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Abstract: Melanocortins exert neuroprotection in a variety of experimental neurodegenerative disorders, including Alzheimer's disease (AD). Further, in previous research we showed that these endogenous peptides stimulate neurogenesis in an acute neurodegenerative disorder such as ischemic stroke. In the present research, we investigated the potential neurogenic effect of melanocortins in AD using APPSwe transgenic mice (Tg2576). To this purpose, 24 week-old animals were prepared for 5-bromo-2'-deoxyuridine (BrdU) labeling of proliferating cells on days 1-11 of the study. Treatment of Tg2576 mice with nanomolar doses of the melanocortin analog [Nle4,D-Phe7]α-melanocyte-stimulating hormone (NDP-α-MSH), administered once daily from day 1 to 50, improved brain histology and cognitive functions relative to saline-treated Tg2576 animals. No signs of toxicity were observed. Immunohistochemical examination of the hippocampus at end of the study (day 50) showed that NDP-α-MSH-treated Tg2576 mice had a greater number of BrdU immunoreactive cells colocalized with NeuN (an indicator of mature neurons) and Zif268 (an indicator of functionally integrated neurons) in the dentate gyrus, relative to saline-treated Tg2576 animals; no newly formed astrocytes were found. Animal pretreatment with the selective melanocortin MC4 receptor antagonist HS024 before each NDP-□-MSH administration prevented all the beneficial effects of the peptide. The present data indicate that MC4 receptor stimulation by a melanocortin prevents cognitive decline in experimental AD not only through a neuroprotective mechanism but also by induction of an intense neurogenesis. MC4 receptor agonists could be innovative and safe candidates to counteract AD progression in humans.

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Modena, February 24, 2015

To Professor Penelope Hallett
Editor
Molecular and Cellular Neuroscience

Dear Prof. Hallett,

thank you for your mail (February 6, 2015) concerning our manuscript (No. MCN-14-195) entitled **"NDP-α-MSH induces intense neurogenesis and cognitive recovery in Alzheimer transgenic mice through activation of melanocortin MC4 receptor".**

We have carefully considered the Reviewers' comments and suggestions, and an accordingly revised version of our manuscript is here appended, with changes marked in red. Moreover, point-by-point responses to the Reviewers are enclosed.

As requested we followed the journal formatting guidelines.

We hope that, in the present revised version, our manuscript can be accepted for publication in *Molecular and Cellular Neuroscience*.

We look forward to hearing from you. Thank you very much for your attention.

Yours Sincerely

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Response to Reviewer 1

Thank you for your careful examination of our manuscript (No. MCN-14-195), and for your useful suggestions. We performed changes throughout the manuscript (marked in red), and these are our replies.

(Specific points)

1. As suggested, quantitative Aβ has been added in Fig. 3.
2. As rightly stated by the Reviewer, no significant difference was observed between WT and Tg2576 mice in BrdU/NeuN positive cells. We expected this result, because WT are healthy mice and neurogenesis is very limited because compensation for possibly significant neuronal loss is unlikely. As suggested, in the Fig. 4 of the revised version of our manuscript we added data on WT mice treated with NDP-alpha-MSH. Moreover, although these mice (31 week old at the end of the study) are no aged, in our opinion they are suitable for investigating cognitive function deficits and neurogenesis. In the present study we planned to investigate young (24 week-old at the start of the study) Tg2576 mice, because unsure of the potential therapeutic efficacy of melanocortins in a condition of severe AD. On the basis of the present exciting results, we have already planned a further research to thoroughly explore neurogenesis (which will be object of an additional paper) with 11-12 month-old transgenic mice. This critical point has been briefly discussed in the previous and present version of our manuscript (page 16, lines 13-14).
3. As suggested, English has been examined by a native speaker.

Response to Reviewer 2

Thank you for your careful examination of our manuscript (No. MCN-14-195), and for your useful suggestions. We performed changes throughout the manuscript (marked in red), and these are our replies.

Methods

1 –Our research group has a long-standing experience in the Morris test, with rats and smaller rodents (gerbils, mice) (see reference list: Giuliani et al., *Endocrinology*, 2006; Giuliani et al., *Eur. J. Pharmacol*., 2006, 2007; Giuliani et al., *Neurobiol. Aging* 2014; Spaccapelo et al., *Eur. J. Pharmacol* , 2011; etc.). Our experience (and that of others groups: eg, Wiard et al., *Stroke*, 26:466-472,1995, etc.) indicate that a water maze of 80 cm in diameter and water at 27°C is suitable for evaluating learning and memory in small rodents. However, it is not ruled out (as hypothesized by the Reviewer) that more difficult conditions could influence the outcome (but in all experimental groups): anyway, with the conditions used by us we found a clear difference between treated and untreated AD mice.

2 – We agree with the Reviewer that repeated learning affects subsequent learning trials and that learning itself also stimulates neurogenesis: indeed, we used this model (see also

our previous paper by Giuliani et al., Acta Neuropathol, 122, 443-453, 2011) to stimulate neurogenesis, by comparing melanocortin-treated and untreated mice, WT and Tg2576.

3 –The number of BrdU/NeuN in Fig 4 is not exactly the same as the number of BrdU/NeuN/Zif268 triple-stained cells. Anyway, surprisingly, in our experimental conditions the number of triple stained cells is very high, and with enthusiasm in Fig. 5 of the previous version (now Fig. 6) we decided to show a field were all BrdU-labeled cells are also colocalized with NeuN and Zif268 (this is specified in the figure caption of the previous and revised version of our manuscript). We agree with the Reviewer that newborn cells usually requires a month or more to migrate; indeed, as stated in methods, results and figure caption, we counted newborn cells at day 50 of the study, that is, 39 days after the last BrdU injection. Anyway, in the previous Fig 5 (now Fig. 6) there are no newborn cells in the outer parts of DG: the 2 right arrowheads in the previous Fig. 5F (now Fig. 6 C, without arrows) belong to DG (see red box in panel A of the new Fig. 5).

4 –As stated in methods and shown in Fig. 1, in our experimental model BrdU treatment was performed on days 1-11 of the study,: therefore, younger cells BrdU positive are 39-day old, and younger cells not BrdU-labeled obviously are not visible. In the previous Fig. 5G (now Fig. 6B) a high number of cells (old and newborn neurons) - but not almost all - is Zif positive (please compare panel A with panel B of Fig. 6). In summary, in the previous Fig. 5H (now Fig. 6F) there are 3 types of cells: a few white cells (BrdU/NeuN/Zif triple-stained, 12%, active neurons born on days 1-11 of the study), cyan cells (NeuN/Zif double-stained, 60%, active neurons born before and after days 1-11 of the study) and green cells (NeuN-stained, 28%, no active neurons born before and after days 1-11 of the study). Anti-apoptotic immunoreactions have not be done, so we are unsure if Zif stained also such cells. All appropriate controls have been done for immunocytochemistry (see methods)..

5 -– See answer to the 1st question of Reviewer 1. Cell number per volume was calculated as indicated in Endocrinology, 147, 1126-1135, 2006. We did not perform stereological evaluation, but only a cell count to allow roughly comparison among experimental groups. Immunocytochemistry for Amyloid was not performed since we preferred the histochemical method that in combination with the polarized light examination prevents false positives. We added further details on histological methods (page 8, lines 10-14). The Reviewer's suggestion “a more convincing morphological (cytoarchitectual) analysis would be welcome including the distribution of amyloid beta deposits over the different hippocampal subregions” was not the goal of this study but it will be in the future.

6 - The figure 3 (now modified) is a representative image depicting the hippocampus situation. As reported in the caption, the Hematoxylin-Eosin staining (C), particularly in the saline treated mice, highlighted nucleus degeneration, pyknosis, cellular shrinkage and pericellular vacuolization.

7 – Our experience indicates that 50 days of ip injection does not cause suffering or appreciable stress. This has not interfered with results, because all groups (including controls) have been ip injected for 50 days. This long-term treatment schedule has been chosen to allow maturation of newborn neurons.

8 –Our very interesting and exciting results on neuroprotection published in 2014 on EJP and NBA encouraged us to investigate also neurogenesis, because (from a scientific and practical point of view) very important and of great novelty.

9 –The possible mechanism of the neurogenic effect of melanocortins was discussed in pages 14-15 of the previous version. In the revised manuscript we discussed some more about MC receptors and AD (page 15, lines 10-20).

10 –Taking into account the Reviewer's comments, in the introduction of the revised manuscript we deleted the reference by Giuliani et al. (2013) about neuroprotection and added Franco and Cedazo-Minguez (2014), and we cited all the relevant key papers related to neurogenesis suggested by the Reviewer.

We thank you for your statement "I like their work and this approach very much" and for your useful criticism. Our work has been now improved as suggested. As stated in the above point 8, we investigated neurogenesis after the important results obtained on neuroprotection. In our opinion, the neurogenic effect is a relevant novelty, because it constitutes a robust framework of the potential therapeutic value of melanocortins against AD (obviously after further studies).

Minor comments

1 –OK: so far, the most appropriate reference is that by Ben Menachem-Zidon et al., 2014 (page 15, line 1).

2 –As stated at page 16, lines 2-6) melanocortins are devoid of appreciable toxicity . Unfortunately, until now we are unable to give further explanations (besides those reported in page 15, lines 10-20) on how melanocortins protect against amyloid overexpression. Further studies are needed.

3 –For many years our results on Morris Water Maze (MWM) have been depicted in the same manner (and published in many important Journals, please see references of our group), because (in our opinion) of easier understanding for readers.

4 -This was probably due to the repeated confocal acquisition to have a well-defined image, without background, that increases contrast.

5 -Microglia increase was seen in isocortex and rarely in the hippocampus. In the revised manuscript, reference to Fig. 3 has been deleted (page 10, line 10).

6 -See answer to the 1st question of Reviewer 1.

7 –OK, the order of figures has been corrected.

8 -The excitation/emission wavelengths have been added in Material and Methods (page 8, line 6).

9 -The figure 5 has been split (Figures 5 & 6) to show better overlap.

10 - As suggested, English has been examined by a native speaker.

NDP-α-MSH induces intense neurogenesis and cognitive recovery in Alzheimer transgenic mice through activation of melanocortin MC_4 receptors

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Keywords: Alzheimer's disease, Tg2576 mice, Melanocortin MC_4 receptors, Learning and memory, Neurogenesis, Functional integration

ABSTRACT

Melanocortins exert neuroprotection in a variety of experimental neurodegenerative disorders, including Alzheimer's disease (AD). Further, in previous research we showed that these endogenous peptides stimulate neurogenesis in an acute neurodegenerative disorder such as ischemic stroke. In the present research, we investigated the potential neurogenic effect of melanocortins in AD using APP_{Swe} transgenic mice (Tg2576). To this purpose, 24 week-old animals were prepared for 5-bromo-2'-deoxyuridine (BrdU) labeling of proliferating cells on days 1-11 of the study. Treatment of Tg2576 mice with nanomolar doses of the melanocortin analog [Nle^4,D-Phe^7]α-melanocyte-stimulating hormone (NDP-α-MSH), administered once daily from day 1 to 50, improved brain histology and cognitive functions relative to saline-treated Tg2576 animals. No signs of toxicity were observed. Immunohistochemical examination of the hippocampus at end of the study (day 50) showed that NDP-α-MSH-treated Tg2576 mice had a greater number of BrdU immunoreactive cells colocalized with NeuN (an indicator of mature neurons) and Zif268 (an indicator of functionally integrated neurons) in the dentate gyrus, relative to saline-treated Tg2576 animals; no newly formed astrocytes were found. Animal pretreatment with the selective melanocortin MC_4 receptor antagonist HS024 before each NDP-α-MSH administration prevented all the beneficial effects of the peptide. The present data indicate that MC_4 receptor stimulation by a melanocortin prevents cognitive decline in experimental AD not only through a neuroprotective mechanism but also by induction of an intense neurogenesis. MC_4 receptor agonists could be innovative and safe candidates to counteract AD progression in humans.

1. Introduction

Alzheimer's disease (AD), both sporadic and genetic, is a chronic disorder characterized by activation of the amyloid/tau cascade in certain brain regions, mainly the hippocampus and isocortex (Bayer and Wirths, 2014; Blennow, 2010; Iqbal and Grundke-Iqbal, 2010). β-Amyloid (Aβ) plaques and intraneuronal tau neurofibrillary tangles trigger pathophysiological pathways leading to synaptic dysfunction, progressive neurodegeneration, and marked neuronal loss. Consequences are progressive cognitive decline and behavioral abnormalities (Giuliani et al., 2014a; Ittner and Götz, 2011; Sperling et al., 2013; Tayeb et al., 2012). In addition to neuroprotective approaches for AD treatment, neurorestorative strategies are presently under investigations (Bayer and Wirths, 2014; Becker et al., 2007; Ben Menachem-Zidon et al., 2014; Franco and Cedazo-Minguez, 2014; Freiherr et al., 2013; Galimberti et al., 2013; Glat and Offen, 2013; Iqbal and Grundke-Iqbal, 2011; Lilja et al., 2013; Tayeb et al., 2012; Wang et al., 2010).

It is well known that neurogenic niches exist also in adult mammals including humans, and a variety of physiological and pathological stimuli can induce neurogenesis. Indeed, new neural stem/progenitor cells are generated mainly in two regions of the central nervous system (CNS), the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG). Other germinal zones occur in the forebrain parenchyma and in the peripheral nervous system (Arvidsson et al,2002; Benarroch, 2013; Giuliani et al., 2011; Lichtenwalner and Parent, 2006; Suh et al., 2009). Neural progenitors migrate and differentiate to mature and functionally integrated neurons and to other neural lineages including astrocytes. A compensatory increase of newborn neurons can occur in neurodegenerative conditions, although this happens in a limited manner depending on the age and disease severity. In particular, there is evidence that neurogenesis is impaired in AD (Becker et al., 2007; Benarroch, 2013; Ben Menachem-Zidon et al., 2014; Boekhoorn et al., 2006; Iqbal and Grundke-Iqbal, 2011; Jin et al., 2004; Lazarov et al., 2010; Lichtenwalner and Parent, 2006; Lilja et al., 2013; Marlatt and

Lucassen, 2010; Mu and Gage, 2011; Perry et al., 2012; Suh et al., 2009; Taupin, 2011;Wang et al., 2010).

The melanocortin system consists of endogenous neuropeptides of the adrenocorticotropin/melanocyte-stimulating hormone (ACTH/MSH) family, acting via five different metabotropic melanocortin receptor subtypes (MC_1-MC_5) (Brzoska et al., 2008; Caruso et al., 2014; Catania et al., 2004; Giuliani et al., 2012; Mountjoy, 2010; Wikberg and Mutulis, 2008). Natural melanocortins and their synthetic analogs contribute to protect the host from damage consequent to a variety of injuries, by targeting multiple pathophysiological mechanisms (Brzoska et al., 2008; Caruso et al., 2014; Catania et al., 2004; Corander et al., 2009; Giuliani et al., 2012; Guarini et al.,1996, 1997; Minutoli et al., 2011; Mioni et al., 2003; Wikberg and Mutulis, 2008). Melanocortins also induce neuroprotection associated with long-lasting functional recovery and prevention of cognitive decline in acute experimental neurodegenerative conditions (Bharne et al., 2011; Bitto et al., 2012; Chen et al., 2008; Gatti et al., 2012; Giuliani et al., 2006, 2007, 2009; Spaccapelo et al., 2011), including a chronic neurodegenerative disease such as AD, as we recently reported (Giuliani et al., 2014a, 2014b). The neuroprotective effects of melanocortins are mediated by CNS melanocortin MC_4 receptors and occur through inhibition of key pathophysiological mechanisms underlying the CNS pathology (Bitto et al., 2012; Giuliani et al., 2006, 2007, 2009, 2012, 2014a, 2014b; Spaccapelo et al., 2011).

We recently discovered that melanocortins, in addition to their neuroprotective action, strongly stimulate neurogenesis in gerbils affected by acute neurodegeneration caused by transient global cerebral ischemia; the data showed that the newborn cells developed properties of mature and functional neurons (Giuliani et al., 2011; Spaccapelo et al., 2013). The availability of drugs that combine both neuroprotective and neuroregenerative influences could be of great clinical relevance for innovative therapeutic approaches in acute and chronic neurodegenerative disorders. Therefore, in order to further assess the potential beneficial effects of melanocortins in AD, we investigated influences of a melanocortin analog on the neurogenic process in APP_{Swe} transgenic mice.

2. Material and methods

2.1 Animals

Twenty-four week-old (at the start of the study) male Tg2576 mice and their wild-type littermates (Taconic; Hudson, NY) were used. These mice, which harbor human transgene APP_{Swe}, overexpress the human APP695 isoform with the Swedish double mutations K670N/M671L (Hsiao et al.,1996; Lilja et al., 2013). Animals were kept in air-conditioned colony rooms (temperature 21 ± 1°C, humidity 60%) on a natural light/dark cycle, with food in pellets and tap water available ad libitum. General health conditions and body weight were recorded throughout the observation period, and rectal temperature was maintained close to 37°C by means of heating lamps; the latter procedure was adopted to avoid an hypothermia-mediated neuroprotection potentially induced by melanocortins (Giuliani et al., 2006, 2012) that could interfere with data interpretation. At the end of the study, animal sacrifice was performed under general anesthesia with i.p. injection of 50 mg/kg sodium pentobarbital (Sigma-Aldrich, St. Louis, MO). Housing conditions and experimental procedures were in strict accordance with the European Community regulations on the use and care of animals for scientific purposes (CEE Council 89/609; Italian D.L. 22-1-92 No. 116), and were approved by the Animal Ethics Committee of Modena and Reggio Emilia University.

2.2 BrdU labeling, drugs and treatment schedules

To label proliferating cells, 5-bromo-2'-deoxyuridine **(**BrdU; Sigma-Aldrich) was dissolved in 0.9% NaCl solution containing 0.007 N NaOH, preparing a 10 mg/ml stock solution. BrdU 50 mg/kg were injected i.p. twice daily on days 1-11 (Arvidsson et al,2002; Giuliani et al., 2011; Lilja et al., 2013; Wang et al., 2004). [Nle^4,D-Phe^7]α-melanocyte-stimulating hormone (NDP-α-MSH), synthetic melanocortin analog with long-lasting biological activity, agonist at melanocortin MC_1, MC_3, MC_4 and MC_5 receptors (Giuliani et al., 2006, 2007, 2009, 2011, 2014a, 2014b) (kindly provided by Prof. Paolo Grieco, University of Naples Federico II), was dissolved in saline (1

ml/kg) and administered i.p. (340 µg/kg, once daily for 50 days). In animals assigned to MC_4 receptor blockade, pretreatment with the selective melanocortin MC_4 receptor antagonist HS024 (Cys-Nle-Arg-His-D-Nal-Arg-Trp-Gly-Cys; cyclic MSH analog with S-S bridge between two Cys) (Giuliani et al., 2006a,b) was performed. HS024 (130 µg/kg, dissolved in saline 1 ml/kg; Tocris; Bristol, UK) (Giuliani et al., 2006, 2007, 2009, 2011, 2014a, 2014b), was injected i.p., 20 min before each administration of NDP-α-MSH. Control animals (Tg2576 and wild-type mice) received an equal volume of saline by the same route of administration. Further control animals were wild-type mice treated with NDP-α-MSH alone or HS024 alone. The doses of NDP-α-MSH and HS024 were chosen on the basis of our previous studies performed in experimental acute and chronic neurodegenerative conditions (Bitto et al., 2012; Giuliani et al., 2006, 2007, 2009, 2011, 2014a, 2014b). Experimental schedule and treatments are depicted in Figure 1.

2.3 Assessment of spatial learning and memory

The mouse ability to learn and recall was evaluated by means of the Morris water-maze test with minor modifications, as previously described in our papers (Bitto et al., 2012; Giuliani et al., 2006, 2007, 2009, 2011, 2013, 2014a, 2014b; Spaccapelo et al., 2011, 2013). Of note, mice used in the present study were 6-8 month old, that is, an age characterized by mild/moderate cognitive decline and AD (Hsiao et al.,1996; Lilja et al., 2013). Briefly, the apparatus consisted of a circular white pool (80 cm in diameter and 55 cm in height) filled to a depth of 15 cm with water (27 °C) rendered opaque with milk. Mice (10 per group) were trained to find the spatial location of a platform of clear perspex hidden by arranging for its top surface (7 cm in diameter) to be 1 cm below the water level. To this end, four cardinal points on the apparatus wall were defined by means of different geometrical figures, and conspicuous cues were placed in a fixed position around the pool. In each daily training, latency to escape onto the hidden platform was recorded. Each mouse received four daily trials starting each time from a different cardinal point in random sequence: in each trial, if the

animal failed to locate the platform within 60 s, escape latency was considered same as 60 s (therefore, the daily maximally possible total escape latency was 240 s). The study was carried out during the twenty-seventh week (starting 14 days after the first BrdU injection) and thirty-first week of age. During the twenty-seventh week of age (study performed mainly for an experience-dependent recruitment of newborn cells into spatial memory networks during the critical period of maturation) (Veyrac et al,2013), mice were subjected to a first 5-day training sequence followed by a second 1-day training 3 days later. Two other similar training sessions of the Morris test were repeated during the thirty-first week of age (last week of the study).

2.4 Histology

Following the last behavioral test (day 50 of the study; 31 week-old mice) and within 90 min after the last behavioral test (to detect Zif268 positivity, indicator of recently activated neurons) (Giuliani et al., 2011; Tashiro et al., 2007), transcardial perfusion of ice-cold 4% paraformaldehyde (phosphate-buffered) was performed and the brains were removed and processed for histologic examination and fluorescence immunohistochemistry.

Isocortex and hippocampus morphology was studied in 7-µm thick paraffin-embedded sections, hematoxylin-eosin stained, as previously described (Giuliani et al., 2006, 2007, 2013, 2014a, 2014b; Spaccapelo et al., 2011); Aβ plaques were detected in 7-µm thick paraffin-embedded sections after ethanol-Congo red/Weigert hematoxylin staining (Giuliani et al., 2013, 2014a, 2014b). Histological analyses were performed using an Axiophot photomicroscope (Carl Zeiss, Jena, Germany) under ordinary and polarized light.

To study neurogenesis, BrdU, NeuN, Zif268 and glial fibrillary acidic protein (GFAP) immunoreactivity was evaluated in hippocampus paraffin sections (7-µm thick), as previously described (Arvidsson et al., 2002; Becker et al., 2007; Giuliani et al., 2011; Lilja et al., 2013; Spaccapelo et al., 2013; Sun et al., 2003). Sections were immunohistochemically treated with the following primary antibodies: rabbit anti-BrdU (1:100, Bioss, Woburn, MA), mouse anti-Zif268

(1:200, Sigma-Aldrich, St. Louis, MO), rabbit anti-NeuN Alexa Fluor 488 conjugated (1:100, Millipore, Billerica, MA), mouse anti-GFAP Cy3 conjugated (1:500, Sigma-Aldrich). As secondary antibodies were used: sheep anti-rabbit Cy3 conjugated (BrdU, 1:100, Sigma-Aldrich), donkey anti-mouse AMCA-conjugated (Zif268, 1:100, Millipore). Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) was used, at the appropriate fluorocrome excitation/emission wavelengths (AMCA 405/450 nm; Alexa Fluor 488/519 nm; Cy3 543/570 nm), to detect fluorescent immunoreactions.

Studies were carried out in three (morphology and Aβ plaque detection) and five (neurogenesis study) serial sections per animal taken every 100 μm starting +2 mm from bregma zero coordinate. Histometry was performed by using an image analyzer and software (analySIS, Soft Imaging System GmbH, Münster, Germany). Morphology and cells positive to BrdU, NeuN and Zif268, were estimated in five randomly selected fields per slide. The density of cells was estimated in a 100 μm-thick band overlapping the pyramidal cell layer of the DG. Extracellular Aβ deposits were estimated on the whole slide.

2.5 Statistical analysis

All data were collected and analyzed by an observer blind to the treatment; they are shown as mean ± SEM. Statistical analysis was performed using either two-way repeated measures ANOVA (behavioral data) or one-way ANOVA (all other data), followed by the Student-Newman-Keuls' test. A value of $p < 0.05$ was considered significant.

3. Results

3.1 NDP-α-MSH improves learning and memory

To investigate the typical AD impairment of learning and memory (Galimberti et al., 2013; Tayeb et al., 2012), the ability of Tg2576 mice to find the spatial location of an hidden platform in

the Morris apparatus was performed in animals prepared for the neurogenesis study (that was executed on day 50). Saline-treated control Tg2576 mice showed impaired ability in platform finding both during the first and second training sessions (days 14-21 of the study; carried out mainly for an experience-dependent recruitment of newborn cells into spatial memory networks during the critical period of maturation; data not shown), as well as during the third and fourth sessions (days 43-50 of the study), relative to wild-type mice (Fig. 2A,B). Conversely, NDP-α-MSH-treated (for 50 days) Tg2576 mice displayed significantly better performance in learning and memory in all sessions of the Morris test, as compared with saline-treated control Tg2576 mice (Fig. 2A,B).

Based on our previous observations, we investigated the role of melanocortin MC_4 receptors mainly expressed in the CNS (Mountjoy, 2010), whose activation induced neuroprotection in acute brain injury and AD (Bitto et al., 2012; Giuliani et al., 2006, 2014a, 2014b) and promoted neurogenesis in stroke (Giuliani et al., 2011). Consistent with our previous findings, the favourable effects of NDP-α-MSH on learning and memory performance in Tg2576 mice was totally prevented by pretreatment with the selective MC_4 receptor antagonist HS024 (Fig. 2A,B).

Of note, neither NDP-α-MSH alone nor HS024 alone significantly affected learning and memory in wild-type mice (not shown). Furthermore, no signs of toxicity were recorded in wild-type and AD animals (ruffled fur, diarrhea, lethargy, aggressiveness, hypothermia, alterations of spontaneous locomotor exploration and grooming), and body weight variations throughout the study were similar in all experimental groups (not shown).

These data confirm our original findings and further suggest that a chronic treatment with melanocortin MC_4 receptor agonists represents a safe strategy to counteract cognitive decline in AD mice.

3.2 NDP-α-MSH preserves histological integrity of the isocortex and hippocampus

Generally, Aβ plaques and morphological alterations first appear in the frontal isocortex and extend thereafter to other isocortex regions and to hippocampus (Giuliani et al., 2014a, 2014b; Oddo et al., 2003). Therefore, to verify existence of brain damage in the mice over the course of AD development, histological examination was performed in the isocortex and hippocampus after the last session of the Morris test (day 50 of the study). In our experimental conditions, in saline-treated Tg2576 mice the histological picture was characterized by significant extracellular Aβ deposits and neurons showing pyknosis, swollen perikaryon and cellular shrinkage, with appreciable neuronal loss, mainly in the isocortex and, in lesser extent, in the hippocampus (Fig. 3), relative to wild-type animals. Microglia increase was also seen in the isocortex and rarely in the hippocampus. Treatment of Tg2576 mice with NDP-α-MSH likewise improved the general morphological picture of the isocortex and hippocampus, with a reduction in Aβ deposits and a greater number of viable neurons, in comparison with saline treatment (Fig. 3). As expected, the favourable effects of NDP-α-MSH on the isocortex and hippocampus were reversed by Tg2576 mouse pretreatment with the MC_4 receptor antagonist HS024 (Fig. 3).

These data provide evidence for a brain damage during AD progression in mice prepared for neurogenesis investigation, and confirm our previous findings that melanocortin MC_4 receptor activation reduces brain histological alterations (Giuliani et al., 2014a, 2014b).

3.3 NDP-α-MSH stimulates neural progenitor proliferation in the dentate gyrus

In subsequent experiments aimed at studying neurogenesis, which was the main aim in the present research , we investigated neural stem/progenitor cell proliferation in the hippocampus. As described in Methods, mice were previously treated with BrdU throughout days 1-11 of the study and sacrificed on day 50 (90 min after the last behavioral test); we chose this time-point as the newborn cells need several weeks to develop into mature and functional neurons (Arvidsson et al.,

2002; Becker et al., 2007; Giuliani et al., 2011; Veyrac et al., 2013). In our study, few BrdU-labeled, newly formed cells were detected on day 50 in the hippocampus DG of saline-treated Tg2576 mice and saline-treated wild-type animals (Fig. 4A). Conversely, NDP-α-MSH treatment of Tg2576 mice was associated with a very high number of BrdU-labeled cells, as compared with saline-treated animals (Fig. 4A and Fig. 6C). The neuroproliferative effects of NDP-α-MSH were prevented by pretreatment with the MC_4 receptor antagonist HS024 (Fig. 4A).

The present data demonstrate that melanocortin MC_4 receptor activation induces neural stem/progenitor cell proliferation in AD.

3.4 Newly generated cells develop properties of functionally integrated neurons

In order to characterize the phenotype of BrdU positive cells, immunoreactivity for the mature neuronal marker NeuN and the astrocyte marker GFAP was evaluated by double-labeling. At confocal microscopy examination, a great number of double-labeled BrdU-NeuN cells, but no double-labeled BrdU-GFAP cells (that is, newly formed astrocytes) was found within the DG of NDP-α-MSH-treated Tg2576 mice (Fig. 4B,C, Fig. 5, Fig. 6).

Colocalization of BrdU incorporation with the neuronal marker NeuN and the early functional gene Zif268 was then examined in the same sections. Zif268 (also known as Egr-1, Krox-24, etc.) is primarily expressed after synaptic activation and is used as an indicator of recently activated neurons (Becker et al., 2007; Giuliani et al., 2011; Tashiro et al., 2007). Impressively, in the DG of NDP-α-MSH-treated Tg2576 mice we counted a number of triple-labeled BrdU-NeuN-Zif268 cells very high and strictly close to that of double-labeled BrdU-NeuN cells, that is, almost all BrdU-NeuN immunoreactive cells colocalized with Zif268 (Fig. 4D, Fig. 6C).

Consistent with a primary role of MC_4 receptors in the neurogenic process, pretreatment of Tg2576 mice with the MC_4 receptors antagonist HS024 prevented the favourable effects of NDP-α-MSH on double (BrdU-NeuN)- and triple (BrdU-NeuN-Zif268)-labeled cell density (Fig. 4C,D).

These data indicate that treatment of AD mice with melanocortin MC_4 receptor agonists shifts newborn cells toward the neuronal phenotype and provides a favourable microenvironment for functional integration.

4. Discussion

An estimated 35.6 million people worldwide were affected by AD in 2012, and this number may triple by 2050. Therefore the aging of human population worldwide and the consequent increase in AD incidence represent a real social and economic alarm (Freiherr et al., 2013; Gustavsson et al., 2011; Ittner and Götz, 2011; Sperling et al., 2013). Indeed, AD remains a major cause of disability and mortality without effective treatment, as current approved therapies (drugs that activate anti-glutamatergic and pro-cholinergic mechanisms) only induce modest and transient improvement of the main symptoms (Galimberti et al., 2013; Tayeb et al., 2012). Novel neuroprotective and neurorestorative strategies for AD, by means of pharmacological therapy, immunotherapy against Aβ, and cell and gene therapy, are under investigation in animals and humans (Bayer and Wirths, 2014; Becker et al., 2007; Ben Menachem-Zidon et al., 2014; Freiherr et al., 2013; Galimberti et al., 2013; Giuliani et al.,.2013, 2014a, 2014b; Glat and Offen, 2013; Iqbal and Grundke-Iqbal, 2011; Lilja et al., 2013; Tayeb et al., 2012; Wang et al., 2010). However, disappointing results are arising from these novel studies (Salloway et al., 2014), and, therefore, effective approaches to slow down AD progression are still unavailable in clinical practice.

Here we report that a 50-day treatment with the melanocortin peptide NDP-α-MSH counteracts learning and memory decline in Tg2576 mice with mild/moderate AD. The NDP-α-MSH-induced improvement in cognitive performance was associated with an improved histological picture within the isocortex and hippocampus, with decreased Aβ deposits, and a very high number of BrdU immunoreactive newly generated cells in the hippocampus DG; few BrdU-labeled cells were detected in saline-treated Tg2576 mice, and in saline-treated wild-type animals (whose good cognitive performance likely depends on an unaltered brain histological picture). The present data

also show that, in NDP-α-MSH-treated Tg2576 mice, almost all BrdU positive cells on day 50 were also NeuN immunoreactive and expressed Zif268, an indicator of functionally integrated neurons. Therefore, melanocortin treatment appear to shift cells toward a neuronal phenotype and to promote functional integration. All these beneficial events occurred at nanomolar doses and seemingly with an involvement of central MC_4 receptors, as NDP-α-MSH failed to protect Tg2576 mice pretreated with the selective MC_4 receptor antagonist HS024.

In our previous studies that investigated neuroprotective effects of melanocortins in transgenic mouse models of mild/moderate AD, $APP_{Swe}/PS1_{M146V}/tau_{P301L}$ mice and Tg2576 mice (Giuliani et al., 2014a, 2014b), we found that melanocortin MC_4 receptor stimulation inhibits the amyloid/tau cascade, oxidative and nitrosative stress, inflammatory and apoptotic responses, with consequent protection against brain morphological alterations and cognitive decline. Our previous results indicate that MC_4 receptor stimulation leads to improved synaptic transmission and plasticity, as strongly suggested by hippocampus overexpression of Zif268, a transcription factor that controls major processes of synaptic activity (Giuliani et al., 2014a, 2014b). Consistently, a full and elegant demonstration that melanocortin MC_4 receptors regulate hippocampal synaptic plasticity has been recently provided by Shen and coworkers (2013).

Our present data demonstrate for the first time that melanocortins, acting at MC_4 receptors, also induce neurogenesis in a transgenic mouse model of AD. This finding strengthens the potential therapeutic value of these endogenous agents. In the present study we investigated the hippocampus neurogenesis, and the present results are consistent with a melanocortin-induced amplification of the neurogenic process in the SGZ; obviously, other established germinal zones may be targeted by melanocortins as well.

Very recently we found that melanocortin treatment of gerbils subjected to experimental stroke strongly stimulates brain generation of new cells, which develop properties of mature and seemingly functionally integrated neurons, with a high proportion of long-term survival (Giuliani et al., 2011; Spaccapelo et al., 2013). Our biomolecular studies on the neurogenic process in stroke

gerbils showed that treatment with the melanocortin NDP-α-MSH induces neural stem/progenitor cell proliferation in the DG. This action was associated with activation of MC_4 receptors and triggering of the canonical Wnt-3A/β-catenin and Sonic hedgehog signaling pathways (Giuliani et al., 2011; Spaccapelo et al., 2013), which play a key role in maintenance and proliferation of stem/progenitor cells and in neuronal fate determination (Benarroch, 2013; Suh et al., 2009). Further, activation of these pathways was associated with early up-regulation of the repair factor Zif268 and the neurogenesis facilitating factor interleukin-10 (IL-10) in the DG (Spaccapelo et al., 2013). It is reasonable to hypothesize that the same molecular mechanisms underlay the neurogenic effects of melanocortins found in AD animals in the present study. In particular, the known ability of melanocortins to induce Zif268 overexpression (Giuliani et al., 2009, 2011, 2014a, 2014b) could have contributed to brain injury repair and improvement of cognitive processes. Indeed, Zif268 is rapidly induced as transcription factor by a variety of physiological and pathological stimuli (such as ischemia, neurodegeneration, learning and memory, etc.), the natural induction of adequate expression level being limited by various causes including the disease severity. The essential role of Zif268 in synaptic plasticity, as well as in long-term survival, maturation and functional integration of newborn neurons, is well established (Giuliani et al., 2009; Tashiro et al., 2007; Veyrac et al., 2013). To allow the Zif268-mediated activity-dependent long-term survival and functional integration of newborn cells, in the present research we subjected mice to two preparatory spatial learning and memory sessions starting 14 days after the first BrdU injection, that is, during the critical period of selection and maturation of a number of new neurons (Veyrac et al.,2013).

Impairment of neurogenesis in AD has been attributed to brain overexpression of the pro-inflammatory cytokine IL-1β signaling. Indeed this cytokine appears to be involved in the generation of AD hallmark lesions (such as increased expression of amyloid precursor protein, tau hyperphosphorylation, over-activity of acetylcholinesterase, etc.). Further, IL-1β may cause an unfavourable microenvironment by reducing expression of neurogenetic factors such as brain-

derived neurotrophic factor (BDNF) (Ben Menachem-Zidon et al., 2014). Interestingly, we previously showed that melanocortin MC_4 receptor agonists inhibit IL-1β expression in the isocortex of AD mice (Giuliani et al., 2014a), and MC_4 receptor activation has been reported to increase brain expression of BDNF (Caruso et al., 2012, 2014). Thus, also these mechanisms may have contributed to the neurogenic effect of melanocortins in AD mice reported in the present research.

Melanocortin MC_4 receptors are mainly expressed in various brain areas including the cortex and hippocampus (Caruso et al., 2014; Catania et al., 2004; Giuliani et al., 2012; Mountjoy, 2010; Wikberg and Mutulis, 2008), and are thought to play a physiological protective role against different types of brain injury. Specifically, experimental evidence suggests that the MC_4 receptor-mediated signal transduction of melanocortins activates pathways that promote the expression of several salutary transcription factors/signaling molecules, including those involved in synaptic plasticity, neuroprotection, neural progenitor cell proliferation and differentiation (Caruso et al., 2014; Giuliani et al., 2012, 2014a, 2014b; Holloway et al., 2011). The possibility of a physiological protective role of melanocortin MC_4 receptor agonists also against AD progression is supported by the following observations: (i) α-MSH produces established neurotrophic effects on central cholinergic neurons, whose loss is a feature of AD (Anderson, 1986); (ii) low ACTH/α-MSH levels were detected in the cerebrospinal fluid/brain of patients with AD-type dementia (Arai et al.,1986; Facchinetti et al.,1984; Rainero et al.,1988). It is presently unknown whether number/activity of brain MC receptors is changed in AD.

It is worth noting that we previously found that melanocortins acting at MC_4 receptors are able to counteract AD progression by inhibiting several AD-related pathophysiological mechanisms up- and down-stream Aβ and tau (Giuliani et al., 2014a, 2014b). The present data also show that this class of endogenous agents induces neurogenesis; further, neuroprotection could ameliorate neurogenesis by providing a favorable microenvironment, also advantageous for survival of newly generated cells. Finally, the potential therapeutic value of melanocortins for AD treatment is

strengthened by the lack of signs of toxicity throughout the treatment period, both in the present (7 weeks) and previous (up to 18 weeks; Giuliani et al., 2014a, 2014b) research. Accordingly, melanocortins have been repeatedly reported to be devoid of appreciable toxicity also in long-term treatments, both in animals and humans, and some melanocortin compounds have already been successfully tested in clinical conditions different from neurodegeneration, with evidence of safety in humans (reviewed by: Brzoska et al., 2008; Catania et al., 2004; Corander et al., 2009; Giuliani et al., 2012; Wikberg and Mutulis, 2008).

In conclusion, the present study demonstrates that melanocortins induce intense neurogenesis via activation of MC_4 receptors in AD transgenic mice when treatment is started at a mild/moderate level of disease severity. The neurogenic effect, together with the neuroprotective action previously demonstrated, represent a robust framework of the potential therapeutic value of melanocortins against AD including cognitive impairment: however, specific correlation and/or link between cognitive performance and neurogenesis should be studied. After further investigations in AD animals ─ also at older ages with progressive severity of AD ─ favourable results would suggest that melanocortins could be an innovative neuroprotective and neuroregenerative strategy to reduce AD progression, as well as to improve aging-impaired neurogenesis in humans.

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Disclosure statement

Dr. Giuliani and Prof. Guarini are inventors on a patent (owner University of Modena and Reggio Emilia) related to the topic of the present paper. The other authors have no disclosure to declare.

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FIGURE CAPTIONS

Fig. 1. Experimental schedule and treatments.

Fig. 2. NDP-α-MSH improves learning and memory in Tg2576 mice. Histograms indicate mean values ± SEM of latency to escape onto the hidden platform (Morris water-maze test; n = 10). Learning/memory session (A) started at the beginning of the 31th week of age (days 43-47 of the study); memory session (B) took place 3 days after the end of the learning session (day 50 of the study). The effects of NDP-α-MSH (340 μg/kg i.p., once daily on days 1-50 of the study) were prevented by pretreatment with the MC_4 receptor antagonist HS024 (130 μg/kg i.p., before each administration of NDP-α-MSH). WT = wild-type mice; Tg = Tg2576 mice; S = saline; NDP = NDP-α-MSH; HS = HS024. *$p < 0.001$ versus the corresponding value of Tg2576 mice treated with saline; $^{\#}p < 0.001$ versus the corresponding value of Tg2576 mice treated with NDP-α-MSH alone.

Fig. 3. NDP-α-MSH reduces isocortex and hippocampus damage in Tg2576 mice. Representative histological pictures on day 50. Histograms (A, B) indicate mean values ± SEM (n = 10) obtained 90 min after the last behavioral test. Notice, in the saline-treated mouse, the greater amount of Aβ deposit under ordinary (C, brown plaques) and polarized (D, birefringent image) light, and of (E) neurons showing nucleus degeneration, pyknosis, cellular shrinkage and pericellular vacuolization, in comparison with NDP-α-MSH-treated one (NDP: 340 μg/kg i.p., once daily on days 1-50 of the study). The effects of NDP-α-MSH were prevented by pretreatment with the MC_4 receptor antagonist HS024 (130 μg/kg i.p., before each administration of NDP-α-MSH). (C, D): ethanol-Congo red/Weigert hematoxylin staining; (E): Hematoxylin-Eosin staining. Tg = Tg2576 mouse. Scale bar = 50 μm.

Fig. 4. NDP-α-MSH induces generation of new cells which develop properties of mature and functional neurons in the dentate gyrus of Tg2576 mice. Histograms indicate mean values ± SEM (*n* = 10) obtained 90 min after the last behavioral test (day 50 after start of BrdU labeling). Total number of (A) BrdU immunoreactive cells, (B) BrdU-GFAP double-labeled cells; (C) BrdU-NeuN double-labeled cells and (D) BrdU-NeuN-Zif268 triple-labeled cells. The effect of NDP-α-MSH (340 μg/kg i.p., once daily on days 1-50 of the study) was prevented by mouse pretreatment with the MC_4 receptor antagonist HS024 (130 μg/kg i.p., before each administration of NDP-α-MSH). WT = wild-type mice; Tg = Tg2576 mice; S = saline; NDP = NDP-α-MSH; HS = HS024. ***p* < 0.001 versus the corresponding value of Tg2576 mice treated with saline; $^{\#}p < 0.001$ versus the corresponding value of Tg2576 mice treated with NDP-α-MSH alone.

Fig. 5. Representative confocal images of the experiments of Fig. 4. NDP-α-MSH-treated Tg2576 mouse. (A) dentate gyrus after anti-NeuN immunoreaction. The white boxed area corresponds to the higher magnification (B, C and D) images, and the red boxed area corresponds to the higher magnification images reported in Fig. 6. The (D) image highlights that there are no NeuN-GFAP double-labeled cells, but only astrocyte processes crossing the pyramidalis stratum. Scale bars: (A) = 500 μm, (B-D) = 50 μm.

Fig. 6. Representative confocal images of the experiments of Fig. 4. NDP-α-MSH-treated Tg2576 mouse. High magnification images of the red boxed area of Fig. 5A. Notice in this field that all BrdU labeled cells (C) are colocalized with NeuN and Zif268 (F): in (F) panel the white cells correspond to BrdU/NeuN/Zif triple-stained cells (about 12%, active neurons born on days 1-11 of the study), the cyan cells correspond to NeuN/Zif double-stained cells (about 60%, active neurons born before and after days 1-11 of the study), and the green cells correspond to NeuN-stained cells (about 28%, no active neurons born before and after days 1-11 of the study). Scale bar = 50 μm.

Fig 1

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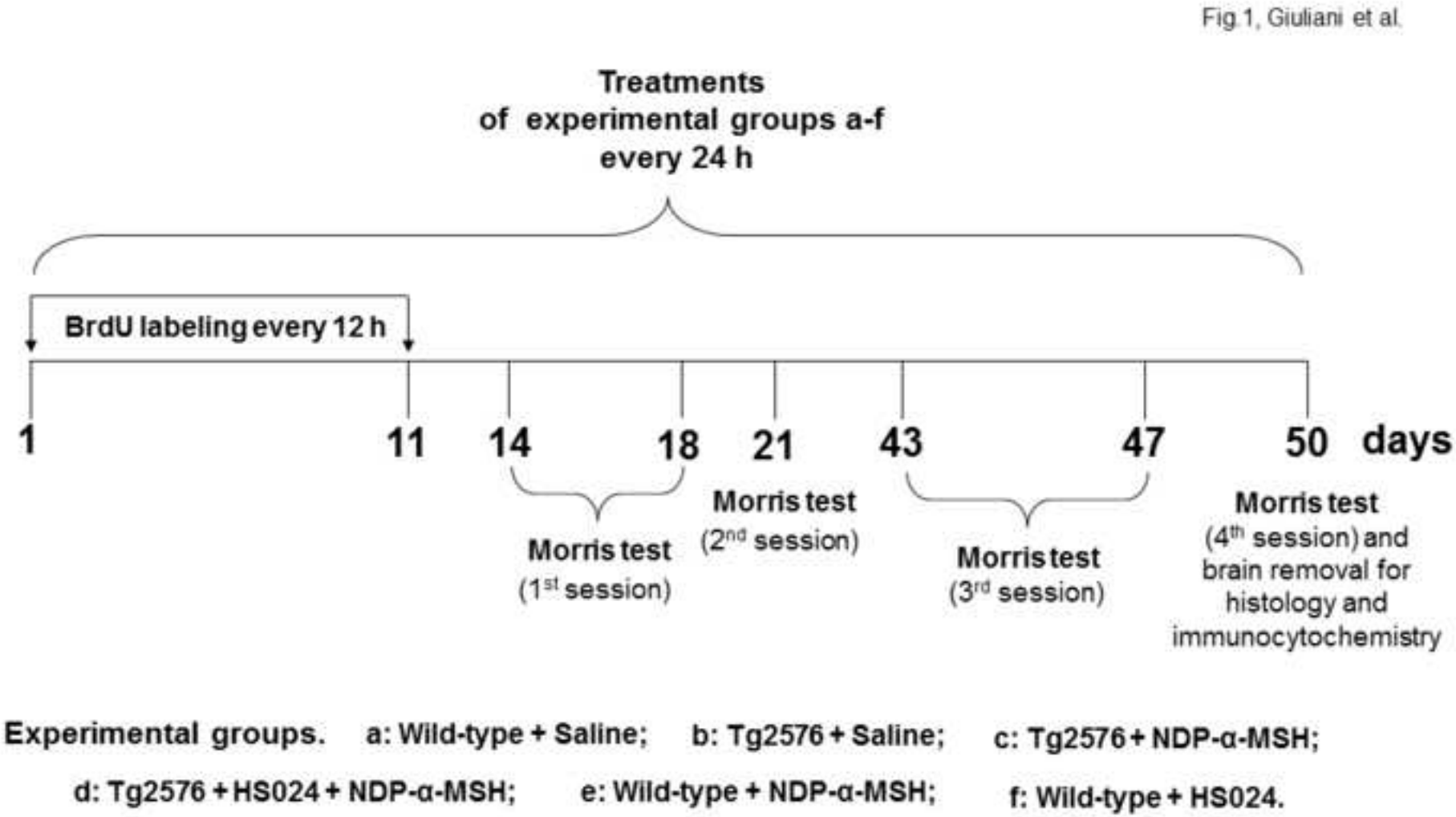

Experimental groups. a: Wild-type + Saline; b: Tg2576 + Saline; c: Tg2576 + NDP-α-MSH; d: Tg2576 + HS024 + NDP-α-MSH; e: Wild-type + NDP-α-MSH; f: Wild-type + HS024.

Fig 2

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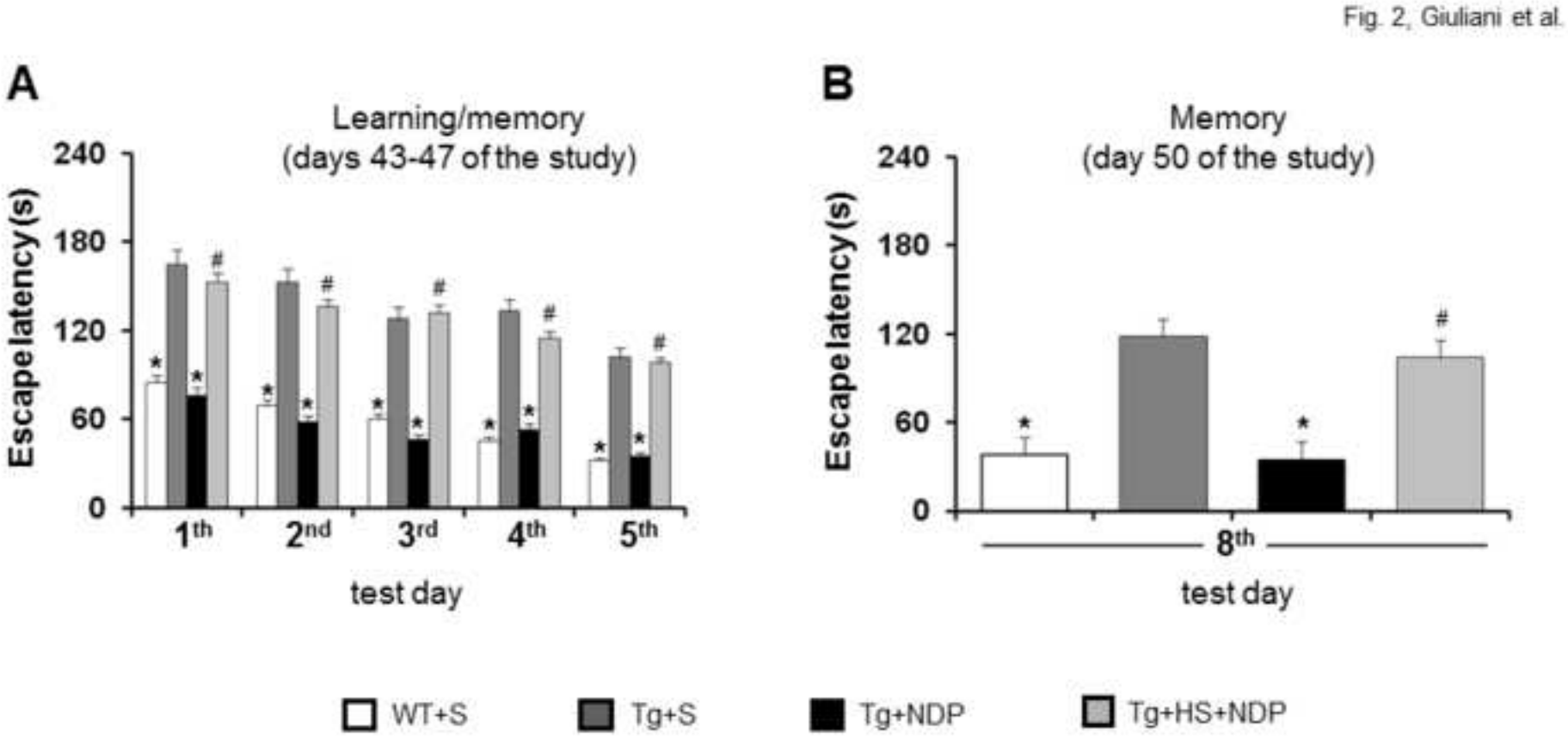

Fig 3
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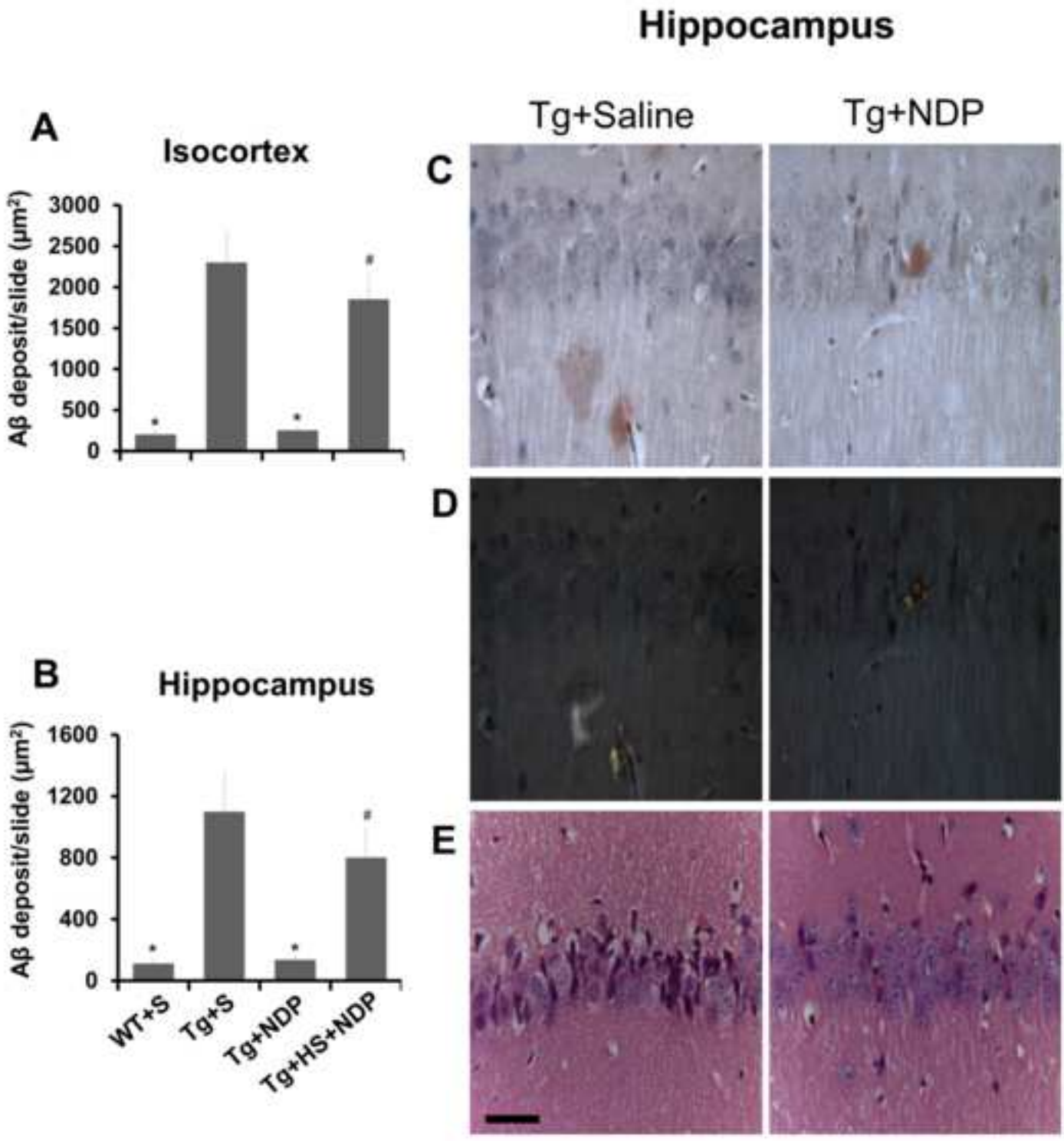

Fig 4

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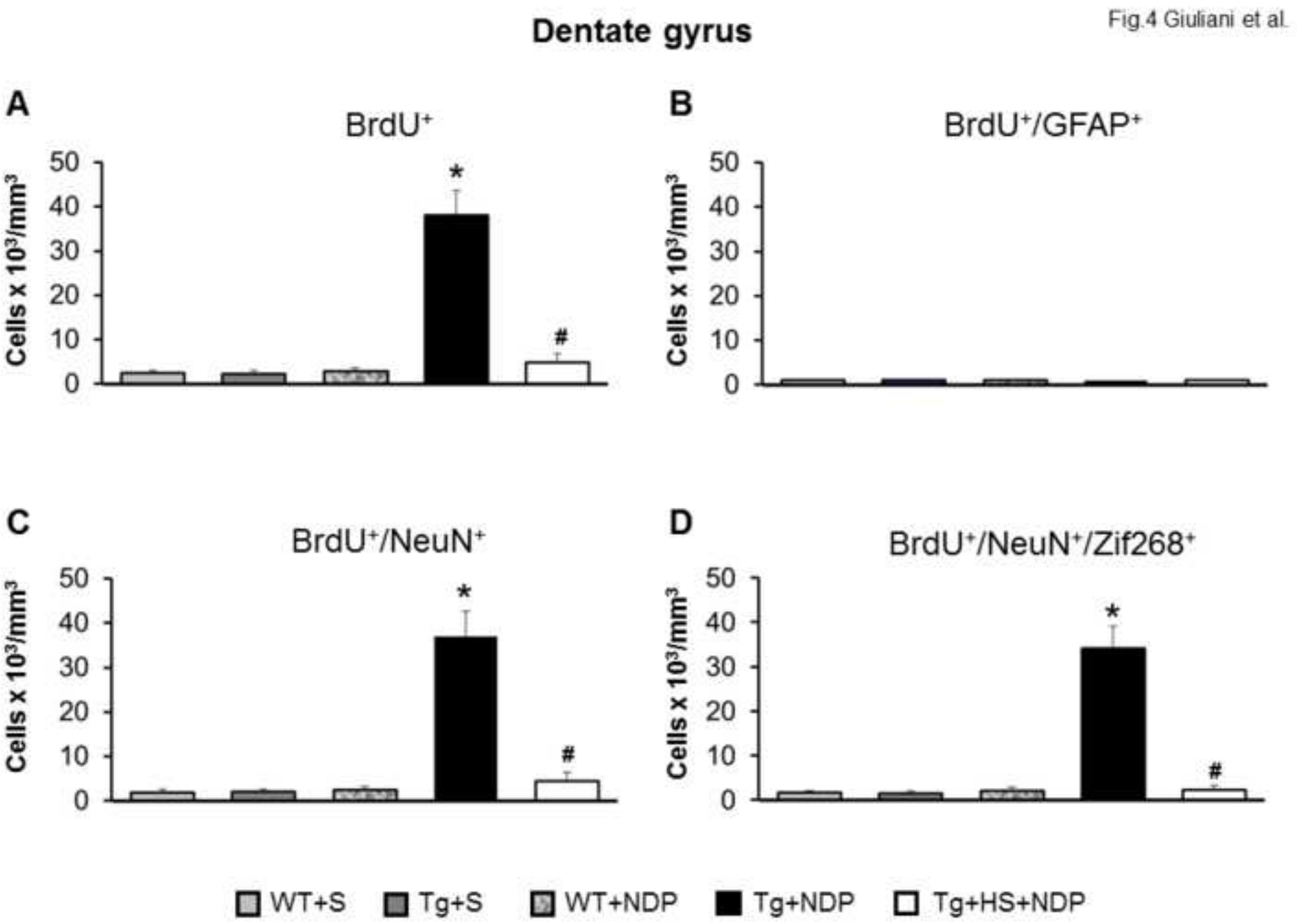

Fig 5
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Figure 5

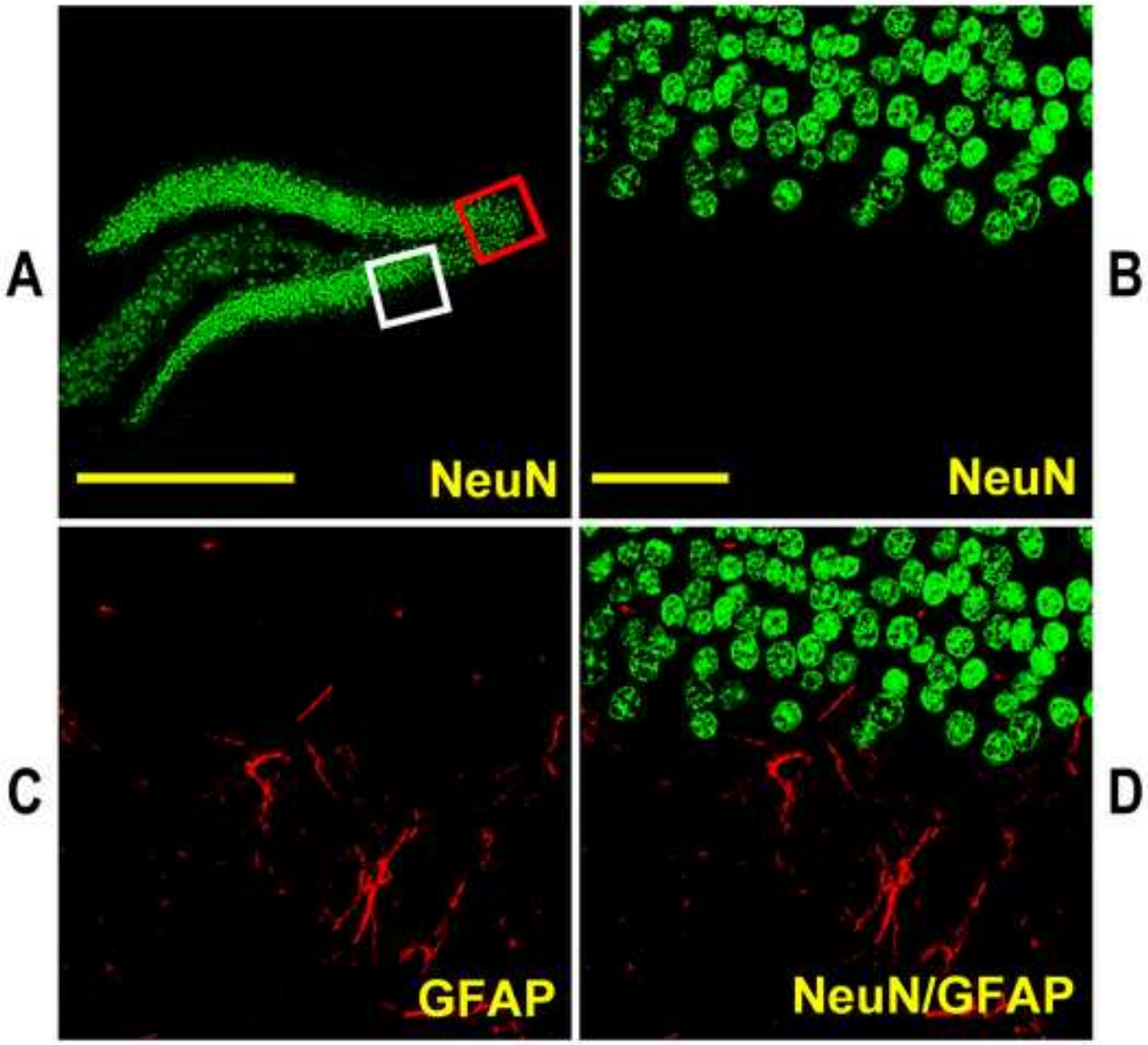

Fig 6

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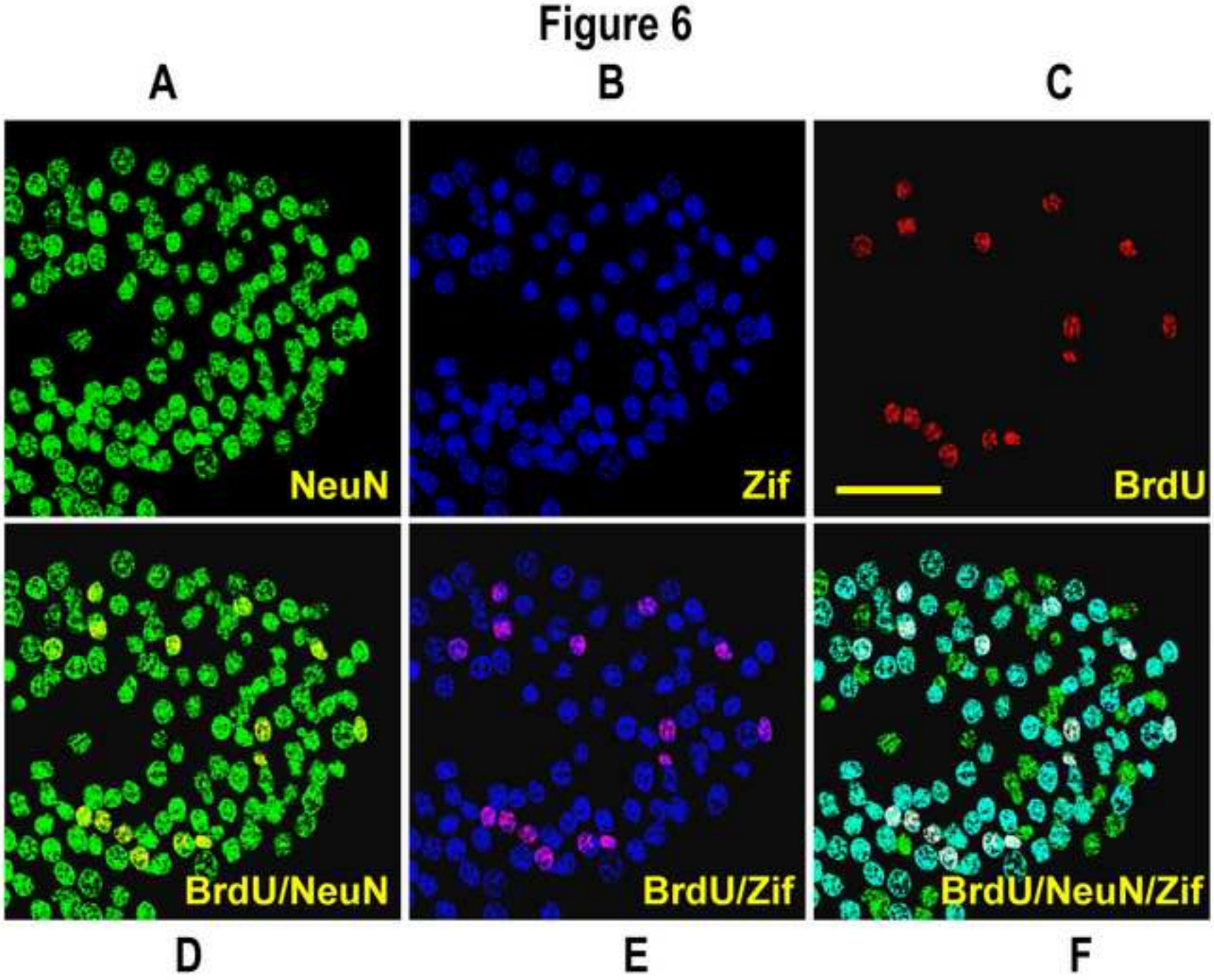

Highlights

1) Melanocortins induce neurogenesis in Alzheimer transgenic mice
2) Newborn neurons functionally integrate
3) Melanocortins counteract cognitive decline
4) These effects are prevented by melanocortin MC4 receptor blockade