the animals were subjected to memory assessment (Morris Water Maze), and then killed. Immunohistochemical procedures were performed on hippocampal tissues.

For the second (ii): the experimental duration was 5 weeks (35 days: Scheme 1c), allowing for the quantitative evaluation of the survival of and the phenotypic appraisal of Aβ peptide-induced proliferated cells (Scheme 1c). Mice were treated similarly to those described above, but were administered drug or vehicle daily for 5 weeks. After 5 weeks, subgroups were subjected to memory tasks and then killed for immunohistochemical evaluation.

Mice received either i.p. vehicle (1% carboxy methyl cellulose solution (Fluka, Cat # 21901) prepared in sterile saline) or i.p. 3,6′-dithiothalidomide, prepared as a suspension in the vehicle at a dose of 56 mg/kg. At the time of Aβ peptide administration, mice were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and positioned in a stereotactic apparatus (Kopf Instruments, Tujunga, CA, USA). $Aβ_{1–42}$ and the reverse peptide $Aβ_{42–1}$ (American Peptide, Sunnyvale, CA, USA) were reconstituted in phosphate-buffered saline (pH 7.4) and aggregated by incubation at 37°C for 7 days prior to administration, as described previously (Choi and Bosetti 2009). $Aβ_{1–42}$ and $Aβ_{42–1}$ (400 pmol) were injected intracerebroventricularly (i.c.v.) into the lateral

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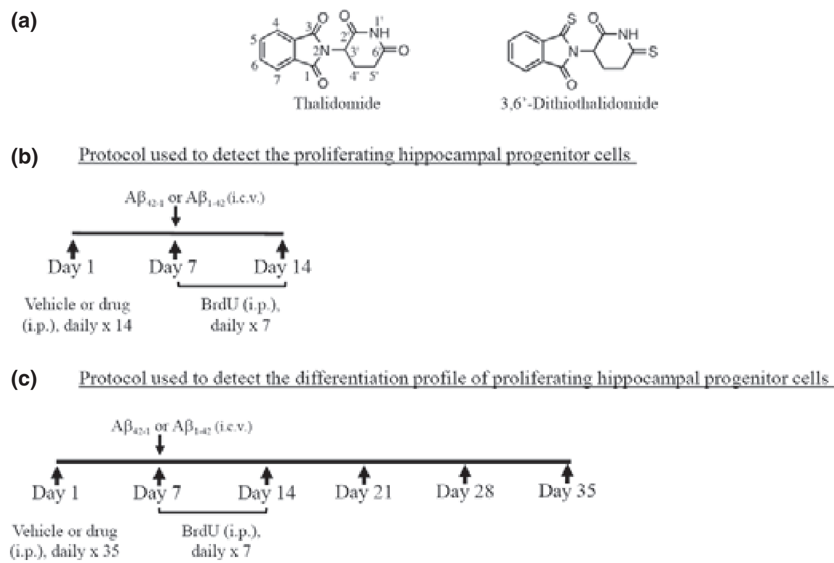

Scheme 1 (a) Chemical structure of thalidomide and 3,6′-dithiothalidomide. (b and c) Animal experimental protocol indicating the time-course for various interventions utilized during the experiment. Drug (56 mg/kg)/vehicle was administered daily, for the indicated time (from 14 up to 35 days). $A\beta_{42-1}$ or $A\beta_{1-42}$ (400 pmol) was administered once at the time point indicated. 5-bromo-2′-deoxyuridine (BrdU) (90 mg/kg) was administered daily for seven consecutive days from day 7 onward of drug administration.

ventricle using a 10-μL syringe (World Precision Instruments, Sarasota, FL, USA) and syringe pump (Stoelting, Wood Dale, IL, USA) at a rate of 1μL/min. Selection of the dose of $A\beta_{1-42}$ and $A\beta_{42-1}$ was based on previous studies (Yan *et al.* 2001; Jhoo *et al.* 2004; Prediger *et al.* 2007). The co-ordinates for the stereotaxic infusion were −2.5 mm dorsal/ventral, −1 mm lateral, and −0.5 mm anterior/posterior from the bregma (Paxinos and Franklin 2001). A marker for cell proliferation, 5-bromo-2′-deoxyuridine (BrdU, 90 mg/kg, 10 mg/mL in 0.9% saline; Sigma Aldrich, St. Louis, MO, USA) was administered i.p. at the time of Aβ peptide administration, and daily for 7 days thereafter. Animals were allowed to recover from surgery and returned to their home cages until the time of their experimental endpoint.

Immunohistochemistry and immunoflourescence

Immunohistochemistry and immunofluorescence triple labeling for BrdU were performed on free-floating 40-μm sagittal sections that were pre-treated by denaturing DNA, as described previously (van Praag *et al.* 1999a). Antibodies used for BrdU immunohistochemistry were mouse anti-BrdU (1 : 100, DAKO, Denmark), for triple labeling immunofluorescence assessments: rat anti-BrdU (1 : 200; Accurate Chemical & Scientific, Westbury, NY, USA), rabbit anti-S-100β (1 : 200; Abcam, Cambridge, MA, USA), mouse anti-NeuN (1 : 1500; Chemicon, Billerica, MA, USA) were used to detect cells with a neuronal phenotype. Mouse anti-synaptophysin (SYN) (1 : 2500; Millipore Corp - Bioscience Division, Billerica, MA, USA) and goat anti-doublecortin (DCX) (1 : 200, Santa Cruz, CA, USA) were used to detect morphological development of new neurons. Rabbit anti-CD11b (1 : 200; Chemicon, Temecula, CA, USA) was used to detect activated microglia cells. For immunohistochemistry, the peroxidase method (ABC system, with biotinylated donkey anti-mouse IgG antibodies and diaminobenzidine as chromogen; Vector laboratories, Burlingame, CA, USA) was used. Images were detected using a light microscope Olympus CKX41, using X40 and X60 objective (Olympus, Center Valley, PA, USA). For immunofluorescence studies, the fluorescent antibodies 488-Alexa Fluor anti-mouse IgG (1 : 500), 594-Alexa Fluor anti-rat IgG (1 : 500), and 405-Alexa Fluor anti-rabbit IgG (1 : 500), 488-Alexa Fluor anti-goat IgG (1 : 200) (Invitrogen, Carlsbad, CA, USA) were used. Fluorescent signals were detected and processed using a Zeiss Axiovert 200 microscope equipped with confocal laser system LSM 510 META. The images were acquired using a 63x oil immersion objective (Zeiss, Thornwood, NY, USA), and were assembled using Adobe Photoshop CS. The images that show the development of new neurons in the hippocampus were obtained by confocal Z-stack 3D acquisition and processed using LSM image examiner software.

Cell counting

BrdU-labeled cells were counted in the SGZ, which was defined as a two-nucleus-wide band below the apparent border between the granule cell layer and the hilus, as previously described (Kempermann *et al.* 2003). We made determinations in one of every six sections, and covered the entire area of DG. The numbers of BrdU-labeled cells in each mouse were calculated by summing the number of labeled nuclei of all slices analyzed. To determine the percentage of neuronal differentiation of newborn cells, one in every six sections was analyzed for the entire area of the DG. For each animal, 50 BrdU-positive cells were randomly selected and analyzed for co-expression of BrdU with NeuN a marker of a neuronal phenotype, or with S100β a marker for a glial phenotype, or neither with NeuN nor S100β (van Praag *et al.* 1999b). The number of activated microglia per section was quantified by counting the number of CD11b-stained cells within 0.3 mm^2 area of the hippocampus. For each measurement, two blinded independent investigators counted three to four brains per group, three sections per brain.

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Morris Water Maze assessment

Spatial learning and memory were assessed using the Morris Water Maze. Briefly, the experimental apparatus consisted of a circular plastic pool (diameter, 97 cm; height, 60 cm) filled with water (23 ± 2°C) that was colored with nontoxic white paint to obscure the location of a submerged platform. A target platform (10 × 10 cm) was submerged 1 cm below the water surface and placed at the midpoint of one quadrant. This platform was located in a fixed position, equidistant from the center and the wall of the pool, and was kept constant for each mouse throughout training. Visual cues were placed around the tank to orient the mice. The water maze test was performed in two sets of animals, 1 and 4 weeks following $A\beta_{1-42}$ administration. The acquisition training session was performed 4 days before the test session and consisted of five trials for 4 days, during which the animals were allowed to search for the platform for 60 s. In the event that a mouse failed to locate the platform in the allotted time, it was manually guided to the location. Mice were allowed to remain on the platform for 30 s for the first day, and then returned to their home cages. On subsequent days, the animals were allowed to remain on the platform for 15 s.

The test session was performed 24 h after the training sessions, on the last day prior to killing the animals. In the test session, the platform was removed from the pool and each mouse was allowed to swim for 60 s. This test session also consisted of five trials, in which the average time spent in the correct quadrant, where the platform was located on the training session, was calculated.

Gene expression

Fresh frozen mouse hippocampus were processed for RNA extraction using the Qiagen RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. Retro-Transcription (RT) was done using the Moloney murine leukemia virus-reverse transcriptase (MMLV-RT) (Invitrogen). A quantity of 2.5 mg of total RNA was mixed with 2.2 μL of 0.2 ng/μL random hexamer (Invitrogen), 10 μL of 56 buffer (Invitrogen), 10 μL of 2 mM dNTPs, 1 μL of 1 mM DTT (Invitrogen), 0.4 μL of 33 U/μL RNasin (Promega, Madison, WI, USA), 2 μL MMLV-RT (200 U/μL), in a final volume of 50 μL. The reaction mix was incubated at 37°C for 2 h and then the enzyme heat inactivated at 95°C for 10 min. Quantitative real-time PCR was performed for TNF-α (TNF-α: Mm00443258_m1), TNFR I (Tnfrsf1b: Mm00441889_m1); TNFR II (Tnfrsf1a: Mm00441883), and phosphoglicerate kinase 1 (pgk1: Mm01225301_m1) (See Supporting information, Figures S1 and S2). PCR reactions were performed using the Applied Biosystems 7500 system Real-time PCR system (Foster City, CA, USA) with Taqman probes following the manufacturer's instructions. Data were analyzed using the comparative threshold cycle (DD Ct) method (Livak and Schmittgen 2001). Results were normalized with Pgk1 as the endogenous control, and expressed as fold difference from the $A\beta_{42-1}$-injected mice, as previously reported (Toscano *et al.* 2007).

Statistical analysis

All data are expressed as means ± SEM. Statistical significance was assessed with a one-way ANOVA followed by a Bonferroni's test using GraphPad Prism. Significance was taken at $p < 0.05$.

Results

In all experiments, we also examined the group of mice injected with the control $A\beta_{42-1}$ peptide and treated with 3,6′-thiothalidomide.When we compared $A\beta_{42-1}$-injected mice treated with 3,6′-thiothalidomide and $A\beta_{42-1}$-injected mice treated with vehicle, there were no significant changes in microglia activation, numbers of BrdU-proliferative cells and BrdU-cells that differentiated in neurons, memory impairment, and memory recovery (data not shown). Since 3,6′-dithiothalidomide treatment alone did not cause any effects in control mice, this group was not included in the graphs.

3,6′-dithiothalidomide treatment decreases microglia activation in response to $A\beta_{1-42}$ –induced neuroinflammation

Mice were treated with 3,6′-dithiothalidomide or vehicle for 14 days, 7 days before and 7 days after injection of $A\beta_{1-42}$ or $A\beta_{42-1}$. $A\beta_{1-42}$ injection caused a robust inflammatory response, characterized by the presence of activated microglia cells within hippocampus in mice injected with vehicle (Fig. 1a and b). Intense CD11b-immunoreactive microglia with enhanced staining intensity, retracted processes, and amoeboid appearance were observed in the hippocampus of these mice (Fig. 1a). In the hippocampus of $A\beta_{1-42}$-injected mice treated with 3,6′-dithiothalidomide and in $A\beta_{42-1}$-injected mice, only a few CD11b-immunoreactive microglia cells were observed (Fig. 1a and b), which retained a resting morphology with small cell bodies, thin, and ramified processes.

3,6′-Dithiothalidomide treatment attenuates the effect of $A\beta_{1-42}$ injection on hippocampal progenitor cell proliferation

To analyze the effects of treatment with 3,6′-dithiothalidomide on $A\beta_{1-42}$ inflammation-induced proliferation of hippocampal progenitor cells, mice were treated for a total of 14 days with 3,6′-dithiothalidomide. Seven days after initiation of drug treatment, the appropriate Aβ peptides were administered. Seven days later, a subset of animals was killed and the numbers of BrdU-labeled cells were counted in the DG of hippocampus (Scheme 1b, Fig. 2a). $A\beta_{1-42}$-induced brain inflammation caused an increase (64%) in the number of BrdU-positive proliferating cells, as compared with mice injected with the control peptide ($A\beta_{42-1}$). Treatment with 3,6′-dithiothalidomide substantially attenuated this effect. Comparing the numbers of BrdU-cells from the drug treated animals with those observed in vehicle-treated animals, there was a 36% reduction in proliferated cells counts induced by $A\beta_{1-42}$ insult (427.6 ± 32.59 for 3,6′-dithiothalidomide-treated animals vs. 547.6 ± 29.91 for non-drug-treated animals, $p < 0.01$, Fig. 2b). The distribution of the BrdU-labeled proliferative cells was found mainly

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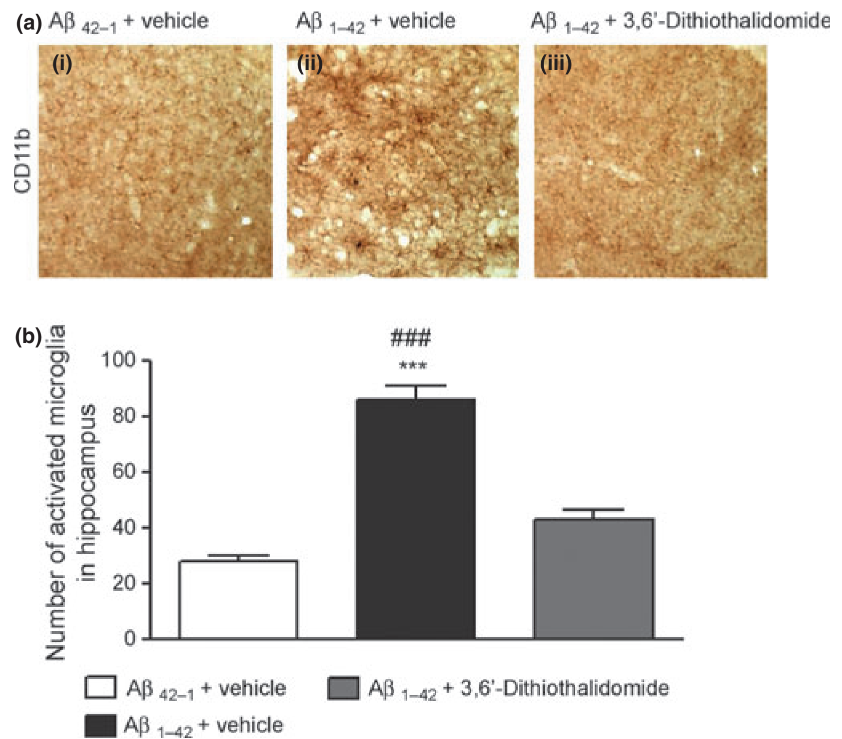

Fig. 1 (a) Representative photomicrographs of CD11b immunoreactivity in the hippocampus subfield (i) $A\beta_{42-1}$ vehicle-injected mice (control), (ii) Vehicle-treated $A\beta_{1-42}$-injected mice, (iii) 3,6′-dithiothalidomide-treated $A\beta_{1-42}$-injected mice. (b) Quantification of CD11b-positive cells in the hippocampus. Mean ± SEM ($n = 4$ per group). ***$p < 0.001$ compared with vehicle-treated - $A\beta_{42-1}$ injected mice; $^{###}p < 0.001$ compared with 3,6′-dithiothalidomide-treated $A\beta_{1-42}$-injected mice. Scale bars = 100 μm.

in clusters at the border between the granule cell layer and the hilus of the DG, with no differences in the distributions being evident among the different treatments.

3,6′-dithiothalidomide treatment represses the effect of $A\beta_{1-42}$ –induced inflammation on BrdU-cells survival at 4 weeks

The effects of 3,6′-dithiothalidomide on the survival of BrdU-cells induced by $A\beta_{1-42}$ mediated neuroinflammation were assessed (Scheme 1c). We analyzed the number of BrdU-cells in the granule cell layer and subgranular zone of the DG of mice treated with vehicle or 3,6′-dithiothalidomide 4 weeks after either $A\beta_{1-42}$ or control peptide administration. $A\beta_{1-42}$ significantly increased the number of BrdU-cells survived compared with the control peptide group (128 ± 1.69 for $A\beta_{1-42}$ vs. 91 ± 2.14 for $A\beta_{42-1}$, a 40% increase in cell number, $p < 0.001$). In mice injected with $A\beta_{1-42,}$ the treatment with 3,6′-dithiothalidomide repressed and normalized the number of surviving BrdU-cells to control peptide levels (91 ± 2.14 for $A\beta_{42-1}$ vs. 93.8 ± 1.7 for drug + $A\beta_{1-42;}$ Fig. 3).

$A\beta_{1-42}$-induced increase in neurogenesis was attenuated by 3,6′-dithiothalidomide treatment

To determine the fate of hippocampal-derived proliferating cells, we examined the phenotype of newly generated BrdU-cells 4 weeks after $A\beta_{1-42}$-induced inflammation by co-immunolabeling for BrdU along with neuronal (NeuN) and glial (S100β) markers (Fig. 4a). The percentage of BrdU-positive cells that co-labeled for NeuN was significantly increased in $A\beta_{1-42}$-injected mice compared with control mice injected with the reverse peptide ($p < 0.001$, Fig. 4b, Table 1). In animals treated with 3,6′-dithiothalidomide and $A\beta_{1-42}$, there was a significant elevation in neurons when compared with control animals, which was less than that induced by $A\beta_{1-42}$ plus vehicle ($p < 0.01$, Fig. 4b, Table 1). $A\beta_{42-1}$-injected mice showed a significantly higher fraction of BrdU-labeled cells that proved neither neuronal nor glial. Indicating that these cells either remained undifferentiated or had differentiated into cells with an alternative phenotype. This was not the case for $A\beta_{1-42}$-injected mice treated with either vehicle or 3,6′-dithiothalidomide ($p < 0.001$; Table 1). Interestingly, there was no significant difference between the groups with regard to percentage of newborn cells differentiated into glia (Table 1).

New hippocampal neurons show normal morphological development 4 weeks after $A\beta_{1-42}$ injection

To determine whether $A\beta_{1-42}$-induced newly generated granule neurons develop normal morphology, we performed DCX-SYN double staining immunofluorescence, as previously described (van Praag *et al.* 2002; Tronel *et al.* 2010). SYN (red), which is expressed by synaptic vesicle membranes, was detected around the newly generated DCX-positive (green) granule cells (Fig. 5), suggesting that the new immature neurons are physically integrated into the

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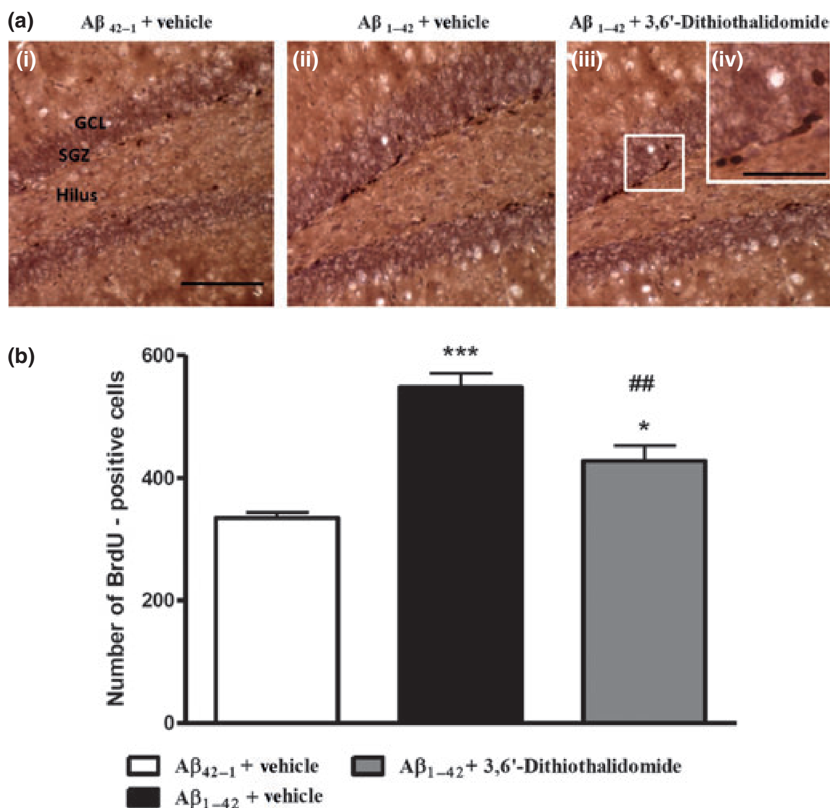

Fig. 2 (a) Effects of 3,6′-dithiothalidomide treatment on hippocampal 5-bromo-2′-deoxyuridine (BrdU) proliferative cells after $A\beta_{1-42}$ administration are presented. Representative photomicrographs of BrdU immunohistochemistry in the DG, 7 days after i.c.v. injection of either the control peptide $A\beta_{42-1}$ (i), or the neurotoxic peptide $A\beta_{1-42}$ (ii and iii) are shown. (iv) Inset showing high-magnification images of BrdU immunostaining cells. Scale bars = 100 μm (i, ii, iii); 50 μm (iv). (b) A quantitative assessment of BrdU-labeled cells as described above, are shown. Data are mean ± SEM ($n = 5$). Data were analyzed using a one-way ANOVA followed by Bonferroni's *post-hoc* test. $^{*}p < 0.05$; $^{***}p < 0.001$ versus $A\beta_{42-1}$-injected mice; $^{*}p < 0.05$ versus $A\beta_{42-1}$- injected mice; $^{\#\#}p < 0.01$ versus β_{1-42}-injected mice treated with vehicle.

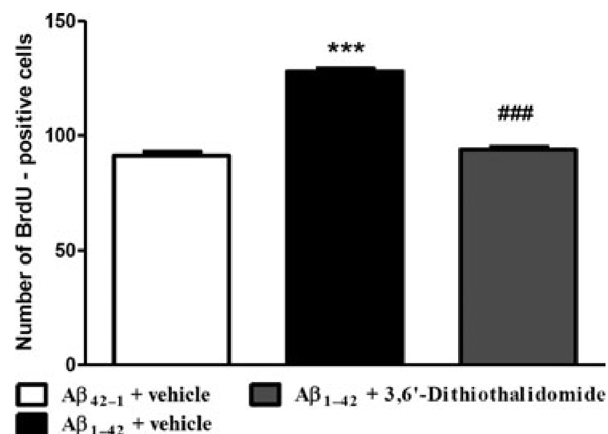

Fig. 3 Effects of 3,6′-dithiothalidomide treatment on hippocampal-derived 5-bromo-2′-deoxyuridine (BrdU)-cell survival at 4 weeks after peptide administration are shown. Presented are quantified data for BrdU-cells in mice administered with the neurotoxic peptide $A\beta_{1-42}$ treated with vehicle or 3,6′-dithiothalidomide and mice administered with control $A\beta_{42-1}$ peptide. Data are mean ± SEM ($n = 5$). Data were analyzed using a one-way ANOVA followed by Bonferroni's *post-hoc* test. $^{***}p < 0.001$ versus $A\beta_{42-1}$-injected mice; $^{\#\#\#}p < 0.001$ versus $A\beta_{1-42}$-injected mice treated with vehicle.

surrounding hippocampal tissue. Co-labeled cells observed in the DG region showed no morphological differences irrespective of treatment group (Fig. 5).

$A\beta_{1-42}$-induced memory deficits are abolished by 3,6′-dithiothalidomide treatment

To explore the effects of 3,6′-dithiothalidomide on memory tasks after $A\beta_{1-42}$ inflammation, mice were subjected to Morris Water Maze assessment at 7 days or 4 weeks after Aβ administration. Training sessions were performed 4 days before the test session, and consisted of five trials for 3 days. The escape latency decreased over the course of the acquisition training in all mice analyzed 7 days after Aβ injection (Fig. 6a). The time to find the platform was significantly increased in mice injected with $A\beta_{1-42}$ peptide treated with vehicle compared with mice injected with the reverse peptide ($p < 0.01$; Fig. 6a). Following 3 days of training, mice were tested using an average of five trials. $A\beta_{1-42}$-induced inflammation significantly impaired memory acquisition compared with control $A\beta_{42-1}$-injected mice (17.09 ± 3.54 vs. 5.48 ± 0.67; $p < 0.01$; Fig. 6b). Treatment with 3,6′-dithiothalidomide prevented memory deficits compared to the respective vehicle-treated mice (7.09 ± 1.05 vs. 17.09 ± 3.54; $p < 0.05$; Fig. 6b). Four weeks after Aβ injection, mice had fully recovered their memory function and there were no differences between the groups (data not shown).

Discussion

Recent studies have shown that neuroinflammation-associated changes in the brain such as microglia activation,

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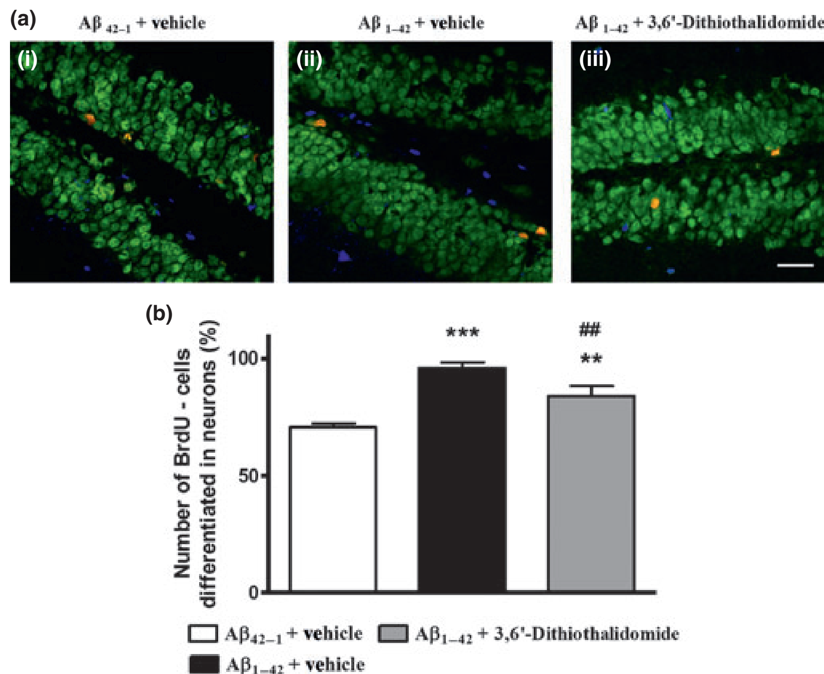

Fig. 4 (a) Effects of 3,6′-dithiothalidomide treatment on the differentiation of hippocampal neuronal progenitor cells, 4 weeks after β-amyloid (Aβ) administration are presented. Representative confocal images of the dentate gyrus (DG) of mice administered with the control peptide $A\beta_{42-1}$ (i), with the neurotoxic peptide $A\beta_{1-42}$ treated with vehicle (ii) or 3,6′-dithiothalidomide (iii) are shown. Hippocampal sections were triple-labeled for 5-bromo-2′-deoxyuridine (BrdU) (red), NeuN indicating neuronal phenotype (green), and S100β selective for glial phenotype (blue), assessments were made with immunofluorescence microscopy, scale bars = 20 μm. (b) The quantification of the percentage of BrdU-cells that differentiated into neurons is described. Data are mean ± SEM ($n = 5$). Data were analyzed using a one-way ANOVA followed by Bonferroni's *post-hoc* test. $p < 0.05$; ***$p < 0.001$ versus $A\beta_{42-1}$-injected mice; **$p < 0.01$ versus $A\beta_{42-1}$-injected mice; ##$p < 0.01$ versus $A\beta_{1-42}$-injected mice treated with vehicle.

Table 1 Phenotype of surviving cells was determined by immunofluorescent triple labeling for 5-bromo-2′-deoxyuridine (BrdU), NeuN (neurons), S100β (glia). The percentage of BrdU-positive cells double labeled for either NeuN or s100β or neither marker is presented

	$A\beta_{42-1}$ + vehicle	$A\beta_{1-42}$ + vehicle	$A\beta_{1-42}$ + 3, 6′-dithiothalidomide
Neuron (%)	70.69 (2.16)	96.01 (3.13)***	85.40 (2.54)** ##
Glia (%)	9.73 (0.55)	3.99 (2)	9.74 (2.32)
Other (%)	19.58 (2.11)	0 (0)***	4.86 (1.6)*** #

All data are means ± SEM and were analyzed using 1-way ANOVA followed by a Bonferroni's post-hoc test). *$p < 0.05$; ***$p < 0.001$; **$p < 0.01$ vs. control group ($A\beta_{42-1}$ + Vehicle. #$p < 0.05$, ##$p < 0.01$ compared with $A\beta_{1-42}$ + Vehicle.

induction of nuclear factor-kappaB transcription factor, and release of inflammatory mediators, including TNF-α, can contribute to impaired neurogenesis (Monje *et al.* 2003; Russo *et al.* 2011a,b). In this study, we investigated whether TNF-α is involved in the neurogenic signaling of hippocampal progenitor cells following $A\beta_{1-42}$-induced inflammation (Akiyama *et al.* 2000; Choi and Bosetti 2009; Ferretti and Cuello 2011) using the potent TNF-α synthesis inhibitor, 3,6′-dithiothalidomide, a small lipophilic experimental drug that readily enters the brain (Tweedie *et al.* 2007, 2009). We report that the treatment with 3,6′-dithiothalidomide normalizes microglia activation, proliferation and survival of progenitor cells, differentiation of new neurons, and cognitive functions to the control conditions, thus repressing the effects of $A\beta_{1-42}$ on neuronal tissue and on the neurogenic hippocampal niche.

TNF-α participates in the regulation of turnover of neural stem/progenitor cells under physiological conditions (Iosif *et al.* 2006), and also has been implicated in the pathogenesis of certain neurodegenerative and neurological disorders like AD, Parkinson's disease, stroke, and head trauma (Hallenbeck 2002; Hirsch *et al.* 2003; Li *et al.* 2004). TNF-α regulates several cellular processes, including inflammation, cell differentiation, cell death, and survival through activation of two TNF receptors: TNFR1 or TNFR2 (Wajant *et al.* 2003). These receptors have been proposed to facilitate distinct TNF-α- mediated effects in the brain: TNFR1, with its intracellular 'death domain', contributes to neuronal death and damage, and primarily responds to soluble TNF-α (Grell *et al.* 1998). In contrast, TNFR2 is associated with cell

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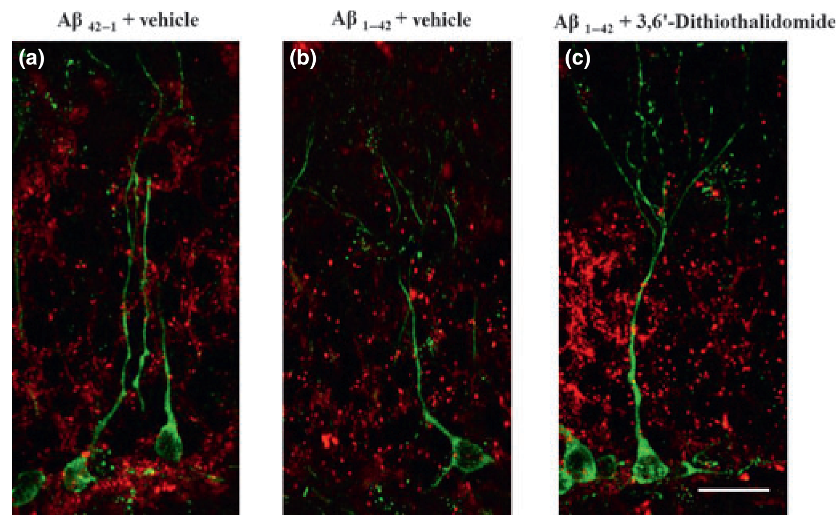

Fig. 5 Representative images of morphological development of new neurons are presented at 4 weeks after administration of either β-amyloid (Aβ) peptide. The degree of neuronal integration appears to be similar between the groups. Confocal images of the dentate gyrus (DG) of mice that receive i.c.v. control $A\beta_{42-1}$ (a) and the neurotoxic $A\beta_{1-42}$ peptide with vehicle (b) or 3,6′-dithiothalidomide (c) are shown. Sections were double-labeled for synaptophysin (SYN) (red) and doublecortin (DCX) (green). Images were obtained using immunofluorescence microscopy, scale bar = 10 μm.

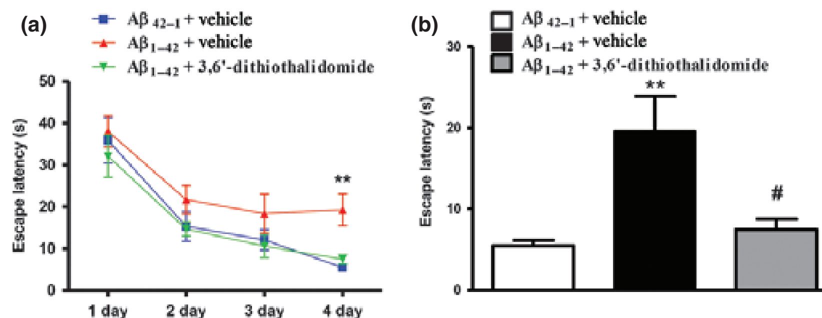

Fig. 6 Effect of $A\beta_{1-42}$ administration and $A\beta_{1-42}$ plus 3,6′-dithiothalidomide treatment on mouse memory performance in the Morris Water Maze test, 7 days after β-amyloid (Aβ) peptide injection are shown. (a) The escape latency during the training and memory assessment for mice that received i.c.v. control peptide and the neurotoxic $A\beta_{1-42}$ peptide treated with vehicle or 3,6′-dithiothalidomide are presented. (b) Quantified probe trial data of mice that received i.c.v. control peptide, or $A\beta_{1-42}$ treated with vehicle or 3,6′-dithiothalidomide 7 days after Aβ peptide administration are shown. Data are mean ± SEM ($n = 5$). Data were analyzed using a one-way ANOVA followed by Bonferroni's *post-hoc* test. Significance was set at $p < 0.05$. $^{**}p < 0.01$ versus $A\beta_{42-1}$ peptide-injected mice; $^{\#}p < 0.05$ versus $A\beta_{1-42}$ peptide-injected mice.

survival and neuroprotection (Fontaine *et al.* 2002; Yang *et al.* 2002; Wajant *et al.* 2003; Marchetti *et al.* 2004), and is primarily activated via membrane-bound TNF-α (Grell *et al.* 1995). The pro-survival or pro-death roles of TNF-α likely depend on which TNF receptor is activated. Under pathological conditions, soluble and membrane-bound TNF-α levels may be differentially elevated and diverse types of neurons may have different expression ratios of the two TNF-α receptors (Yang *et al.* 2002). Furthermore, the net effect of TNF-α is dependent on several factors, such as the site, degree, and duration of neuroinflammation, the level and cellular origin of the cytokine, the type of target cells, the expression level of the two receptors and their affinity to TNF-α (Fontaine *et al.* 2002; Hallenbeck 2002; Heldmann *et al.* 2005). Because of the diverse bioactivities of TNF-α, it remains unclear under which conditions TNF-α promotes beneficial or deleterious effects on neuronal tissue and on the hippocampal neurogenic niche.

The cellular synthesis of TNF-α, similar to other inflammatory cytokines, is closely regulated at the post-transcriptional level of mRNA stability through its 3′-UTR (Moreira *et al.* 1993), which allows for rapid alterations in TNF-α production in response to exogenous and endogenous-induced changes in the brain microenvironment. The presence of adenylate-

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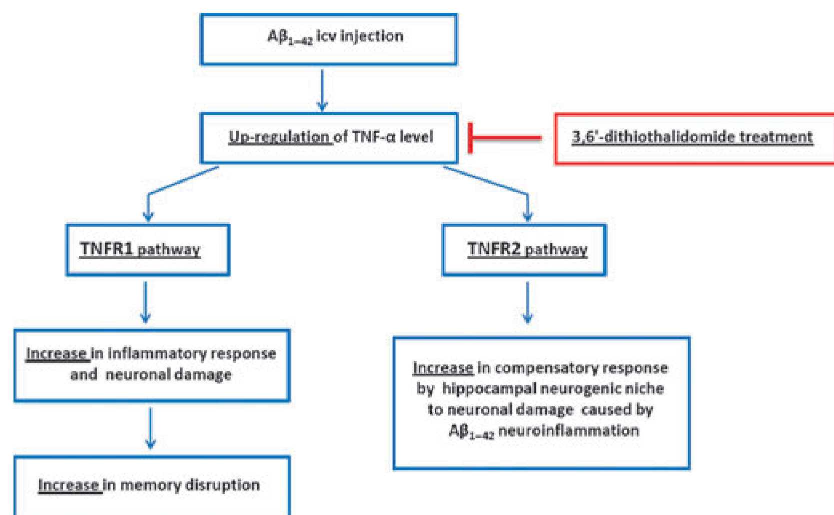
uridylate-rich elements within the 3-UTR of TNF-α mRNA permits post-transcriptional repression that, through interaction with specific RNA-binding proteins, can either promote its stability and thereby increase its synthesis or, alternatively, target it for rapid degradation or inhibition of translation, to lower its rate of generation (Patil *et al.* 2008; Khera *et al.* 2010). In this regard, thalidomide has been shown to increase translational blockade and thereby reduce the rate of TNF-α protein synthesis (Sampaio *et al.* 1991; Moreira *et al.* 1993). 3,6′-dithiothalidomide, like thalidomide, regulates TNF-α mRNA stability via its 3′-UTR (Zhu *et al.* 2003), but with the isosteric replacement of carbonyl groups by thiocarbonyls has incremental increases in TNF-α inhibitory activity of up to 30-fold, without toxicity (Tweedie *et al.* 2009). A recent study showed how, using the same concentration, 3,6′-dithiothalidomide but not thalidomide lowered TNF-α protein level following lipopolysaccharide-challenged RAW 264.7 cells (Tweedie *et al.* 2009). Thus, the potent action of 3, 6′-dithiothalidomide allows the administration of lower and better tolerated doses during anti-inflammatory treatment (Baratz *et al.* 2011; Belarbi *et al.* 2012; Tweedie *et al.* 2012, in press).

Neuronal progenitor cells express both TNFR1 and TNFR2, suggesting that TNF-α can modulate the formation of new hippocampal neurons under pathological conditions by acting on these two receptors with differential effects on proliferation and survival (Iosif *et al.* 2006). TNF-α is also released by neuronal progenitor cells (Heldmann *et al.* 2005), and can act directly on the neuronal progenitor cells via either autocrine mechanisms or by release from neighboring microglia cells (Heldmann *et al.* 2005).

TNF-α, mainly via TNFR2 signaling, has been shown to promote neurogenesis in various models of injury, such as ischemia, demyelination, and status epilepticus. Specifically, immunotherapy with an antibody to TNF-α attenuates the increase in neurogenesis after middle cerebral artery occlusion (Heldmann *et al.* 2005). Furthermore, studies in TNFR2 knock-out mice subjected to cuprizone-induced demyelination indicated that TNF-α *via* TNFR2 is critical to oligodendrocyte regeneration by promoting proliferation of oligodendrocyte progenitors (Arnett *et al.* 2001). TNFR2 knock-out mice show reduced neurogenesis also after status epilepticus, which is associated with inflammation and elevated TNF-α level (Iosif *et al.* 2006). In this study, we showed that following $A\beta_{1-42}$ injection and 3,6′-dithiothalidomide treatment, TNF receptors mRNA are not modulated (Supporting information, Fig. S2), although TNF-α mRNA level is drastically up-regulated also after drug treatment (Supporting information, Fig. S1). As 3,6′-dithiothalidomide inhibits TNF-α protein synthesis (Tweedie *et al.* 2009; Baratz *et al.* 2011; Belarbi *et al.* 2012; Tweedie *et al.* 2012; in press) by a mechanism related to the destabilizing of its mRNA (Greig *et al.* 2004), one probable reason is that microglial cells respond with a robust increase in transcription of TNF-α mRNA to compensate for any reduction in TNF-α protein synthesis.

Several studies reported that an increase in neurogenesis is associated with cognitive improvement (Stone *et al.* 2011; Marlatt *et al.* 2012); conversely, impairment in neurogenesis is associated with cognitive deficits (Kim *et al.* 2011; Mishra *et al.* 2012). Taking into account the effect of inflammation on neurogenesis and memory tasks, in this study, we showed that $A\beta_{1-42}$-induced neuroinflammation is accompanied by enhanced neurogenesis and cognitive impairment.

Altered neurogenesis has been shown in various AD transgenic mouse models, with reporting of both decreased and increased neurogenesis (Lazarov and Marr 2010; Marlatt and Lucassen 2010). Possible explanations for these apparently conflicting data could be ascribed to neurodegenerative stage-dependent effects as well as strain, genetic background, and specific transgene mutations, which could differentially affect the different stages of proliferation, survival, and



Scheme 2 Mechanism linking hippocampal neurogenesis and memory tasks in response to $A\beta_{1-42}$-induced neuroinflammation and after 3,6′-dithiothalidomide treatment. 3,6′-dithiothalidomide treatment leads to a reduction in TNF-α protein level and consequently, attenuates the neuroinflammatory response and neuronal damage after $A\beta_{1-42}$ injection. The hippocampal neurogenic niche, in turn, responds with an attenuated compensatory increase in nurogenesis.

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differentiation of newly formed cells (Verret *et al.* 2007). In this regard, several studies have demonstrated that hippocampal neurogenesis *in vivo* is significantly increased at early stages of neurodegeneration, and although neurogenesis and the differentiation of newly generated neurons into a mature phenotype are still significantly increased at late stages of neurodegeneration, these neurons are impaired in comparison with the early stage neurons *(Jin et al.* 2004b). In addition, it has been reported that Aβ levels and/or conformation may affect neurogenesis, for instance, oligomeric $A\beta_{42}$ has been shown to enhance neuronal differentiation of embryonic and postnatal neuronal stem cells *in vitro* (Lopez-Toledano and Shelanski 2004). Furthermore, studies from human post-mortem brain of AD patients showed increased hippocampal neurogenesis (Jin *et al.* 2004a,b). Our data suggest that increased neurogenesis occurs perhaps as an endogenous compensatory response to neuroinflammation and at early neurodegenerative events following $A\beta_{1-42}$ injection, where these events are likely mediated by TNFR2 signaling actions (Heldmann *et al.* 2005; Iosif *et al.* 2006).

In this study, we found that treatment with 3,6′-dithiothalidomide attenuates the memory disruption, and at the same time attenuates the compensatory response and the number of new neurons generated by hippocampal neurogenic niche after $A\beta_{1-42}$ injection (Scheme 2). Thus, it is possible that TNF-α plays a dual role and is involved in mediating at the same time neurodegeneration (memory disruption) and regeneration (increase of neurogenesis), through TNFR1 and TNFR2 signaling respectively, and that there is a cooperative action of both receptors during an inflammatory response (Fontaine *et al.* 2002). TNF-α also has a homeostatic role in limiting and controlling the extent and duration of an inflammatory response (Hallenbeck 2002; Heldmann *et al.* 2005), probably through modification of the TNF-α core signaling framework and by generating feedback responses that suppress inflammation (Hallenbeck 2002).

Understanding which factors regulate hippocampal neurogenesis and its effects on cognition and memory could have important therapeutic implications to help gain insight on the cellular mechanisms underlying AD and to development of new therapeutic strategies. A recent study from our groups showed that chronic 3,6′-dithiothalidomide administration to an elderly symptomatic cohort of 3xTg AD mice reduced multiple hallmark features of AD (Tweedie *et al.* 2012; in press). The data presented in this manuscript indicate that while treatment with 3,6′-dithiothalidomide attenuates neurogenesis caused by $A\beta_{1-42}$–induced inflammation, importantly it prevents the subsequent memory impairments most likely as a result of reduced neuronal cell damage and an attenuated neuroinflammatory response related to lower TNF-α protein levels (Scheme 2), suggesting that the anti-inflammatory effect is a critical component in the improvement of memory function. Here, we can hypothesize that the new neurons generated in a brain with less neuroinflammation could have a higher probability to integrate and synaptically participate to the hippocampal circuitry, as neuroinflammation and its propagation limit the integration of new neurons (Jin *et al.* 2004a).

In conclusion, our data indicate that TNF-α synthesis inhibition by 3,6′-dithiothalidomide treatment prevents memory impairments induced by a $A\beta_{1-42}$-neuroinflammation and suggest that this drug should be further investigated as a therapeutic in AD as well as other animal models of neurodegenerative disease with an inflammatory component.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figure S1. Quantitative real-time PCR analysis of TNF-α mRNA in Aβ42-1 injected mice (control), Vehicle-treated Aβ1-42-injected mice, and 3,6′-dithiothalidomide-treated Aβ1-42 -injected mice.

Figure S2. Quantitative real-time PCR analysis of TNFR1 And TNFR2 mRNA in Vehicle-treated Aβ1-42-injected mice and 3,6′-dithiothalidomide-treated Aβ1-42-injected mice.

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