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Anti-inflammatory and cognitive effects of interferon- β 1a (IFN β 1a) in a rat model of Alzheimer's disease

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Abstract

Background: A β ₁₋₄₂ peptide abnormal production is associated with the development and maintenance of neuroinflammation and oxidative stress in brains from Alzheimer disease (AD) patients. Suppression of neuroinflammation may then represent a suitable therapeutic target in AD. We evaluated the efficacy of IFN β 1a in attenuating cognitive impairment and inflammation in an animal model of AD.

Methods: A rat model of AD was obtained by intra-hippocampal injection of A β ₁₋₄₂ peptide (23 μ g/2 μ l). After 6 days, 3.6 μ g of IFN β 1a was given subcutaneously (s.c.) for 12 days. Using the novel object recognition (NOR) test, we evaluated changes in cognitive function. Measurement of pro-inflammatory or anti-inflammatory cytokines, reactive oxygen species (ROS), and SOD activity levels was performed in the hippocampus. Data were evaluated by one-way ANOVA with Fisher's Protected Least Significant Difference (PLSD) test.

Results: We showed that treatment with IFN β 1a was able to reverse memory impairment and to counteract microglia activation and upregulation of pro-inflammatory cytokines (IL-6, IL-1 β) in the hippocampus of A β ₁₋₄₂-injected rats. The anti-inflammatory cytokine IL-10, significantly reduced in the A β ₁₋₄₂ animals, recovered to control levels following IFN β 1a treatment. IFN β 1a also reduced ROS and lipids peroxidation and increased SOD1 protein levels in the hippocampus of A β ₁₋₄₂-injected rats.

Conclusion: This study shows that IFN β 1a is able to reverse the inflammatory and cognitive effects of intra-hippocampal A β ₁₋₄₂ in the rat. Given the role played by inflammation in AD pathogenesis and the established efficacy of IFN β 1a in the treatment of inflammatory diseases of the central nervous system such as multiple sclerosis, its use may be a viable strategy to inhibit the pro-inflammatory cytokine and oxidative stress cascade associated with A β deposition in the hippocampus of AD patients.

Keywords: IFN β 1a, Neuroinflammation, ROS, Pro-inflammatory cytokines, SOD, A β ₁₋₄₂, IL-10, NF- κ B, Hippocampus

Background

Alzheimer disease (AD), an age-dependent neurodegenerative disorder and the most common cause of dementia, is a multifactorial disease with a complex interplay of genetics and environmental factors [1, 2]. The

pathological hallmarks of AD include the deposition of extracellular neuritic plaques of β -amyloid (A β) peptide and the formation of intracellular neurofibrillary tangles in the brain [3]. A β peptides are neurotoxic and may cause neurodegenerative changes, including apoptosis, oxidative stress, and neuroinflammation. Neuroinflammation has been widely recognized as a possible pathological contributor to AD, usually including activation of glial cells, such as microglia and astrocytes [4], and release of cytotoxic compounds, e.g., cytokines and ROS, able to cause neuronal damage and death [3, 5]. Since

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suppression of neuroinflammation could represent an interesting therapeutic target for AD, several approaches have been tested to smolder inflammatory processes by using anti-inflammatory drugs [5–8] or other drugs with anti-inflammatory effects in AD models [9]. However, although all anti-inflammatory strategies tried so far in AD patients have not achieved satisfactory results, indicating the need for a better understanding of the role of the immune system in cerebral proteinopathies and how to modulate it [10], intervention with drugs modulating pro-inflammatory cytokine production is still considered a potentially useful strategy to slow down the disease course of this dreadful disease.

Interferons (IFNs) are a super-family of cytokine proteins that play an important role in the host immune response to infections and immune-mediated diseases [11]. Interferon- β 1a (IFN β 1a) is the prototypical regulatory cytokine with anti-inflammatory properties largely used in the past two decades to slow down pathological and clinical features of central nervous system (CNS) immune-mediated diseases, such as multiple sclerosis [12–17] and its animal model experimental autoimmune encephalomyelitis [18, 19]. IFN β -1a displays several cellular and humoral immune effects, including inhibition of pro-inflammatory cytokine (IL-6, IL-1 β , TNF- α , IFN- γ) and downregulation of glial cells and oxidative stress [20]. Interestingly, IFN β 1a significantly prevented cognitive decline in a large cohort of patients with multiple sclerosis, thus suggesting that modulation of neuroinflammatory pathways may prevent cognitive decline in humans [21, 22], as well as cortical atrophy associated with cognitive impairment in patients with multiple sclerosis [23]. We also preliminarily evaluated the safety and efficacy of IFN β 1a in subjects affected by mild-to-moderate AD in a phase 2a study mainly aimed to evaluate the safety of the drug in this elderly population [24], and although not statistically significant, we observed a reduction in disease progression during follow-up as measured by the AD Physical Self-Maintenance Scale.

Taken together, these human studies support the hypothesis that IFN β 1a could attenuate the inflammatory response in AD and led us to look for an experimental confirmation by assessing the efficacy of IFN β 1a in attenuating inflammation in an animal model of AD obtained by intra-hippocampal injection of A β ₁₋₄₂ peptide. Intra-hippocampal injection of A β ₁₋₄₂ peptide is considered a suitable animal model of AD [25] with several pathological and behavioral features of AD patients, including cognitive impairment and inflammatory reactivity [26]. Therefore, we first evaluated in rats the cognitive impairment induced by intra-hippocampal injection of A β ₁₋₄₂ peptide and the therapeutic effects of IFN β 1a treatment. The anti-inflammatory effects of IFN β 1a were then assessed by examining in post-mortem rat brains several

inflammation markers, such as pro- and anti-inflammatory cytokines, and oxidative stress responses.

Methods

Animals

Adult female Wistar rats (3 months old) were used. Rats were housed in a specific pathogen-free environment, three per polypropylene cages in controlled temperature ($23 \pm 2^\circ\text{C}$), humidity (50–55%), and light (12-h light/dark cycle), with access to food and water ad libitum. Procedures involving animal were carried out in accordance with the Italian institutional guidelines (D. LGS. no. 26, GU n.61, March 2014). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. No other methods to perform the described experiments (3Rs) were found.

Experimental design

For this study, we used the following experimental groups: (1) a control group treated with saline only (control); (2) a sham-operated control group and treated with saline (sham); (3) an A β ₁₋₄₂ peptide-treated group; (4) an interferon- β 1a (IFN β 1a)-treated group; and (5) an A β ₁₋₄₂ peptide + IFN β 1a-treated group. Rats received two bilateral intra-hippocampal injections of 23 $\mu\text{g}/2\ \mu\text{l}$ of A β ₁₋₄₂ peptide dissolved in 0.9% physiological saline solution. IFN β 1a was given subcutaneously (s.c.) at a dose of 3.6 μg (1 M Units, Rebif, Merk Serono, London) in a volume of 0.1 ml of 0.9% of saline solution. We used female rats since in AD disproportionately the female/male ratio is 2:1, although the biological basis of these sex-based differences in AD onset and progression remains elusive [27, 28].

A β ₁₋₄₂ peptide preparation and toxicity

A β ₁₋₄₂ protein was produced according to Carrotta et al. [29]. For the kinetics of aggregation, the sample of A β ₁₋₄₂ protein was loaded in a 96 black multi-well and added with 8 μM of thioflavin-T. The multi-well was read to the plate reader every 30 s at 450–485 nm wavelength for 8 h at 37 $^\circ\text{C}$. After the 8 h of incubation, the formation of A β ₁₋₄₂ protein aggregates was also evaluated at the fluorescence microscope (Leica Microsystems, Heidelberg, Germany). In addition, the mean size of the A β ₁₋₄₂ plates was measured by fluorescence microscopy software (Leica QFluoro V1.1 software, Heidelberg, Germany).

LAN5 cells were cultured with RPMI 1640 medium (Celbio srl, Milan, Italy) supplemented with 10% fetal bovine serum (Gibco-Invitrogen, Milan, Italy) and 1% antibiotics (50 mg mL⁻¹ penicillin and 50 mg mL⁻¹ streptomycin). Cells were maintained in a humidified 5% CO₂ atmosphere at $37 \pm 0.1^\circ\text{C}$. For dose-effect studies of A β ₁₋₄₂ toxicity, cells were treated with 50, 75, and 100 μM of A β ₁₋₄₂ for 24 h, and thereafter, their viability was evaluated by MTS assay ([3-(4,5-dimethylthiazol-

2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2-H-tetrazolium]; Promega Italia, S.r.l., Milan, Italy) and morphological analyses. After 24 h of cell treatment with A β_{1-42} , 20 μ l of the MTS solution was added to each well for 3 h at 37 °C, 5% CO₂. The absorbance was read at 490 nm on the Microplate reader (WallacVictor 2 1420 Multilabel Counter) (PerkinElmer, Inc. Monza, Italy).

A β_{1-42} intra-hippocampal injection

The A β_{1-42} peptide intra-hippocampal injection was performed as described by Muddò et al. [30]. Shortly, rats were anesthetized with mixture of 1:1 of Zolazepam + tiletamine 15 mg/kg b.w. (Zoletil, Virbac) and xilazine 9 mg/kg b.w. (Nerfasin, ATI, Italy), placed in a David Kopf stereotaxic apparatus, and received two bilateral intra-hippocampal injections of 23 μ g/2 μ l of A β_{1-42} peptide, using the following stereotaxic coordinates from the Bregma, according to Paxinos and Watson (1998): first injection AP = - 3.6, L = 2, and V = 4.5; second injection AP = - 4.2, L = 2.4, and V = 4.5. The sham group was intra-hippocampal-injected with 2 μ l of 0.9% physiological saline. Injections were performed by 30-gauge injector cannula that was connected by a piece of polyethylene tube to the 10 μ l Hamilton syringe. Each injection was performed over 3 min, and following injection, the needle remained in the target location for 3 min to avoid A β_{1-42} peptide reflux along the needle tract and to achieve a proper diffusion of the drug. After surgery, each rat was treated with penicillin (100,000 U/i.m.) to prevent infection.

Behavioral testing

Using the novel object recognition (NOR) test, we evaluated changes in cognitive function of the experimental animals. Rats were tested in an open field Plexiglas square box, in a mean light intensity (100 lx) illuminated chamber. All experimental groups were subjected to a 5-min training session when they were presented two identical non-toxic objects (i.e., two metal cans) placed against a wall in the open field arena. To prevent coercion to explore the objects, rats were released against the opposite wall with the back to the objects. The time spent on exploring each object was recorded using ANY MAZE Video Tracking System (Ugo Basile, Italy); a 2-cm² area surrounding the objects was defined such that nose entries were recorded as time exploring the objects. After the training session, animals were placed in their home cage for a 24-h retention interval. Then, they were returned to the arena containing two objects: one was identical to the familiar one but previously unused (to prevent olfactory cues and the necessity to wash objects during experimentation) and the other was a novel object (metal, glass, or hard plastic items). Time spent on exploring each object was recorded along

5-min session. Objects were randomized and counterbalanced across animals. The objects and arena were thoroughly cleaned at the end of each experimental session. The recognition index (RI), which is the time spent on investigating the novel object, divided by the total amount of exploration time of the novel (TN) and familiar objects (TF), [RI = TN/(TN + TF)], is a measure of novel object recognition and the main index of retention. If RI percentage is higher than 50%, it indicates more time spent on inquiring into the novel object, whereas less than 50% indicates that time was prevalently spent on exploring the familiar object, and 50% indicates a null preference.

Immunohistochemistry

The rats under deep anesthesia were perfused through the aorta with 0.9% saline and brain dissected. The left side of the brain was used for histological investigations and the right side for molecular analysis. Immuno-histochemical investigation was performed as described by Di Liberto et al. [31]. The left side of brain was fixed with 4% of paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 days and then immersed in the sucrose 10% solution for 1 day and in sucrose 20% for 2 days. Subsequently, brains were frozen in cooled isopentane and 20- μ m-thick coronal sections at hippocampal level were prepared and processed for immunohistochemistry as free-floating sections. Sections were washed for 5 min in 0.1 M PBS and incubated for 15 min with BSA (5 mg/ml) and Triton X-100 (0.3%) in PBS 0.1 M. Mouse monoclonal antibody anti-glial fibrillary acidic protein (anti-GFAP; diluted 1:400; MAB360 Chemicon) or rabbit anti-ionized calcium-binding adapter molecules-1 (anti-Iba-1; diluted 1:300 Wako Catalog No. 019-19741) was added to sections that were then incubated at 4 °C overnight. After two washing steps with PBS for 5 min, the sections were incubated at RT for 1 h with specific Cy2-conjugated secondary antibodies diluted 1:250 (711225152 and 115-165-003; Jackson Immuno Research, West Grove, PA, USA). Following two washing steps with PBS, the sections were counterstained by incubation for 10 min in 0.5 mg/ml of the fluorescent nuclear dye Hoechst 33258 (bisbenzimidazole, Sigma-Aldrich, Seelze, Germany). Following a short washing with PBS, sections were coverslipped in a glycerol-based medium and slides were examined under a fluorescence microscope (DMRBE, Leica Microsystems, Wetzlar, Germany).

Western blotting

Rats were sacrificed at the end of experimental procedures by an overdose of anesthesia, and the hippocampi were rapidly removed from their brain, collected, and stored at - 70 °C for later use. Dissected hippocampal tissue was homogenized in cold radio-immunoprecipitation assay (RIPA) buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1%

Triton, SDS 0.1%), supplemented with protease inhibitor cocktail (Sigma-Aldrich P8340) and phosphatase inhibitor cocktail (Sigma-Aldrich P5726). Samples were sonicated (30 pulsations/min), quantified by the Lowry method [32], and stored at -80°C . Western blotting was performed as previously described by Frinchi et al. [33]. Protein samples (50 μg per lane) and molecular weight marker (161-0376 BIO-RAD) were run on 10% or 12% polyacrylamide gel and electrophoretically transferred onto nitrocellulose membrane (RPN303E, Hybond-C-extra, GE Healthcare Europe GmbH). The membranes were incubated for 1 h in blocking buffer, 1x TBS, 0.1% Tween-20, and 5% *w/v* nonfat dry milk, with gentle shaking overnight at -4°C with specific antibody in blocking buffer. For detection of superoxide dismutase-1 (SOD1) and superoxide dismutase-2 (SOD2), the following antibodies were used: rabbit polyclonal anti-SOD1 1:1000 (Sc-11407 Santa Cruz Biotechnology); mouse anti-SOD2 1:500 (SOD2; sc-137254, Santa Cruz Biotechnology). For detection of GFAP and Iba-1, the following antibodies were used: mouse monoclonal antibody anti-GFAP 1:2000 (MAB360 Chemicon), rabbit anti-Iba-1 1:1000 (Wako Catalog No. 019-19741). The day after, the membranes were washed three times for 10 min with TBS/T and then incubated for 1 h at room temperature with goat anti-rabbit IgG (sc-2004 Santa Cruz Biotechnology) or goat anti-mouse IgG (Sc.7076 Cell Signaling Technology) horseradish peroxidase-conjugated diluted 1:10000. After three washings with TBS-T, immune complexes were visualized with a chemiluminescence reagent (RPN2236, GE Healthcare Europe GmbH) according to the manufacturer's instructions. The Hyperfilm (ECL-films 28906837, GE Healthcare Europe GmbH) were developed using Kodak developer and fixer (catalog no. 1900984 and 1902485, Kodak GBX, Eastman Kodak). For the normalization of quantitative evaluation of bands, each membrane was stripped at 65°C for 30 min in buffer containing NaCl 137 mM, TrisHCl 20 mM pH 7.6, and β -mercaptoethanol 0.01%. After two washings with TBST, the membranes were reprobed with an anti- β -actin antibody (sc-47778, Santa Cruz Biotechnology). The densitometric evaluation of bands was performed by measuring the optical density (O.D.) using the Image J software (Rasband WS, ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, <https://imagej.nih.gov/ij/>, 1997–2018).

Measurement of pro-inflammatory or anti-inflammatory cytokines by ELISA assay

Concentrations of interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-10 (IL-10), and transforming growth factor-beta1 (TGF- β 1) were measured in the hippocampus homogenates (20 mg of tissue sample) using enzyme-linked immunosorbent assay (ELISA) kits for rat

(Cloud-Clone Corp, Wuhan, Hubei) according to the manufacturers' protocols and as reported by Zizzo et al. [34].

Reactive oxygen species analysis

To assess reactive oxygen species (ROS) generation by fluorimeter analysis, 10 mg of tissue from rat hippocampus was weighed and suspended on 1000 μl of PBS1X with 10 μl of protease inhibitors (Amersham Life Science, Munich, Germany). Samples were then incubated with 1 mM dichlorofluorescein diacetate (DCFH-DA) for 10 min at room temperature in the dark. The conversion of non-fluorescent DCFH-DA to the highly fluorescent compound 20,70-dichlorofluorescein (DCF) by esterase activity was used to monitor the presence of peroxides due to the oxidative burst in the brain [34]. The samples were analyzed by fluorimeter (Microplate reader WallacVictor 2 - 1420 Multilabel Counter; PerkinElmer, Inc.), using the excitation filter set at 485 nm and the emission filter set at 530 nm. Relative ROS production was expressed as the change in fluorescence of the experimental groups compared with that of the control group (100%).

SOD activity levels

The hippocampus of rats was homogenized in PBS with protease inhibitors (Amersham Life Science, Munich, Germany). To remove insoluble material, tissue lysates were sonicated and centrifuged (14,000 rpm, at 4°C , for 30 min). In the supernatant, total proteins were quantified by the Lowry method [32]. Volume corresponding to 50 μg of protein was used for total SOD enzymatic activity measurement, by using the SOD assay kit (Sigma-Aldrich) according to manufacturer's instructions [34]. Absorbance was measured by using the iMark™ Microplate Absorbance Reader at 450 nm.

Lipid peroxidation assay

The lipid peroxidation assay kit (Sigma Aldrich) was used to detect the concentration of malondialdehyde (MDA), a final product of lipids peroxidation. Ten milligrams of hippocampal tissues were homogenized in 300 μl of MDA lysis buffer (supplied in the kit), and colorimetric reaction with thiobarbituric acid (TBA) was read on an iMark™ Microplate Absorbance Reader at 532 nm, according to manufacturer's instructions [34].

Cell counting

The number of Iba-1 and GFAP-positive cells was estimated by counts made by systematic sampling of brain sections, every third section of total 10 sections, in the hippocampal region of rat brain. All counts were made in four rats for each group and were carried out double-blindly. Labeled cells were evaluated using ImageJ software (Cell Counter plugin; Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland,

USA, <http://imagej.nih.gov/ij/>, 1997–2018). The cell count values obtained from three to five random fields per section, in 10 sections examined, were expressed as means \pm SEM values per square millimeter of tissue.

Cortisol levels

Rats under anesthesia were sacrificed between 11:00 and 12:00 am, and blood was taken by intracardiac puncture and collected in tubes coated with EDTA. Blood samples were centrifuged at $4000\times g$ at 4°C for 10 min, and the supernatant was stored at -80°C . The plasma levels of cortisol were measured using an automated electrochemiluminescence immunoassay (Roche Diagnostics Elecsys Cortisol II assays and COBAS E801), and values were expressed in nanograms per milliliter. The minimum level of detection for assays of cortisol was 0.15 ng/ml [35].

Statistical analysis

Data analysis was performed using the GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Results are presented as mean \pm SE, and in some cases are expressed as arbitrary units, with controls equal to 1, or as percentage of control. For the *novel object recognition task*, the parameter chosen to assess rats' ability to discriminate novelty from familiar features was the recognition index (RI) and was calculated using the

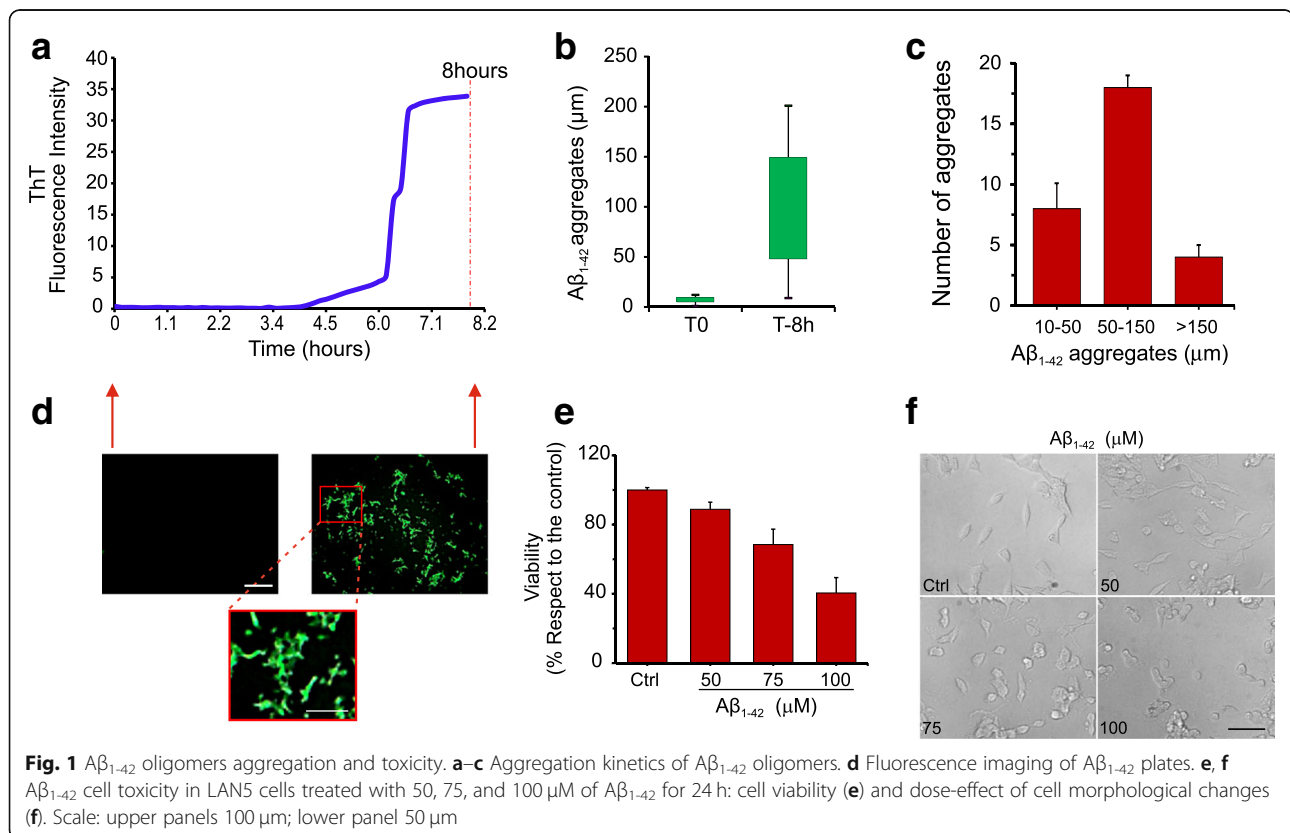
following formula: $[\text{RI} = \text{TN}/(\text{TN} + \text{TF})]$. Statistical evaluations were performed by one-way ANOVA, followed by Fisher's Protected Least Significant Difference (PLSD) test, with the exclusion of behavioral data for which we used the Tukey's multiple comparison test. Differences in P value less than 0.05 were considered statistically significant.

Results

AD rat model

$\text{A}\beta_{1-42}$ oligomers aggregation and toxicity

$\text{A}\beta_{1-42}$ oligomers, prepared as reported in Carrotta et al. [29], were aggregated by incubation for 8 h at 37°C . The results of aggregation kinetics of $\text{A}\beta_{1-42}$ oligomers are shown in Fig. 1a–c. The $\text{A}\beta_{1-42}$ oligomers aggregation was also evaluated by fluorescence microscope, and the $\text{A}\beta_{1-42}$ plate's mean size was measured by fluorescence microscopy software; results are shown in Fig. 1d. For $\text{A}\beta_{1-42}$ cell toxicity, LAN5 cells were treated with 50, 75, and $100\ \mu\text{M}$ of $\text{A}\beta_{1-42}$ for 24 h and the cell viability was evaluated using MTS assay; results are shown in Fig. 1e, f. Based on the present aggregation and toxicity data of $\text{A}\beta_{1-42}$ oligomers and on data of neurotoxicity dependent on the types and sizes of $\text{A}\beta_{1-42}$ oligomers [36, 37], it was decided to inject $\text{A}\beta_{1-42}$ oligomers aggregates, formed after 8 h of incubation and at concentration of $75\ \mu\text{M}$, in the dorsal hippocampus.



A β ₁₋₄₂ intra-hippocampal injection and IFN β 1a treatment

In Fig. 2a, the stereotaxic position of injection site in the dorsal hippocampus is shown. Fig. 2b shows the scheme of treatment performed. The amount of A β ₁₋₄₂ protein aggregates injected was 23 μ g/2 μ l according to toxicity data of previous experimental models [38, 39]. The scheme of dose and time of IFN β 1a treatment was based on previous work using a similar experimental rat model [20] or a rat model of autoimmune encephalomyelitis [40–42].

IFN β 1a treatment rescues cognitive performances impaired by intra-hippocampal injection of A β ₁₋₄₂ peptide

Using the NOR test, we evaluated changes in cognitive function induced by intra-hippocampal injection of A β ₁₋₄₂ peptide. At scheduled time (Fig. 2b), rats were tested in an open field arena in order to assess declarative memory as assessed by the recognition index of novel objects from familiar ones. When rats were trained with two identical objects, a one-way ANOVA did not show (Fig. 3a) any statistical variation in the RI% ($F_{(4,35)} = 0.6122$; $p = 0.6566$) among the experimental groups. Twenty-four hours after the training, rats' preference toward a novel object was evaluated. A one-way ANOVA showed (Fig. 3b) a significant effect of treatment ($F_{(4,35)} = 5.971$; $p < 0.001$). The post hoc analysis conducted by Tukey's multiple comparison test showed a significant reduction of RI% in the A β ₁₋₄₂ group as compared to the control ($p < 0.01$), to A β ₁₋₄₂ + IFN β 1a ($p < 0.01$), and to IFN β 1a ($p < 0.05$) groups (Fig. 3b). In the A β ₁₋₄₂ + IFN β 1a group, the treatment with IFN β 1 fully counteracted the RI% reduction observed in the A β ₁₋₄₂ group. In

the IFN β 1a and sham groups, the RI% did not change as compared to control.

Body weight and cortisol levels

Rats' body weight was measured at the beginning and the end of the experimental period (Fig. 2b). Two-way repeated ANOVA measurements showed no significant differences in body weight (Fig. 4a) both among the various experimental groups and in each group at the end of the experimental period as compared to the beginning of the experiment.

Although corticosterone is considered the main glucocorticoid involved in the regulation of stress responses in rodents, we preferred to measure cortisol in consideration of procedure availability in our laboratory and of good correlation between serum cortisol and corticosterone [43]. The results are reported in Fig. 4b: one-way ANOVA did not show significant change in cortisol level among experimental groups.

IFN β 1a effects on glial cell activation by A β ₁₋₄₂ protein injection in the hippocampus

Since one of the features of AD pathology is activation of microglia and astrocytes induced by A β deposits, we analyzed by immunohistochemistry and Western blot Iba-1 and GFAP markers for microglia and astrocyte activation. As shown in Fig. 5a, b, the count of Iba-1-positive cells was significantly increased in the hippocampus of the A β ₁₋₄₂-treated group ($F_{(4,22)} = 26.97$, $p < 0.0001$) as compared to the control ($p < 0.0001$) and sham groups ($p < 0.001$). However, in the A β ₁₋₄₂ + IFN β 1a group, the treatment with IFN β 1a significantly counteracted the effect of A β ₁₋₄₂ injection as shown by the Iba-1-positive cell number significantly reduced as compared to the A β ₁₋₄₂ group ($p = 0.02$). However, the

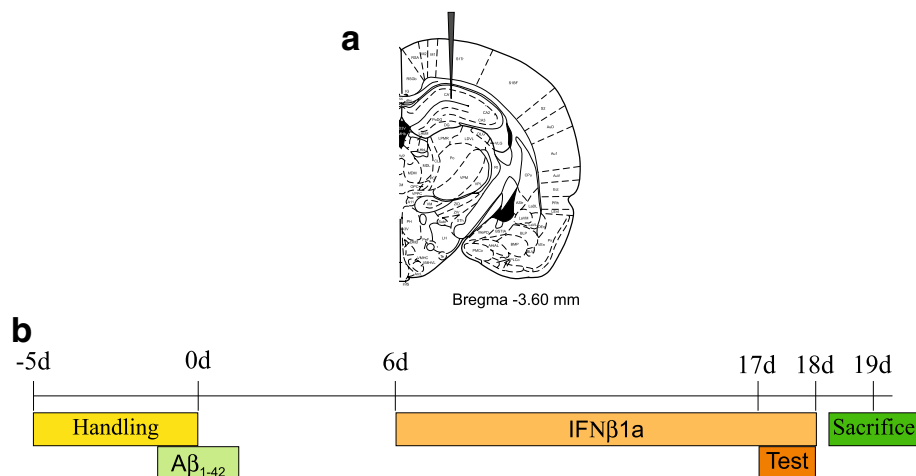


Fig. 2 A β ₁₋₄₂ intra-hippocampal injection and IFN β 1a treatment. **a** Stereotaxic position of A β ₁₋₄₂ injection in the dorsal hippocampus. **b** Scheme of A β ₁₋₄₂ and IFN β 1a treatment performed

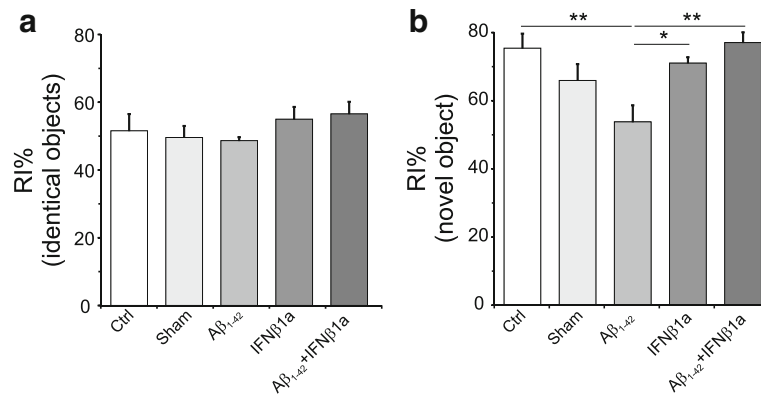


Fig. 3 Cognitive evaluation by NOR test. Rats ($n = 8$ per group) exposed to the training with two identical objects did not show any statistical variation in the RI% ($F_{(4,35)} 0.6122$; $p = 0.6566$) among the experimental groups (a). Twenty-four hours after the training was the evaluation of rats' preference toward novel object. The results of one-way ANOVA showed b a significant effect of treatment ($F_{(4,35)} = 5.971$; $p < 0.001$). The post hoc analysis conducted by Tukey's multiple comparison test showed a significant reduction of RI% in the $A\beta_{1-42}$ group with respect to control ($p < 0.01$), $A\beta_{1-42} + IFN\beta 1a$ ($p < 0.01$), and $IFN\beta 1a$ ($p < 0.05$) groups (b). In the $A\beta_{1-42} + IFN\beta 1a$ group, the treatment with $IFN\beta 1a$ full counteracted the RI% reduction observed in the $A\beta_{1-42}$ group. In the $IFN\beta 1a$ and sham groups, the RI% did not change as compared to control. * $P < 0.05$, ** $P < 0.01$

cell number in the $A\beta_{1-42} + IFN\beta 1a$ group was still significantly increased when compared to the control group ($p < 0.0001$) but not compared to the sham group (Fig. 5a, b). The sham group, but not the $IFN\beta 1a$ group, showed a significant increase of Iba-1-positive cell number as compared to the control group ($p < 0.001$). Quantitative Western blot analyses of Iba-1 protein levels clearly showed a significant increase in the $A\beta_{1-42}$ group as compared to both control ($p < 0.05$) and sham ($p < 0.05$) groups. In the $A\beta_{1-42} + IFN\beta 1a$ group, the treatment with $IFN\beta 1a$ counteracted the $A\beta_{1-42}$ effect on Iba-1 protein levels. Indeed, $A\beta_{1-42} + IFN\beta 1a$ Iba-1 protein levels are not significant when compared to the $A\beta_{1-42}$ group, but they are also not significant when compared both to

control and sham groups (Fig. 5c). This means that treatment with $IFN\beta 1a$ in the $A\beta_{1-42} + IFN\beta 1a$ group counteracted the increase of Iba-1 protein levels induced by $A\beta_{1-42}$, as shown by its loss of significance with respect to controls, although it does not bring them back to the levels detected in the control and sham groups (Fig. 5c). Iba-1 protein levels in the $IFN\beta 1a$ and sham groups did not show significant difference as compared to the control group.

The immunohistochemistry analysis of GFAP marker showed a not significant trend toward an increase in the astrocytes num in all experimental groups as compared to control group (Fig. 6a, b). By contrast, the quantitative Western blot analyses of GFAP protein levels

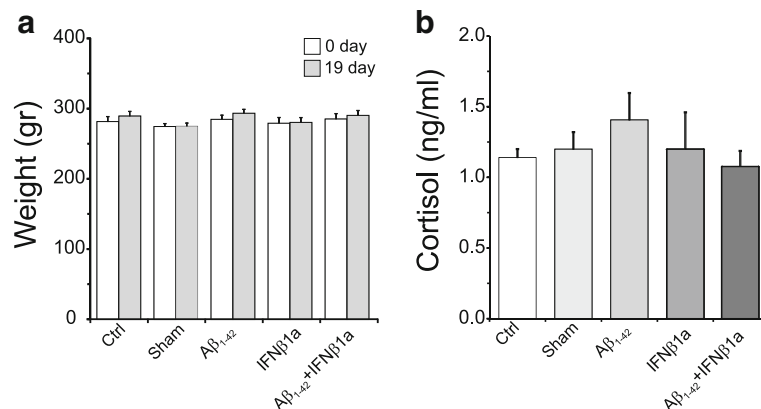


Fig. 4 Body weight and cortisol levels. Two-way repeated measurement ANOVA showed not significant differences in the body weight, both among the experimental groups and each group at the end of experimental period as compared to starting body weight (a). One-way ANOVA did not show significant change in cortisol level among the experimental groups (b)

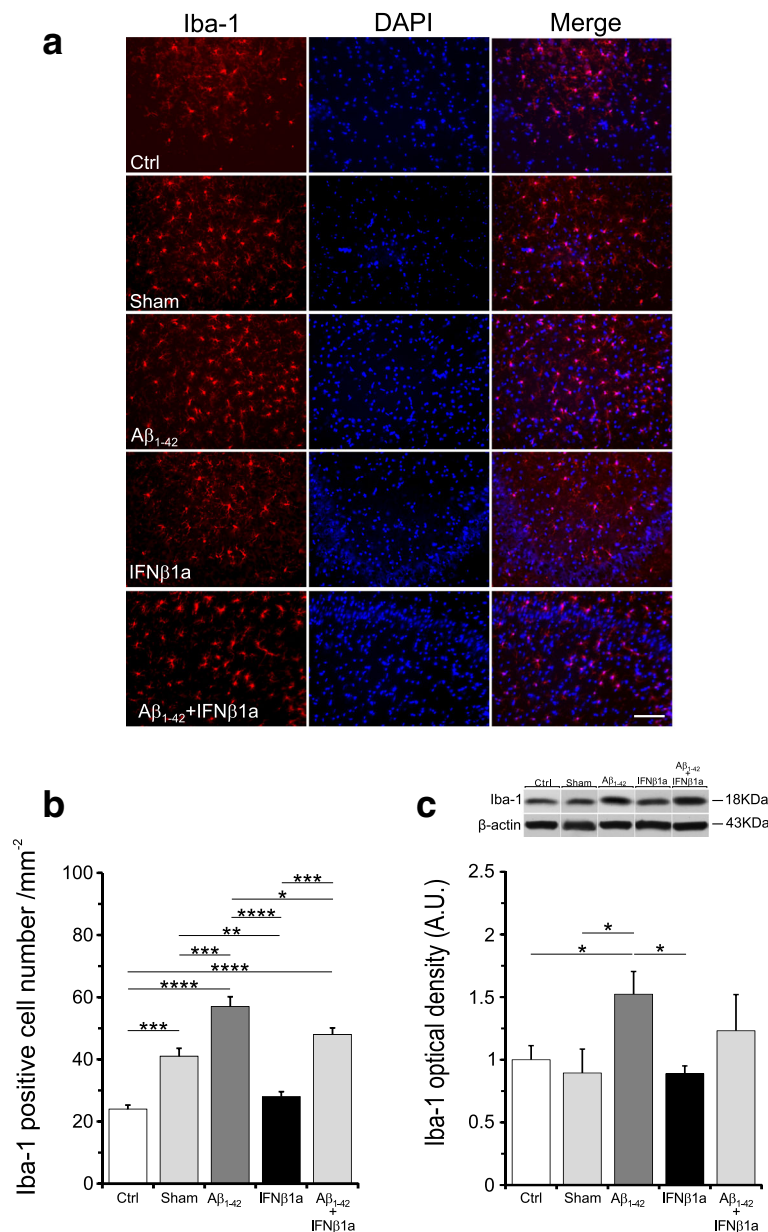


Fig. 5 IFNβ_{1a} effects on microglial cell activation by Aβ₁₋₄₂. **a** Iba-1 immunohistochemistry in the hippocampus. **b** Count of Iba-1-positive cells and **c** quantitative Western blot analyses of Iba-1 protein levels. **b** The count of Iba-1-positive cells showed a significant increase of cell number in the hippocampus region of the Aβ₁₋₄₂-treated group (*n* = 6) and treatment with IFNβ_{1a} (*n* = 6) significantly counteract this effect in the Aβ₁₋₄₂+IFNβ_{1a} group (*n* = 6) as compared to sham group but not to the control group. **c** Similarly, quantitative Western blot analyses of Iba-1 protein levels showed that in the Aβ₁₋₄₂ group, Iba-1 protein levels were significantly increased and treatment with IFNβ_{1a} significantly counteract this effect in the Aβ₁₋₄₂+IFNβ_{1a} group. Scale: 100 μm. **P* < 0.05, ****P* < 0.01, *****P* < 0.001, ******P* < 0.0001

showed that in the Aβ₁₋₄₂ and in the Aβ₁₋₄₂+IFNβ_{1a} groups, GFAP protein levels were significantly increased (both *p* < 0.0001) as compared to the control group and in the Aβ₁₋₄₂+IFNβ_{1a} group as compared to the sham group (Fig. 6c). Surprisingly, the treatment with IFNβ_{1a} alone significantly reduced (*p* < 0.01) the GFAP protein levels as compared to the control group. The cell number of the sham group was not significantly different from the control group.

IFNβ_{1a} inhibits pro-inflammatory cytokines increase induced by Aβ₁₋₄₂ protein injection in the hippocampus

We also analyzed by ELISA the hippocampal levels of pro-inflammatory cytokines, IL-1β and IL-6, and anti-inflammatory cytokines, IL-10 and TGF-β1. As shown in Fig. 7a, b, we found that both IL-1β and IL-6 levels were significantly increased in the Aβ₁₋₄₂ group as compared to the control (*p* < 0.0001 and *p* < 0.01

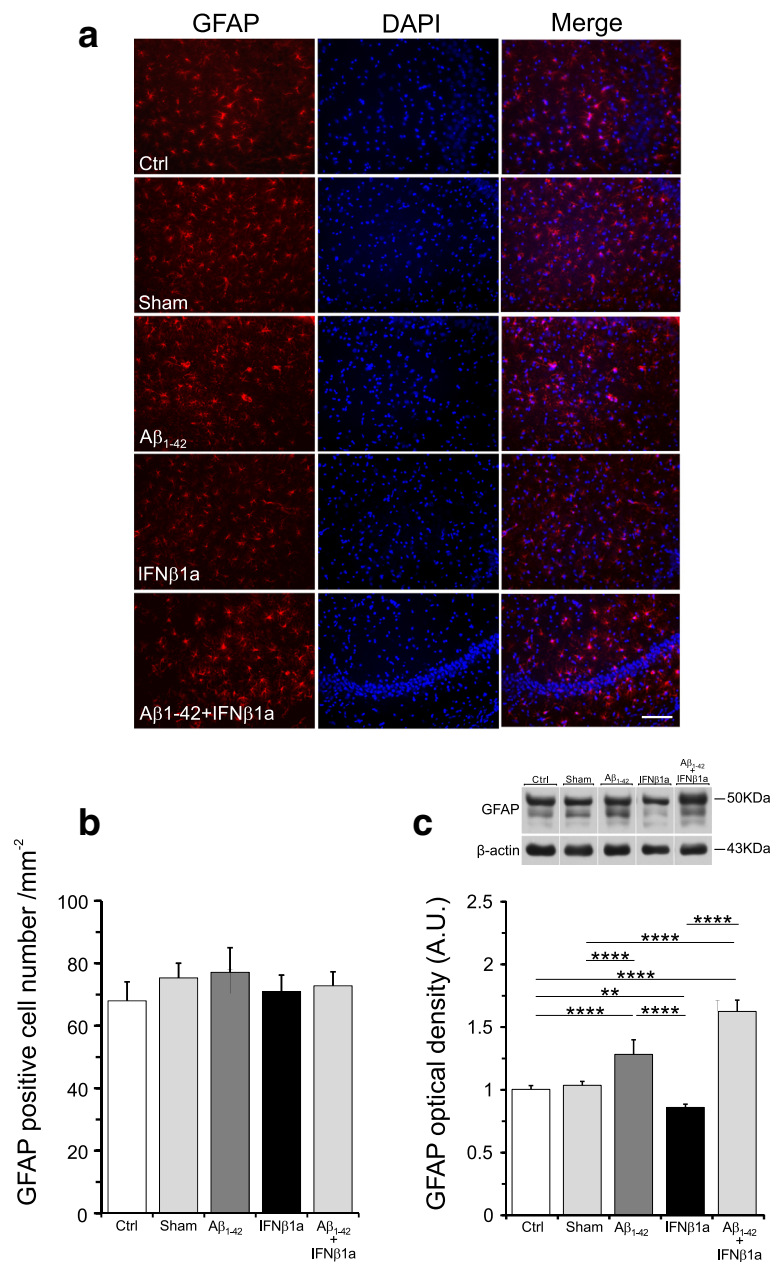


Fig. 6 IFNβ_{1a} effects on astroglial cell activation by Aβ₁₋₄₂. **a** GFAP immunohistochemistry in the hippocampus. **b** Count of GFAP positive cells and **c** quantitative Western blot analyses of GFAP protein levels. **b** Count of astrocytes number showed no significant changes in all experimental groups. **c** By contrast, the quantitative Western blot analyses of GFAP protein levels revealed that in the Aβ₁₋₄₂ group ($n = 6$), GFAP protein levels were significantly increased and this increase was not counteracted by treatment with IFNβ_{1a} ($n = 6$) in the Aβ₁₋₄₂ + IFNβ_{1a} group ($n = 6$). Note IFNβ_{1a} alone significantly reduced the GFAP protein levels as compared to the control group. Scale: 100 μm * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

respectively) and sham ($p < 0.0001$ and $p < 0.001$ respectively) groups. In the Aβ₁₋₄₂ + IFNβ_{1a} group, the treatment with IFNβ_{1a} counteracted this Aβ₁₋₄₂ effect on IL-1β and IL-6 levels ($p < 0.0001$ and $p < 0.01$ respectively), bringing them back to the levels of control and sham groups. In the IFNβ_{1a} group, the treatment with IFNβ_{1a} did not change the IL-1β levels as compared to the control, whereas it produced a substantial reduction

of IL-6 levels as compared to the control and sham groups ($p < 0.05$).

In contrast to hippocampal upregulation of IL-1 and IL-6 levels, the anti-inflammatory cytokine IL-10 was found significantly reduced in the Aβ₁₋₄₂ group as compared to the control ($p < 0.001$) and sham ($p < 0.001$) groups (Fig. 7c). Following IFNβ_{1a} treatment in the Aβ₁₋₄₂ + IFNβ_{1a} group, the IL-10 levels recovered to control

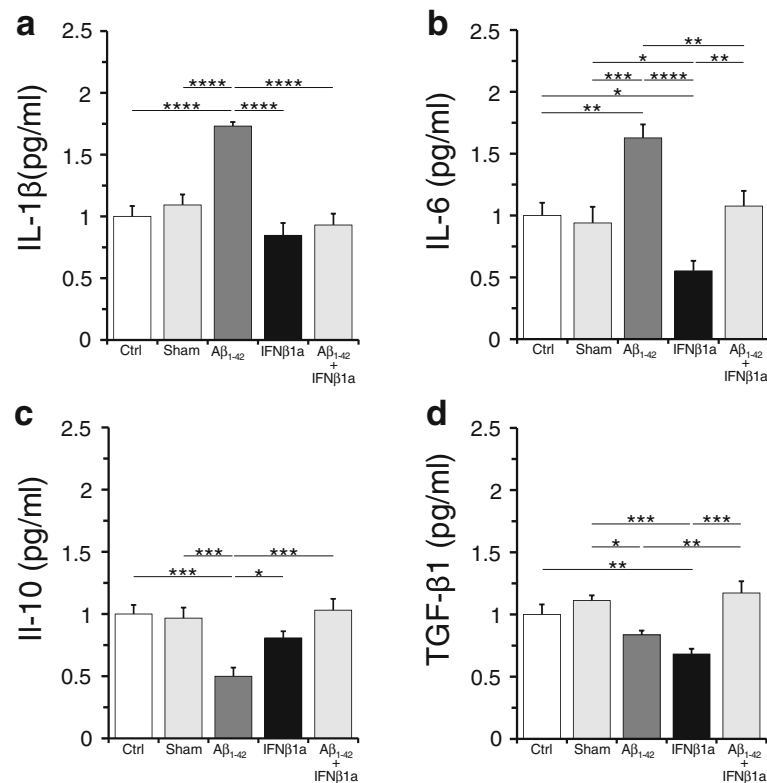


Fig. 7 IFNβ_{1a} effects on inflammatory cytokines in the hippocampus. **a, b** Hippocampal levels of pro-inflammatory cytokines, IL-1β and IL-6. Note that IL-1β and IL-6 levels were significantly increased in the Aβ₁₋₄₂ group ($n = 5$ respectively) and this increase was counteracted by treatment with IFNβ₁ ($n = 5$ respectively) in the Aβ₁₋₄₂ + IFNβ_{1a} group ($n = 5$ respectively). **c, d** Hippocampal levels of anti-inflammatory cytokines, IL-10 and TGF-β₁. Note IL-10 and TGF-β₁ levels showed significant reduction in the Aβ₁₋₄₂ group ($n = 5$ respectively) and following IFNβ_{1a} ($n = 5$, respectively) treatment in the Aβ₁₋₄₂ + IFNβ_{1a} group ($n = 5$, respectively) were recovered to control ($n = 5$ and $n = 6$, respectively) levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

levels, strengthening the anti-inflammatory property of IFNβ_{1a}. The TGF-β₁ levels were significantly reduced in the IFNβ_{1a} group as compared to control or sham group ($p < 0.01$ and $p < 0.001$ respectively) and Aβ₁₋₄₂ group as compared to sham ($p < 0.05$) group (Fig. 7d), whereas surprisingly in the Aβ₁₋₄₂ + IFNβ_{1a} group, TGF-β₁ levels recovered to the control group levels. In the sham group, we did not find significant changes in both IL-10 and TGF-β₁ levels as compared to the control group.

IFNβ_{1a} treatment effects on ROS levels and SOD1 or SOD2 proteins and activity levels

SOD1 levels (Fig. 8a) were increased in the Aβ₁₋₄₂ + IFNβ_{1a} group as compared to the control ($p < 0.05$) and sham ($p < 0.05$) groups, whereas SOD2 levels (Fig. 8b) were significantly reduced in the same group as compared to the control ($p < 0.05$) and sham ($p < 0.01$) groups. The SOD2 levels were significantly lower in the IFNβ_{1a} group as compared to the sham group ($p < 0.05$)

but not to the control group, suggesting that IFNβ_{1a} per se may negatively regulate the SOD2 levels. In the sham group, we did not find significant changes in both SOD1 and SOD2 levels as compared to the control group.

Concerning the SOD activity (Fig. 8c), we found that SOD activity levels were significantly decreased in the Aβ₁₋₄₂ group as compared to control ($p < 0.01$). In the Aβ₁₋₄₂ + IFNβ_{1a} group, the treatment with IFNβ_{1a} counteracted the decrease of SOD activity level bringing it back to the control level. In the IFNβ_{1a} group, the treatment with IFNβ_{1a} did not change the SOD activity levels as compared to the control and sham groups. In the sham group, we did not find significant changes in SOD activity levels as compared to the control group.

Concerning the oxidative stress analysis (Fig. 8d), we found that ROS levels were significantly increased in the Aβ₁₋₄₂ group as compared to control ($p < 0.0001$) and sham ($p < 0.01$) groups. In Aβ₁₋₄₂ + IFNβ_{1a} group, the

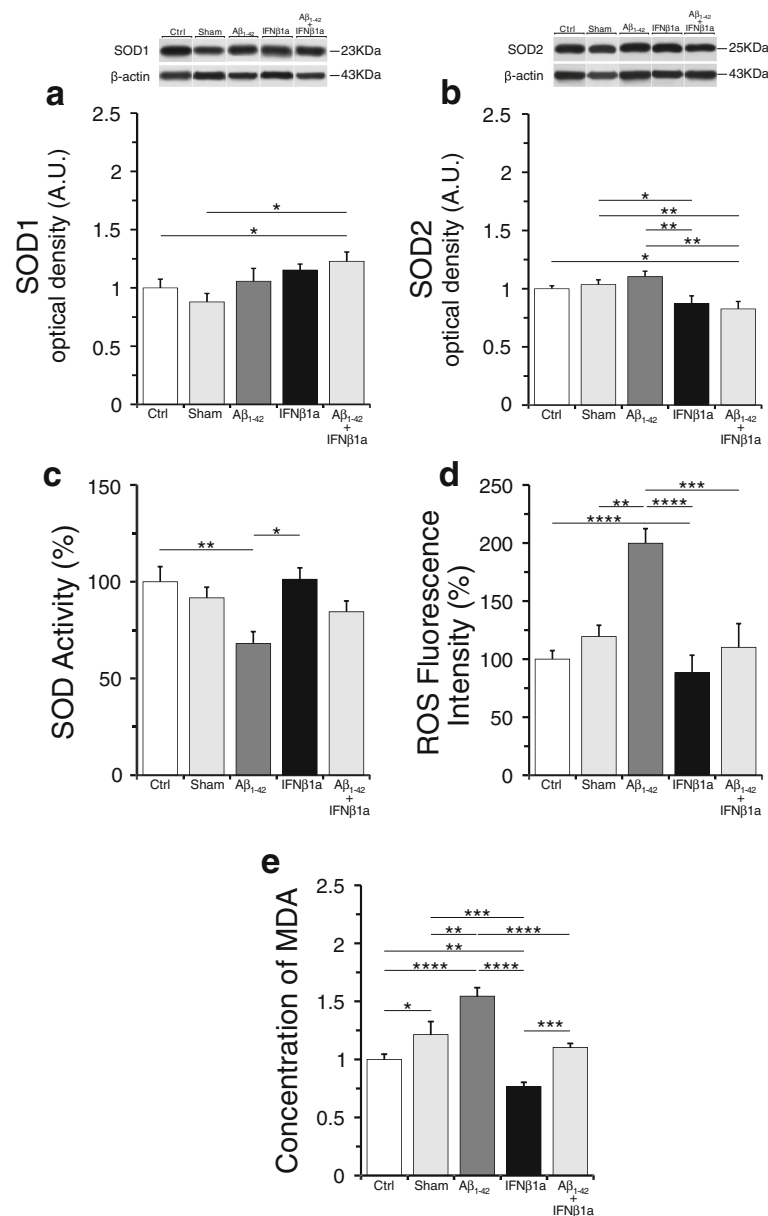


Fig. 8 IFNβ_{1a} effects on ROS and lipid peroxidation levels and SOD1 or SOD2 proteins and activity levels in the hippocampus. **a, b** The levels of SOD1 showed significant increase in the Aβ₁₋₄₂ + IFNβ_{1a} group ($n = 5$), whereas those of SOD2 appear significantly reduced in the same group ($n = 5$). **c** Total SOD activity levels showed significant decrease in Aβ₁₋₄₂ group ($n = 5$), and this decrease was counteracted in the Aβ₁₋₄₂ + IFNβ_{1a} group ($n = 5$) by treatment with IFNβ_{1a}. **d** ROS levels were significantly increased in the Aβ₁₋₄₂ group ($n = 5$), and this increase was counteracted by treatment with IFNβ_{1a} ($n = 5$). **e** The analysis of lipid peroxidation levels, measured as concentration of MDA, revealed a significant increase of MDA levels in the Aβ₁₋₄₂ group ($n = 5$) that was completely blocked by IFNβ_{1a} treatment in the Aβ₁₋₄₂ + IFNβ_{1a} group ($n = 5$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$

treatment with IFNβ_{1a} counteracted the increase of ROS levels bringing them back to the control level. ROS levels were substantially similar in the IFNβ_{1a}, control, and sham groups.

Lipid peroxidation is the degradation of lipids that occurs as a result of oxidative damage, typically by reactive oxygen species, resulting in a well-defined chain reaction with the production of end products such as MDA.

According to previous data [44] showing that Aβ₁₋₄₂ injection induces ROS levels, we analyzed the level of lipid peroxidation in the hippocampus by measuring the concentration of MDA. The analysis (Fig. 8e) revealed a significant increase of MDA levels in the Aβ₁₋₄₂ group as compared to control ($p < 0.0001$) and sham ($p < 0.01$) groups. This increase of MDA levels was positively correlated with the increased levels of ROS. In the

$A\beta_{1-42}$ + IFN β 1a group, the treatment with IFN β 1a completely blocked the increase of MDA levels observed in the $A\beta_{1-42}$ group. Interestingly, in the IFN β 1a group, the MDA levels were found significantly reduced as compared to control ($p < 0.01$) and sham ($p < 0.001$) groups. However, the MDA levels were found also significantly increased in the sham as compared to control group ($p < 0.05$).

Discussion

In our rat model of AD, the administration of $A\beta_{1-42}$ oligomer aggregates in the dorsal hippocampus led to neuroinflammation, via the activation of glial immune system, and memory impairment as evaluated by the NOR test. The short treatment with IFN β 1a was able to reverse memory impairment and to suppress microglia activation and the upregulation of pro-inflammatory cytokine (IL-6, IL-1 β) levels and oxidative stress in the hippocampus. All together, these data suggest a protective effect of IFN β 1a against $A\beta_{1-42}$ -induced functional alterations in the hippocampus of our rat AD model.

AD is characterized by an insidious clinical onset, by a progressive cognitive and memory decline, and by accumulation of $A\beta$ plaques and neurofibrillary tangles leading to loss of neurons in the hippocampus and in the cerebral cortex [1–3]. In a previous pilot study, we evaluated the safety and efficacy of IFN β 1a in subjects affected by mild-to-moderate AD and detected a reduction in disease progression and improvements in the instrumental activities of daily living and physical self-maintenance scales [24]. Apart for this limited human study, IFN β 1a had never been previously tested in AD, unlike its long and widespread use in patients with multiple sclerosis, where it has been clearly shown that subcutaneous IFN β 1a protects against cognitive decline [15, 21, 22, 41, 45]. Although most of its pleiotropic effects occur in the peripheral immune system, a direct anti-inflammatory effect of IFN β 1a within the brain has also been proposed [23]. We actually found an anti-inflammatory effect of IFN β 1a in the CNS of experimental rats that reasonably explains the recovery from memory impairment caused by hippocampal $A\beta_{1-42}$ peptide injection. Improvement of memory deficit in AD animal model after treatments targeting inflammation has already been reported [46, 47]. However, it has been reported that microglia of adult mice following chronic exposure to IFN β 1a express an aging-like phenotype and negatively affect learning abilities [48, 49].

Histological studies of brains from individuals with AD have revealed a direct relationship between $A\beta_{1-42}$ peptide abnormal production and the development and/or maintenance of neuro-inflammation and oxidative stress [5, 50]. Accordingly, exposure of the brain to

$A\beta_{1-42}$ peptide causes inflammation, by activating microglia as well as astrocytes, and promotes the production of cytotoxic molecules, such as pro-inflammatory cytokines and reactive oxygen species, that contribute to dysfunction, injury, and ultimately neuronal loss [3, 51–55]. In addition, the release of inflammatory mediators may in turn increase $A\beta_{1-42}$ peptide production, which may further contribute to plaque formation and progression to neuronal loss [3, 52]. Besides this consolidated evidence of the close relationship between plaques and activated glial cells, neuro-inflammation is increasingly believed to be an early player in the pathological cascade leading to AD rather than a mere consequence.

Although its role is still debated, the recognition of an inflammatory contribution to AD pathogenesis has led to therapeutic attempts using several anti-inflammatory agents [9]. Therapeutic approaches using phenols, phytoestrogens, neuro-steroids, and other natural phytochemicals have been explored in AD and experimental models, with some promising results such as cognitive improvements and attenuation of neuro-inflammation. Several non-steroidal anti-inflammatory drugs have also been tested in the attempt to prevent the onset or to slow down progression of AD [1, 2, 56, 57]. Flavonoids have been tested in AD models and seem to be able to reduce AD severity by modulating the production of microglia pro-inflammatory cytokines (TNF- α and IL-1 β) or by reducing their $A\beta$ -induced cytokine production [3]. In the present work, we tested IFN β 1a in an AD rat model and showed that its anti-inflammatory ability is mainly associated to the block of IL-1 β and IL-6 upregulation levels in the hippocampus. Deposition of $A\beta$ peptide may also activate astrocytes inducing astrogliosis with release of pro-inflammatory agents, such as IL-1, IL-6, and IL-10, and oxidative stress with production of reactive oxygen and nitrogen species [58, 59]. Changes in astrocyte function have been observed in brains from individuals with AD, as well as in AD in vitro and in vivo animal models [60]. However, in the present work, we could not observe a reduction of astrocytes activation in the $A\beta_{1-42}$ group treated with IFN β 1a, as evaluated by the GFAP-positive cell number and GFAP protein levels.

IL-1 β , synthesized and released by both activated microglia and astrocytes, is considered to be a major pro-inflammatory cytokine in the brain and play a key role in the progression of AD [61]. Similarly, IL-6 is also a pro-inflammatory cytokine mainly produced by activated microglia and when increased in brain of AD and AD animal models [5, 52] may impair cognitive processes, such as spatial learning and memory [62, 63], and stimulate the synthesis of $A\beta$ precursor protein. Indeed, active microglia constitute the core immune

system in the brain and release pro-inflammatory cytokines and free radicals that may elicit neurodegenerative processes, and since the pro-inflammatory mediators, IL-1 β and IL-6, are produced primarily by microglia in the brain, the IFN β 1a inhibition of IL-1 β and IL-6 in the hippocampus of A β ₁₋₄₂-treated group suggests a direct effect on microglial cells. This is supported by our observation of a reduction of microglial activation in the A β ₁₋₄₂ group treated with IFN β 1a, as reflected by the decrease in number of microglia cells and Iba-1 protein levels. Previously, following chronic exposure to IFN β 1a, Deczkowska et al. [48] reported modification in microglia morphology and expression of genes present in aged microglia, suggesting that microglia can contribute to the pro-inflammatory response of the brain in AD or aging, thereby exacerbating cognitive loss and disease pathology. In our rat model, we did not observe alterations in the microglia or astrocytes morphology, probably because the treatment time with IFN β 1a was shorter compared to those given by Deczkowska et al. [48]. In addition, in the present study, we did not explore gene expressed in aged microglia.

In contrast to hippocampal upregulation of IL-1 and IL-6 levels, the anti-inflammatory cytokines, IL-10 and TGF- β 1, were significantly reduced in the A β ₁₋₄₂ group and recovered to control levels following IFN β 1a treatment. This result further supports the anti-inflammatory properties of IFN β 1a.

Although the numerous mechanisms underlying the IFN β 1a anti-inflammatory effects have been clearly defined within the peripheral immune system, its role in the central nervous system function has been little explored. Indeed, the only data available in this field, in addition to already mentioned anti-inflammatory effect of IFN β 1a in patients with multiple sclerosis [17, 18, 64], derived from studies on experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. Indeed, IFN β 1a treatment prevents and reduces the progression of the experimental CNS demyelination [42, 65, 66] by inhibiting pro-inflammatory cytokines (IL-6, IL-1 β , TNF- α , IFN- γ), astrocytes activation, and inducible nitric oxide synthase expression [20]. However, adverse effect of type I interferon (IFN-I) has been reported that in the aging brain chronically elevated IFN-I activity contributes to the pathology of various human CNS diseases and in animal models, including aging microglial phenotype, neurodegeneration, and microgliosis [48].

The present and above-listed data showed beneficial effects of IFN β 1a in a model of AD pathology, but it is still unclear how it exerts this effect within the CNS, since its passage from the bloodstream to the brain

parenchyma is significantly restricted by the blood–brain and blood–cerebrospinal fluid barriers [67–70]. In this context, the effects of IFN β 1a in the brain have been associated with possible modulation of brain inflammatory events at capillaries level [41] or with a decreased permeability of the blood–brain barrier to inflammatory cell entry into to the brain [71], thus reducing CNS inflammatory response. Several studies have demonstrated anti-inflammatory effect of IFN β 1a outside the CNS [17, 18, 64]. Indeed, IFN β 1a regulates several immunological functions, including decrease in T cell activation, induction of cytokine shifts in favor of an anti-inflammatory effect, prevention of T cell adhesion, and extravasation across the blood–brain barrier, as well as induction of T-regulatory cells, all occurring within the peripheral immunological organs [12–20]. Recently, several pathways for the transport of cytokines from systemic circulation into the brain have been reviewed [70], but the mechanisms by which IFN β 1a treatment may affect pro-inflammatory response induced by A β ₁₋₄₂ peptide injection in the hippocampus need further investigations. Anyway, in the present work, it is possible to exclude a role of glucocorticoid in mediating anti-inflammatory effects, since we did not observe any significant change in cortisol levels among the experimental groups.

Another particular hypothesis that has received considerable interest in the pathogenesis and progression of AD is the oxidative damage in the brain [72, 73]. Therefore, strategies aimed to reduce oxidative stress in AD have been proposed, also supported by reports of beneficial effects in AD of various antioxidants treatments [74, 75]. The present study indicated potential neuroprotective effects of IFN β 1a possibly mediated also by its ability to reduce ROS and lipids peroxidation and to increase SOD1 protein levels, although we did not find a parallel increase in SOD activity. A recent report actually showed that treatment with IFN β 1a inhibits oxidative stress in an animal model of multiple sclerosis, but at present, there are no reports of similar findings in AD or AD models [20].

Conclusions

Based on the hypothesis that A β ₁₋₄₂-induced inflammation plays an important role in AD pathogenesis, using a rat model of AD, we provided evidence that IFN β 1a treatment may be a viable strategy to inhibit pro-inflammatory cytokines and oxidative stress. Therefore, IFN β 1a treatment, used for decades to contain inflammatory-mediated diseases of the brain, could be effective in AD patients, contributing to smolder the progression of this devastating disease.

Abbreviations

AD: Alzheimer disease; DCF: Dichlorofluorescein; DCFH-DA: Dichlorofluorescein diacetate; ELISA: Enzyme-linked immunosorbent assay; IFN β 1a: Interferon- β 1a; MDA: Malondialdehyde; NF- κ B: Transcription factor nuclear factor- κ B; PLSD: Fisher's Protected Least Significant Difference; pNF- κ B: Phosphorylated NF- κ B; ROS: Reactive oxygen species; TBA: Thiobarbituric acid; TGF- β 1: Transforming growth factor-beta1; A β ₁₋₄₂: Amyloid β Protein Fragment 1-42

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GM, MF, and PS performed the experiments in vivo and analysis of immunostaining data. GC and LC performed the experiments in vivo and animal care. CC and FP planned and performed behavioral testing. MFM performed detection of cortisol levels. DN and MDC prepared A β ₁₋₄₂ oligomers aggregates and performed analysis of cytokines and oxidative stress. MR provided the IFN β 1a and planned the treatment. GM and MF performed the statistical analysis and figures. NB, LMG, and GM conceived and planned the work and contributed in writing the text. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The experiments were carried out in accordance with the National Institute of Health Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (The National Academics Press, WA, USA), with the rules and principles of the European Communities Council Directive 2010/63/EU revising Directive 86/609/EEC, in accordance with the national D.L. March 4, 2014, no. 26, and were approved by the local Animal Care Committee (OPBA) of University of Palermo, Italy and Ministry of Health, Italy. The *consent to participate* is not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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