International Immunopharmacology 52 (2017) 290–296

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

N-Stearoylethanolamine protects the brain and improves memory of mice treated with lipopolysaccharide or immunized with the extracellular domain of α7 nicotinic acetylcholine receptor

Olena Lykhmus[a], Kateryna Uspenska[a], Lyudmyla Koval[a], Daria Lytovchenko[a], Larysa Voytenko[a], Tetyana Horid'ko[b], Halyna Kosiakova[b], Nadiya Gula[b], Serhiy Komisarenko[a], Maryna Skok[a,*]

[a] Laboratory of Cell Receptors Immunology, Palladin Institute of Biochemistry, 9, Leontovycha str, 01030 Kyiv, Ukraine
[b] Department of Lipid Biochemistry, Palladin Institute of Biochemistry, 9, Leontovycha str, 01030 Kyiv, Ukraine

ARTICLE INFO

Keywords:
Neuroinflammation
α7 nicotinic acetylcholine receptor
N-stearoylethanolamine
Alzheimer disease
Mitochondria

ABSTRACT

Neuroinflammation is an important risk factor for neurodegenerative disorders like Alzheimer's disease. Nicotinic acetylcholine receptors of α7 subtype (α7 nAChRs) regulate inflammatory processes in various tissues, including the brain. N-stearoylethanolamine (NSE) is a biologically active cell membrane component with anti-inflammatory and membrane-protective properties. Previously we found that mice injected with bacterial lipopolysaccharide (LPS) or immunized with recombinant extracellular domain (1–208) of α7 nAChR subunit possessed decreased α7 nAChR levels, accumulated pathogenic amyloid-beta peptide Aβ(1–42) in the brain and demonstrated impaired episodic memory compared to non-treated mice. Here we studied the effect of NSE on behavior and brain components of LPS- treated or α7(1–208)-immunized mice. NSE, given *per os*, non-significantly decreased LPS-stimulated interleukin-6 elevation in the brain, slowed down the α7(1–208)-specific IgG antibody production and prevented the antibody penetration into the brain of mice. NSE prevented the loss of α7 nAChRs and accumulation of α7-bound Aβ(1–42) in the brain and brain mitochondria of LPS-treated or α7(1–208)-immunized mice and supported mitochondria resistance to apoptosis by attenuating Ca^{2+}-stimulated cytochrome *c* release. Finally, NSE significantly improved episodic memory of mice impaired by either LPS treatment or immunization with α7(1–208). The results of our study demonstrate a therapeutic potential of NSE for prevention of cognitive disfunction caused by neuroinflammation or autoimmune reaction that allows suggesting this drug as a candidate for the treatment or prophylaxis of Alzheimer's pathology.

1. Introduction

Neuroinflammation is an important risk factor for neurodegenerative disorders like Alzheimer disease (AD). It was shown not only to accompany but to precede the development of cognitive symptoms in AD patients [1–2]. Correspondingly, non-steroid anti-inflammatory drugs (NSAID) were shown to decrease the risk of AD development in humans [3] and to prevent accumulation of amyloid-beta peptide Aβ(1–42) and appearance of cognitive symptoms in transgenic mice bearing the AD-related mutations [4].

Nicotinic acetylcholine receptors of α7 subtype (α7 nAChRs) expressed in the cells of monocyte and related origin are known to attenuate pro-inflammatory cytokine production [5]. Correspondingly, cholinergic anti-inflammatory pathway was found important in regulating inflammatory processes in various tissues, including the brain [5–7]. In addition, α7 nAChR subunits interact directly with amyloid-beta precursor protein (APP) and its processing products to ensure their proper metabolism [8–9]. Therefore, α7 nAChR deficiency favors inflammatory reactions and aberrant APP processing [10–11]. The α7 nAChRs are also expressed in the brain mitochondria to regulate the early events of mitochondria-driven apoptosis, and their deficiency decreases cell sustainability against apoptogenic stimuli that favors neurodegeneration [12–13].

Previously we found that neuroinflammation caused by regular injections of bacterial lipopolysaccharide (LPS) results in the decrease of α7 nAChR density and accumulation of Aβ(1–42) in the brain of mice accompanied by the impairment of episodic memory – symptoms characteristic for the early-stage AD. Similar symptoms were observed

Abbreviations: AD, Alzheimer disease; APP, amyloid-β precursor protein; BBB, blood-brain barrier; cyt *c*, cytochrome *c*; LPS, lipopolysaccharide; NAE, N-acylethanolamine; nAChR, nicotinic acetylcholine receptor; NSE, N-stearoylethanolamine

* Corresponding author.
E-mail address: skok@biochem.kiev.ua (M. Skok).

http://dx.doi.org/10.1016/j.intimp.2017.09.023
Received 14 June 2017; Received in revised form 12 September 2017; Accepted 25 September 2017

in mice immunized with recombinant extracellular domain α7(1–208) suggesting a critical role of α7 nAChRs [13]. Mitochondria of LPS-injected or α7(1–208)-immunized mice were less sustainable against Ca^{2+} apoptogenic effect and less sensitive to normalizing effect of α7 nAChR agonist PNU282987 [14]. This data formed a basis for the search of approaches to prevent neuropathogenic effects of either inflammation or α7(1–208)-specific immune reaction in the brain.

N-stearoylethanolamine (NSE) and other N-acylethanolamines (NAE) are biologically active cell membrane components possessing cannabimimetic activity [15–17]. Normally, mammals contain picomolar quantities of NAE, which can increase 10- to 100-fold upon pathological conditions [15,18–19]. NSE is of considerable interest for pharmacologists, because of its multiple therapeutic effects: ability to modulate immune functions [20], cause anti-inflammatory [21], membranoprotective and anxiolytic effects [19,22–23]. Other NAE were found to regulate permeability of the blood-brain barrier [24–26] and to influence the activity of various cell plasma membrane receptors and channels [27–29]. Interestingly, production of endogenous NAE was shown to be dependent on α7 nAChR activation [29]. Several studies describe the protective effects of NAE in the brain, in particular, against excitotoxic neuronal death [30], pain [31–33] and depression [23,34]. Our previous experiments demonstrated anti-inflammatory effect of NSE in the model of LPS-induced pulmonary inflammation [35–36], thermal skin burn [37] and neuroprotective effect in the model of chronic morphine dependence [38–39], as well as its anti-acetylcholine esterase and pro-cognitive activity in the model of cholinergic deficiency caused by scopolamine [40].

Taking into account the described pharmacological effects of NSE, we put an aim to test this drug in LPS-treated or α7(1–208)-immunized mice in order to study its potential protective effect against neuroinflammation, accumulation of amyloid-beta peptides and cognitive impairment caused by these interventions.

2. Materials and methods

2.1. Animals

We used female C57BL/6 J mice starting from 2 months of age. The mice were kept in the animal facility of Palladin Institute of Biochemistry, were housed in quiet, temperature-controlled room and were provided with water and food pellets *ad libitum*. Before removing the brain mice were sacrificed by cervical dislocation. All procedures complied with the ARRIVE guidelines, were carried out in accordance with the Directive 2010/63/EU for animal experiments and were approved by the Animal Care and Use Committee of Palladin Institute of Biochemistry.

2.2. Reagents

All reagents were of chemical grade and were purchased from Sigma-Aldrich unless specially indicated. NSE was synthesized in the Department of Lipid Biochemistry at Palladin Institute of Biochemistry as described previously [20]. Briefly, ethanolamine and stearic acid were co-condensed in argon athmosphere and upon defined temperature regime and NSE obtained was purified by re-crystallization in ethanol. The end product was identified as a single spot corresponding to NSE standard (S8439 from Sigma-Aldrich) by the thin layer chromatography in the solvent system chloroform: methanol: 25% ammonia (80: 20: 2).

Recombinant nAChR extracellular domain α7(1–208) produced in yeast was a kind gift of Prof. Socrates Tzartos from Hellenic Pasteur Institute, Athens, Greece. It was characterized previously [41,42] to display a significant ^{125}I-alpha-bungarotoxin-binding affinity, a similar secondary structure composition to that of the acetylcholine-binding protein, Torpedo α-nAChR-extracellular domain, and mouse α1-nAChR-extracellular domain and a well-defined tertiary structure. Antibodies against cytochrome *c*, α7(179–190) or α7(1–208) nAChR fragments were obtained and characterized in our laboratory [43–45]. The antibodies were biotinylated according to standard procedure [46]. Antibody against Aβ(1–42) and IL-6-specific ELISA kit were from Novex (Life Technologies), USA.

2.3. Animal treatment and brain samples preparation

To study the effect of NSE on LPS-induced cognitive and biochemical changes, two groups of mice, 10 animals per group, were injected intraperitoneally with 2 mg kg^{-1} LPS (*E. coli* strain 055:B5, purchased from Sigma-Aldrich) in 0.1 ml of saline, twice, with the interval of four weeks. NSE was given *per os* to one of the groups, 50 mg kg^{-1}, 50 μl per mouse during 9 days: 4 days before and 5 days after LPS injection. The dose of NSE, as well as the scheme of treatment have been optimized in previous experiments [36].

To study the effect of NSE in α7(1–208)-immunized mice, three groups of mice, 9 animals in each, were immunized intraperitoneally with 50 μg of recombinant α7(1–208) emulsified in 0.2 ml of complete Freund's adjuvant. The second immunization was performed after 21 days with the same dose of the antigen emulsified in incomplete Freund's adjuvant. One group of mice obtained NSE (50 mg kg^{-1}, 50 μl per mouse) *per os* 9 days daily before each immunization. Another group of animals obtained similar doses of NSE during 7 days after each immunization. The third group of mice got no NSE, and the fourth group was intact (no immunizations or LPS injections). The blood samples (20 μl) were taken from the tail vein of immunized mice on days 10 and 15 after the first immunization and on days 8 and 16 after the second immunization.

After the end of immunization/injection cycle, mice were examined in behavioral test and then sacrificed by cervical dislocation. The brains were removed and homogenized in a glass homogenizer. The primary preparation was fractionated into mitochondria and non-mitochondria by standard procedure of differential centrifugation as described [13,47]. Live mitochondria were further examined in functional test of cytochrome *c* (cyt *c*) release (see below), while the pellets of both mitochondria and non-mitochondria fractions were used to prepare the detergent lysates, as described previously [13]. Protein content was measured with the BCA kit (Thermo Scientific, France).

To study the antibody penetration into the brain mice were injected intraperitoneally with LPS (1.5 mg kg^{-1}) and the next day were injected intravenously with rabbit biotinylated α7(1–208)-specific antibody (200 μg per mouse). Another group of mice was pretreated with NSE (50 mg kg^{-1}, 50 μl per mouse) four days before and an hour after injecting LPS. Mice were sacrificed 15 min or 3 h after the antibody injection; their brains were removed and cut into two halves. One half was fixed in 4% paraformaldehyde and cut by vibratome into coronal 40 μm sections to be further studied by confocal microscopy. Another half was homogenized, lysed in detergent-containing buffer, as described elsewhere [13], and studied by ELISA for the presence of α7(1–208)-specific antibodies.

2.4. ELISA assays

To determine the level of α7 nAChR subunits within the brain or mitochondria preparations, the immunoplates (Nunc, Maxisorp) were coated with rabbit α7(1–208)-specific antibody (20 μg/ml), blocked with 1% BSA, and the detergent lysates of brain tissue or mitochondria were applied into the wells (1 μg of protein per 0.05 ml per well) for 2 h at 37 °C. The plates were washed with water and the second biotinylated α7(179–190)-specific antibody was applied for additional 2 h being revealed with Streptavidin-peroxidase conjugate and *o*-phenylendiamine-containing substrate solution. The specificity of such assay has been previously demonstrated by using the corresponding preparations of α7 −/− mice [12].

To determine the level of Aβ(1–42) bound to α7 nAChR, the plates

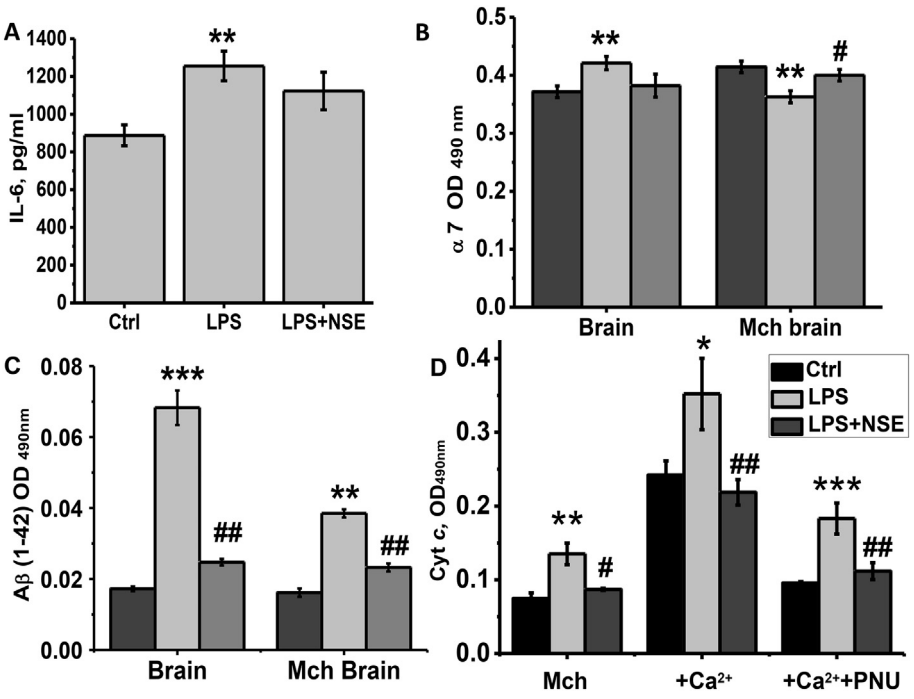

Fig. 1. The effect of NSE on the IL-6 level in the brain (A), α7 nAChR (B) or α7-bound Aβ(1–42) (C) levels in the brain and brain mitochondria of LPS-treated mice and on cyt c release from the brain mitochondria of LPS-treated mice stimulated with 9 μM Ca^{2+} in the presence or absence of 30 nM PNU282987. Columns correspond to Mean ± SE values, * - $p < 0.05$; ** - $p < 0.005$; *** - $p < 0.0005$ compared to the data of non-treated mice (Ctrl); # - $p < 0.05$; ## - $p < 0.005$ compared to the data of LPS-treated mice; $n = 6$.

were coated with α7(1–208)-specific antibody, and the α7-Aβ complex from the brain preparation applied as described above was revealed with biotinylated Aβ(1–42)-specific antibody, Streptavidin-peroxidase conjugate and *o*-phenylendiamine-containing substrate solution. The optical density was read at 490 nm using Stat-Fax 2000 ELISA Reader (Advanced Technologies, USA).

To determine the presence of α7(1–208)-specific antibodies in the mouse blood sera the immunoplates were coated with recombinant α7(1–208), 10 μg/ml in PBS, blocked with 1% BSA and washed by tap water before application of the sera diluted 1:50 in 0.05% Tween 20-containing PBS overnight at 4 °C. The bound antibodies were detected with peroxidase conjugate of anti-mouse IgG and *o*-phenylendiamine-containing substrate solution; the optical density was read at 490 nm.

To determine the presence of injected biotinylated α7(1–208)-specific antibodies in the brain preparations, the immunoplates were coated with recombinant α7(1–208), the brain detergent lysates were applied at 100 μg/ml and the bound antibodies were revealed with Streptavidin-peroxidase conjugate and *o*-phenylendiamine-containing substrate solution.

2.5. Cyt c release studies

The purified live mitochondria (120 μg of protein per ml) were incubated with either 9.0 μM $CaCl_2$ or 9.0 μM $CaCl_2$ and 30 nM PNU282987 for 2 min at room temperature and were immediately pelleted by centrifugation (10 min, 7000 g) at 4 °C. The incubation medium contained 10 mM HEPES, 125 mM KCl, 25 mM NaCl, 5 mM sodium succinate and 0.1 mM K_2PO_4, pH 7.4. The α7 nAChR agonist PNU282987 was applied just prior to Ca^{2+}. The mitochondria supernatants were collected and tested for the presence of cyt *c* by sandwich assay as described previously [45,47].

2.6. Measuring IL-6

The primary mouse brain homogenates were tested for the presence of IL-6 using the Mouse IL-6 Antibody Pair kit from Novex (Life Technologies), according to manufacturer's instructions.

2.7. Immunohistochemistry and confocal microscopy studies

The floated sections of mouse brains (presumably containing biotinylated antibodies) were incubated with Extravidin-Cy3 (Sigma-Aldrich, 1:200) in 1% BSA-containing PBS for 1 h, placed onto microscopic slides and embedded in Vectashield to be examined under Zeiss LSM 510 Meta confocal laser scanning microscope. The brain regions were identified according to Paxinos and Franklin [48].

2.8. Behavioral studies

Mice of all groups were tested in the "Novel Object Recognition" (NOR) behavioral test [13,49]. The results of NOR test are presented as Discrimination Index (DI) calculated as the difference in the number of "novel" and "famous" object explorations divided by the total number of explorations.

2.9. Statistical analysis

ELISA experiments have been performed in triplicates and mean values for individual mice were used for statistical analysis assessed using either the Student's *t*-test or Mann-Whitney test. Behavioral tests were also performed in triplicate for each mouse and mean values for individual mice were taken for statistical analysis. The data are presented as Mean ± SE and the difference was considered significant at $p < 0.05$. The data of Student's and Mann-Whitney tests were similar.

3. Results

3.1. The effect of NSE on LPS-induced changes in the brain of mice

LPS injections resulted in increase of IL-6 level in the brain of mice and treatment with NSE did not significantly decrease it (Fig. 1A). The level of α7 nAChR was slightly (about 13%) increased in the brain of LPS-treated mice and slightly (about 12%) decreased in the brain mitochondria; both changes tended to be prevented by NSE treatment (significantly for the brain mitochondria, Fig. 1B). In contrast, LPS-treated mice possessed dramatically elevated levels of α7-bound Aβ(1–42) in both mitochondria and non-mitochondria brain fractions;

they were diminished almost to the control level in LPS + NSE-treated mice (Fig. 1C). In functional assay, the brain mitochondria of LPS-treated mice released much more cyt *c* in response to 9 μM Ca^{2+} and were less sensitive to attenuating effect of α7-specific agonist PNU282987 than mitochondria of control mice; moreover small amount of cyt *c* was released even without Ca^{2+} demonstrating impaired integrity of mitochondria under LPS effect. NSE treatment prevented all these changes and returned the studied parameters to control levels (Fig. 1D).

3.2. The effect of NSE on the integrity of the blood-brain barrier disturbed by LPS

The brain is normally protected from penetration of high molecular weight substances, like antibodies, by tightly connected vascular endothelial cells forming the blood-brain barrier (BBB). Inflammatory stimuli use to loosen the tight intercellular junctions making the BBB permeable and allowing the antibody penetration into the brain parenchyma [13]. To study whether NSE affects the BBB integrity, we injected biotinylated α7(1–208)-specific antibodies into the tail vein of mice, either intact of pre-injected with LPS a day before; a group of LPS-treated mice obtained NSE before and just after the LPS injection. Mice were sacrificed in either 15 min or 3 h after the antibody injection and their brains were examined for the presence of biotinylated antibodies by ELISA and immunohistochemistry. As shown in Fig. 2A, no antibody was found by ELISA in the brain preparations of non-treated mice. LPS pre-treatment resulted in the antibody appearance in the soluble brain fraction (supernatant) 15 min after injection and in the brain tissue (detergent lysate of the pellet) 3 h after injection. NSE significantly decreased the detected antibody levels in both brain fractions.

To check which brain regions were mostly affected by NSE, we performed biotin-specific staining and confocal microscopy examination of the brain slices of antibody-injected mice. As shown in Fig. 2 B-D, biotinylated α7(1–208)-specific antibodies were detected in various brain regions 3 h after the antibody injection. The non-specific rabbit IgG injected into LPS-treated mice also penetrated through the walls of the brain vessels but did not stain the cells within the brain parenchyma ([13], data not shown here). NSE prevented antibody penetration into *striatum radiatum* and *striatum oriens* area of the hippocampus (Fig. 2B), and to the frontal cortex internal granular layer (Fig. 2C) and also slightly decreased the antibody staining in *striatum* and *medulla* (data not shown). In contrast, no visible difference between NSE-treated and non-treated mice was found in pyramidal layer of the hippocampus CA3 zone (Fig. 2B), in internal pyramidal layer of the frontal cortex (Fig. 2D) and in *thalamus-habenula* region (data not shown).

3.3. The effect of NSE on the antibody production and on immunization-induced changes in the brain and behavior of mice

NSE slowed down the antibody production in α7(1–208)-immunized mice. However, by day 35, the level of α7(1–208)-specific antibodies in the blood serum was similar in NSE-treated and non-treated mice (Fig. 3A). α7(1–208)-immunized mice demonstrated decreased α7 nAChR levels in the brain and brain mitochondria; the decrease was significantly attenuated by NSE pre-treatment (Fig. 3B). The brains and brain mitochondria of α7(1–208)-immunized mice contained elevated amount of α7-bound Aβ(1–42), which was decreased to almost control level by NSE pre-treatment and, less, post-treatment (Fig. 3C). In contrast to LPS treatment, immunization with α7(1–208) did not affect the brain mitochondria sensitivity to 9 μM Ca^{2+}; nevertheless, NSE decreased the level of cyt *c* released under the effect of Ca^{2+} or Ca^{2+} + PNU282987 and even in mitochondria of control mice (Fig. 3D).

Finally, mice either treated with LPS or immunized with α7(1–208) demonstrated significantly worse episodic memory compared to non-treated mice. NSE restored the memory of immunized mice (pre-treatment being again more effective than post-treatment) and tended to restore it in LPS-treated mice (Fig. 4A–B).

4. Discussion

The data presented here demonstrate that either LPS treatment or immunization with extracellular domain of α7 nAChR subunit resulted in significant changes in the brain and behavior of mice. This is in accord with our previously published data showing that α7(1–208)-specific antibodies generated *in vivo* produced an inflammation-like effect

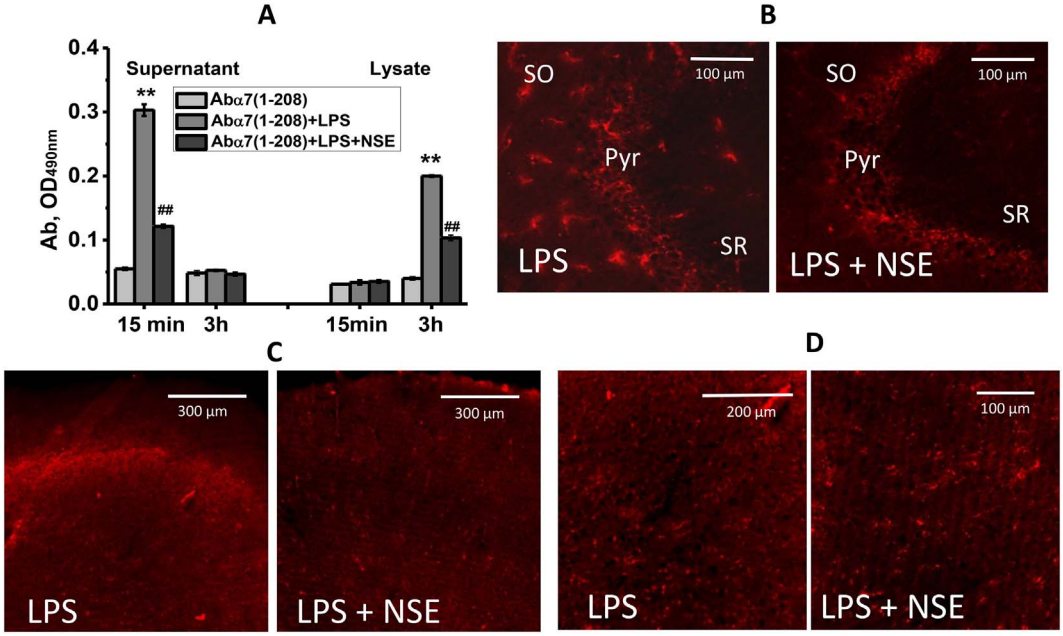

Fig. 2. The effect of NSE on the α7(1–208)-specific antibody penetration into the brain studied by ELISA (A) or confocal microscopy (B–D) in the hippocampus (B) and frontal cortex internal granular (C) or pyramidal layer (D) 3 h after the antibody injection. SO – *striatum oriens*, SR – *striatum radiatum*, *Pyr* – *pyramidal layer of the hippocampus*. Columns in (A) correspond to Mean ± SE values. ** - $p < 0.005$ compared to LPS-non-treated mice; ## - $p < 0.005$ compared to LPS-treated mice; $n = 3$.

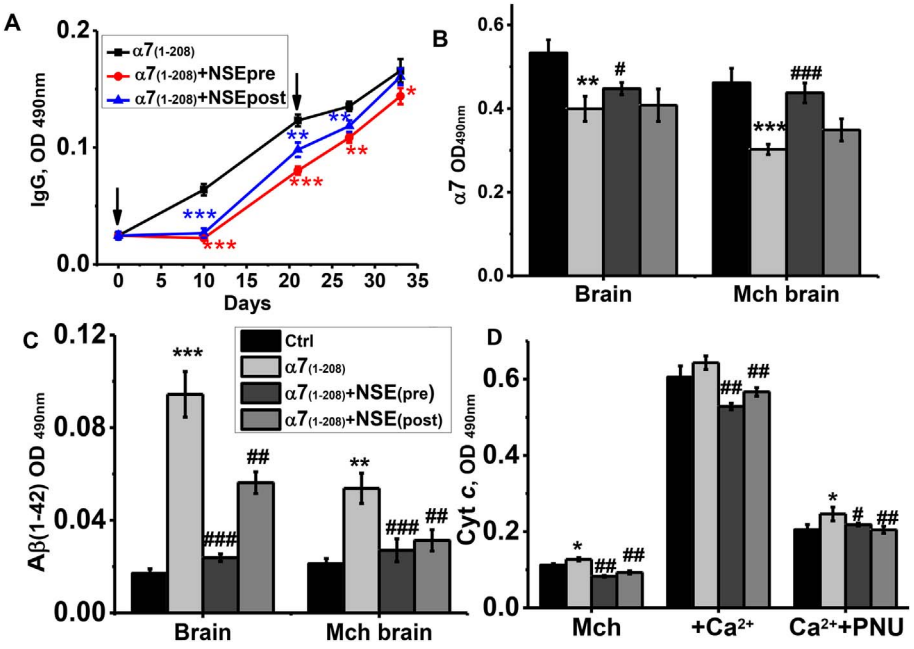

Fig. 3. The effect of NSE pre- or post-treatment on the α7(1–208)-specific IgG level in the blood sera of α7(1–208)-immunized mice (A), on the α7 nAChR (B) and α7-bound Aβ(1–42) (C) levels in the brain or brain mitochondria of α7(1–208)-immunized mice and on the sensitivity of brain mitochondria to 9 μM Ca^{2+} in the presence or absence of 30 nM PNU282987 (D). Points (A) and columns (B-D) correspond to Mean ± SE values, $n = 9$. A: * - $p < 0.05$; ** - $p < 0.005$; *** - $p < 0.0005$ compared to corresponding values of NSE-non-treated mice. Arrows indicate the time points of the antigen injections. B-D: * - $p < 0.05$; ** - $p < 0.005$; *** - $p < 0.0005$ compared to corresponding values of non-immunized mice (Ctrl); # - $p < 0.05$; ## - $p < 0.005$; ### - $p < 0.0005$ compared to corresponding values of α7(1–208)-immunized NSE-non-treated mice.

in mice resulting in accumulation of Aβ peptides, mitochondria damage and episodic memory impairment [13–14]. The α7 nAChRs are known to be involved in regulating pro-inflammatory cytokines production [5–6], and high LPS doses resulted in the decrease of both α7 RNA and protein levels [50]. The α7(1–208)-specific antibody could decrease the level of α7 nAChR protein by inducing its internalization and catabolism; in addition, as it was shown with single-chain α7-specific antibodies, the antibody binding stimulated pro-inflammatory IL-6 production in astrocyte-derived human cell line [51].

The LPS dose used in these experiments (2 mg kg^{-1}) affected significantly, but not dramatically, the α7 nAChR expression in the brain and brain mitochondria and made mitochondria much more sensitive to apoptogenic effect of Ca^{2+}. In contrast, immunization with α7(1–208) dramatically decreased α7 nAChR levels in the brain and brain mitochondria, but did not affect mitochondria sensitivity to Ca^{2+}. These data indicate that inflammation caused by LPS injection or immune reaction stimulated by α7(1–208) injection affected mice in different ways. Nevertheless, both treatments stimulated considerable accumulation of α7-bound Aβ(1–42) and dramatically decreased episodic memory of mice. Therefore, memory was mostly affected by Aβ(1–42) accumulation in the brain and was less dependent on the level of α7 nAChR or the state of mitochondria.

Numerous data demonstrate that Aβ peptides are involved in pathogenesis of AD [52–53]. They are oligomerized to form extracellular senile plaques affecting interneuronal contacts in the brain [54]. Being accumulated intracellularly, they affect mitochondria functions leading to reactive oxygen species formation, mitochondrial membrane depolarization, mitochondria swelling and cytochrome *c* release [55–58]. Aβ(1–42) was shown to interact directly with α7 nAChRs [8–9]. In our experiments, the level of α7-bound Aβ(1–42) was increased in both the brain and brain mitochondria of LPS-treated or α7(1–208)-immunized mice that is in accord with our previously published data [13–14]. Given almost similar α7 nAChR levels found in LPS-treated and non-treated mice and decreased α7 levels in α7(1–208)-immunized mice, this data demonstrate a significant increase in Aβ(1–42) production under the effect of either LPS or immunization, independently on the α7 nAChR level.

NSE effectively prevented the Aβ(1–42) accumulation and subsequent memory decline in both experimental schemes used.

NSE is a natural component of mammalian cell membranes, along with other NAE, like N-palmithoylethanolamine, N-oleylethanolamine and N-linoleylethanolamine, which are important and physiologically relevant mediators of cell protection against various pathological conditions [15–6,18,20,36–37]. The mechanisms of NAE activity include either direct incorporation into the cell membranes or interaction with specific cell receptors: cannabinoid, vanyloid, nuclear PPAR [59–61]. As a result, NAE can stabilize cell membranes and influence the activity of multiple calcium, potassium, chloride and ligand-gated ion channels, including α7 nAChRs [27–29]. However, NSE effects found here don't seem to be α7 nAChR-specific, because similar improvements were found in another experimental model of cognitive pathology caused by scopolamine treatment and, therefore, related to muscarinic

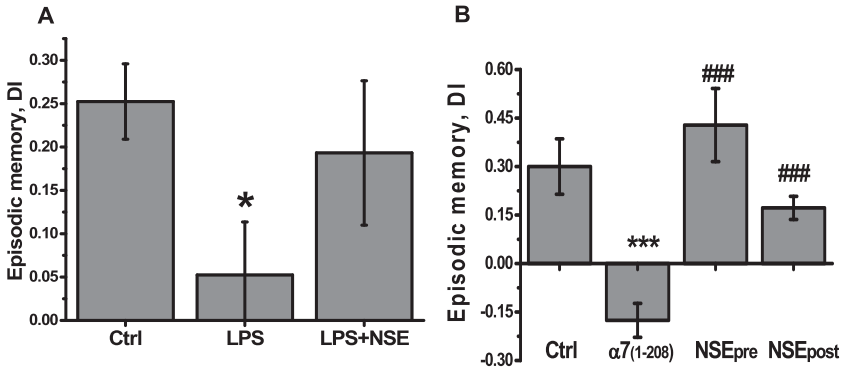

Fig. 4. The effect of NSE on episodic memory of LPS-treated ($n = 10$, A) or α7(1–208)-immunized mice ($n = 7$, B). DI – discrimination index. Columns correspond to Mean ± SE values; * - $p < 0.05$; *** - $p < 0.0005$ compared to non-treated mice (Ctrl); ### - $p < 0.0005$ compared to α7(1–208)-immunized NSE-non-treated mice.

acetylcholine receptors [40]. Most probably, positive effects of NSE are due to its anti-inflammatory and membrane stabilizing activities, which is quite evident from the data presented here.

We did not observe a significant decrease of pro-inflammatory IL-6 in the brain under the effect of NSE. However, previous experiments demonstrated its peripheral anti-inflammatory action [35–37]. Here we show that NSE pre-treatment slowed down the antibody response in α7(1–208)-immunized mice. The α7(1–208)-specific IgG antibody appeared in the blood of non-treated mice quite early (in 10 days) after the primary antigen injection, suggesting that immunization activated memory B lymphocytes. The extracellular domain of α7 nAChR subunit is highly homologous between mammalian species [62], therefore, human recombinant α7(1–208) introduced at the inflammatory background could stimulate “sleeping” autoimmune B cell clones. NSE prevented or at least slowed down such stimulation, obviously, due to anti-inflammatory effect.

The membrane-stabilizing effect of NSE was evident in experiments dealing with the integrity of the BBB. At the time of brain examination NSE-treated and non-treated α7(1–208)-immunized mice contained almost equal amounts of α7(1–208)-specific IgG antibodies. However, negative effects of these antibodies were observed only (mainly) in NSE-non-treated mice. We found that NSE prevented antibody penetration into the frontal cortex and hippocampus, the areas involved in cognition and memory, that could explain undisturbed episodic memory of NSE-treated compared to non-treated α7(1–208)-immunized mice.

In addition, NSE was shown to possess cannabimimetic activity, although its effects were not attributed to activation of cannabinoid receptors [17]. Nevertheless, the results of our study are strikingly similar to the described ability of phytocannabinoid cannabidol to reduce cognitive AD symptoms in pharmacological and transgenic models in rodents by exhibiting anti-inflammatory, anti-oxidant and neuroprotective properties [63–64]. Moreover, dysregulation of endocannabinoid-eicosanoid network supported pathogenesis in a mouse model of Alzheimer's disease [65]. These data indicate that cannabimimetic properties of NSE should also be taken into account in relation to its pharmacological activity. Similarly, we cannot exclude the anti-acetylcholine esterase activity of NSE found in previous experiments [40] that would favor stimulation of both muscarinic and nicotinic AChRs in the brain.

5. Conclusion

The results of our study demonstrate a therapeutic potential of NSE for prevention (prophylaxis) of cognitive disfunction caused by neuroinflammation or autoimmune reaction that allows suggesting this drug for further pre-clinical studies in AD models.

Funding information

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgements

We are grateful to Dr. S. Karakhim for the help in confocal microscopy studies.

References

[1] M.T. Heneka, M.J. Carson, J.E. Khoury, et al., Neuroinflammation in Alzheimer's disease, Lancet Neurol. 14 (4) (2015) 388–405.
[2] F.L. Heppner, R.M. Ransohoff, B. Becher, Immune attack: the role of inflammation in Alzheimer disease, Nat. Rev. Neurosci. 16 (6) (2015) 358–372.
[3] C.A. Szekely, T. Town, P.P. Zandi, NSAIDs for the chemoprevention of Alzheimer's disease, Subcell. Biochem. 42 (2007) 229–248.
[4] C. Cunningham, D.T. Skelly, Non-steroidal anti-inflammatory drugs and cognitive function: are prostaglandins at the heart of cognitive impairment in dementia and delirium? J. NeuroImmune Pharmacol. 7 (1) (2012) 60–73.
[5] W.J. De Jonge, L. Ulloa, The alpha7 nicotinic acetylcholine receptor as a pharmacological target for inflammation, Br. J. Pharmacol. 151 (2007) 915–929.
[6] L.D. Truong, J. Trostel, G.E. Garcia, Absence of nicotinic acetylcholine receptor α7 subunit amplifies inflammation and accelerates onset of fibrosis: an inflammatory kidney model, FASEB J. 29 (8) (2015) 3558–3570.
[7] N. Terrando, T. Yang, J.K. Ryu, et al., Stimulation of the α7 nicotinic acetylcholine receptor protects against neuroinflammation after tibia fracture and endotoxemia in mice, Mol. Med. 20 (2015) 667–675.
[8] H.Y. Wang, D.H. Lee, M.R. D'Andrea, P.A. Peterson, R.P. Shank, A.B. Reitz, β-Amyloid 1–42 binds to α7 nicotinic acetylcholine receptor with high affinity. Implications for Alzheimer's disease pathology, J. Biol. Chem. 275 (2000) 5626–5632.
[9] H.R. Parri, K.T. Dineley, Nicotinic acetylcholine receptor interaction with β-amyloid: molecular, cellular, and physiological consequences, Curr. Alzheimer Res. 7 (1) (2010) 27–39.
[10] C.M. Hernandez, R. Kayed, H. Zheng, J.D. Sweatt, K.T. Dineley, Loss of alpha7 nicotinic receptors enhances beta-amyloid oligomer accumulation, exacerbating early-stage cognitive decline and septohippocampal pathology in a mouse model of Alzheimer's disease, J. Neurosci. 30 (7) (2010) 2442–2453.
[11] R. Ni, A. Marutle, A. Nordberg, Modulation of α7 nicotinic acetylcholine receptor and fibrillar amyloid-β interactions in Alzheimer's disease brain, J. Alzheimers Dis. 33 (3) (2013) 841–851.
[12] O. Lykhmus, G. Gergalova, L. Koval, M. Zhmak, S. Komisarenko, M. Skok, Mitochondria express several nicotinic acetylcholine receptor subtypes to control various pathways of apoptosis induction, Int. J. Biochem. Cell Biol. 53 (2014) 246–252.
[13] O. Lykhmus, L. Voytenko, L. Koval, S. Mykhalskiy, V. Kholin, K. Peschana, et al., α7 Nicotinic acetylcholine receptor-specific antibody induces inflammation and amyloid β42 accumulation in the mouse brain to impair memory, PLoS One 10 (3) (2015) e0122706.
[14] O. Lykhmus, G. Gergalova, M. Zouridakis, S. Tzartos, S. Komisarenko, M. Skok, Inflammation decreases the level of alpha7 nicotinic acetylcholine receptors in the brain mitochondria and makes them more susceptible to apoptosis induction, Int. Immunopharmacol. 29 (1) (2015) 148–151.
[15] H.H.O. Schmid, P.C. Schmid, V. Natarajan, N-acylated glycerophospholipids and their derivatives, Prog. Lipid Res. 29 (1990) 1–43.
[16] H.H.O. Schmid, Cannabinoid receptor-inactive N-acylethanolamines and other fatty acid amides: metabolism and function, Prostaglandins Leukot. Essent. Fatty Acids 66 (2, 3) (2002) 363–376.
[17] M. Maccarrone, A. Cartoni, D. Parolaro, A. Margonelli, P. Massi, M. Bari, et al., Cannabimimetic activity, binding, and degradation of stearoylethanolamide with in the mouse central nervous system, Mol. Cell. Neurosci. 21 (1) (2002) 126–140.
[18] H.S. Hansen, B. Moesgaard, H.H. Hansen, A. Schousboe, G. Petersen, Formation of N-acyl-phosphatitylethanolamine and N-acylethanolamine (including anandamide) during glutamate - induced neurotoxicity, Lipids 34 (1999) S327–S330.
[19] E.V. Berdyshev, P.C. Shmid, Z. Dong, H.H.O. Schmid, Stress - induced generation of N - acylethanolamines in mouse epidermal JB6 P^+ cells, Biochem. J. 346 (2) (2000) 369–374.
[20] N.M. Hula, A.A. Chumak, O.F. Mehed, T.M. Horid'ko, N.L. Kindruk, A.H. Berdyshev, Immunosuppressive characteristics of N-stearoylethanolamine a stable compound with cannabimimetic activity, Ukr. Biokhim. Zh. 80 (1) (2008) 57–67 (Article in Ukrainian).
[21] J. Lo Verme, J. Fu, G. Astarita, G. La Rana, R. Russo, A. Calignano, et al., The nuclear receptor peroxisome proliferator-activated receptor-alpha mediates the anti-inflammatory actions of palmitoylethanolamide, Mol. Pharmacol. 67 (1) (2005) 15–19.
[22] N.M. Gulaya, A.I. Kuzmenko, V.M. Margitich, N.M. Govseeva, S.D. Melnichuk, T.M. Goridko, et al., Long-chain N-acylethanolamines inhibit peroxidation in rat liver mitochondria under acute hypoxic hypoxia, Chem. Phys. Lipids 97 (1998) 49–54.
[23] R. Crupi, I. Paterniti, A. Ahmad, Effects of palmitoylethanolamide and luteolin in an animal model of anxiety/depression, CNS Neurol. Disord. Drug Targets 12 (7) (2013) 989–1001.
[24] L. Mestre, P.M. Inigo, M. Mecha, F.G. Correa, M. Hernangómez-Herrero, F. Loría, et al., Anandamide inhibits Theiler's virus induced VCAM-1 in brain endothelial cells and reduces leukocyte transmigration in a model of blood brain barrier by activation of CB(1) receptors, J. Neuroinflammation 8 (2011) 102–107.
[25] W.H. Hind, C. Tufarelli, M. Neophytou, S.I. Anderson, T.J. England, S.E. O'Sullivan, Endocannabinoids modulate human blood-brain barrier permeability in vitro, Br. J. Pharmacol. 172 (12) (2015) 3015–3027.
[26] P.S. Katz, J.K. Sulzer, R.A. Impastato, S.X. Teng, E.K. Rogers, P.E. Molina, Endocannabinoid degradation inhibition improves neurobehavioral function, blood-brain barrier integrity, and neuroinflammation following mild traumatic brain injury, J. Neurotrauma 32 (5) (2015) 297–306.
[27] M. Oz, Receptor-independent effects of endocannabinoids on ion channels, Curr. Pharm. Des. 12 (2) (2006) 227–239.
[28] S.P. Alexander, D.A. Kendall, The complications of promiscuity: endocannabinoid action and metabolism, Brit. J. Pharmacol. 152 (5) (2007) 602–623.
[29] M. Melis, S. Scheggi, G. Carta, C. Madeddu, S. Lecca, A. Luchicchi, et al., PPARα regulates cholinergic-driven activity of midbrain dopamine neurons via a novel mechanism involving α7 nicotinic acetylcholine receptors, J. Neurosci. 33 (14) (2013) 6203–6211.
[30] S.D. Skaper, A. Buriani, R. Dal Toso, L. Petrelli, S. Romanello, L. Facci, et al., The ALIAmide palmitoylethanolamide and cannabinoids, but not anandamide, are

protective in a delayed postglutamate paradigm of excitotoxic death in cerebellar granule neurons, Proc. Natl. Acad. Sci. U. S. A. 93 (9) (1996) 3984–3989.
[31] A. Calignano, G. La Rana, A. Giuffrida, D. Piomelli, Control of pain initiation by endogenous cannabinoids, Nature 394 (6690) (1998) 277–281.
[32] N. Ghafouri, B. Ghafouri, B. Larsson, N. Stensson, C.J. Fowler, B. Gerdle, Palmitoylethanolamide and stearoylethanolamide levels in the interstitium of the trapezius muscle of women with chronic widespread pain and chronic neck-shoulder pain correlate with pain intensity and sensitivity, Pain 154 (9) (2013) 1649–1658.
[33] J.M. Hesselink, Evolution in pharmacologic thinking around the natural analgesic palmitoylethanolamide: from nonspecific resistance to PPAR-α agonist and effective nutraceutical, J. Pain Res. 8 (6) (2013) 625–634.
[34] I. Smaga, B. Bystrowska, D. Gawliński, B. Pomierny, P. Stankowicz, M. Filip, Antidepressants and changes in concentration of endocannabinoids and N-acylethanolamines in rat brain structures, Neurotox. Res. 26 (2) (2014) 190–206.
[35] E. Berdyshev, E. Boichot, M. Corbel, N. Germain, V. Lagente, Effects of cannabinoid receptor ligands on LPS-induced pulmonary inflammation in mice, Life Sci. 63 (8) (1998) 125–129.
[36] A.G. Berdyshev, H.V. Kosiakova, O.V. Onopchenko, R.R. Panchuk, R.S. Stoika, N.M. Hula, N-Stearoylethanolamine suppresses the pro-inflammatory cytokines production by inhibition of NF-κB translocation, Prostaglandins Other Lipid Mediat. 121 (A) (2015) 91–96.
[37] O.D. Zhukov, A.H. Berdyshev, H.V. Kosiakova, V.M. Klimashevs'kyĭ, T.M. Horid'ko, O.F. Mehed, et al., N-stearoylethanolamine effect on the level of 11-hydroxycorticosteroids, cytokines IL-1, IL-6 and TNFalpha in rats with nonspecific inflammation caused by thermal burn of skin, Ukr. Biokhim. Zh. 86 (3) (2014) 88–97 (Article in Ukrainian).
[38] N.M. Hula, V.M. Marhitych, M.V. Artamonov, O.D. Zhukov, T.M. Horid'ko, V.M. Klimashevs'kyĭ, Neuroprotective effect of N-acylethanolamines in chronic morphine dependence. I. Rat brain phospholipids as a target of their action, Ukr. Biokhim. Zh. 76 (5) (2004) 123–131 (Article in Ukrainian).
[39] N.M. Hula, M.F. Huliĭ, N.K. Kharchenko, T.M. Horid'ko, V.M. Marhitych, Neuroprotective effect of N-acylethanolamines in chronic morphine dependence. III. Influence on the content of neurotransmitters in the rat brain, Ukr. Biokhim. Zh. 7 (1) (2005) 47–51.
[40] T.M. Horid'ko, O.F. Mehed, H.V. Kosiakova, A.H. Berdyshev, V.O. Kholin, K.O. Peschana, et al., The effect of N-stearoylethanolamine on the antioxidant enzymes, content of nitric oxide and the activity of acetylcholinesterase in the blood plasma and different parts of the brain of female rats under the scopolamine action, Ukr. Biokhim. Zh. 86 (5, Supplement 2) (2014) 10–11.
[41] M. Zouridakis, P. Zisimopoulou, E. Eliopoulos, K. Poulas, S.J. Tzartos, Design and expression of human α7 nicotinic acetylcholine receptor extracellular domain mutants with enhanced solubility and ligand-binding properties, Biochim. Biophys. Acta 1794 (2009) 355–366.
[42] O. Lykhmus, L. Koval, D. Pastuhova, M. Zouridakis, S. Tzartos, S. Komisarenko, M. Skok, The role of carbohydrate component of recombinant α7 nicotinic acetylcholine receptor extracellular domain in its immunogenicity and functional effects of resulting antibodies, Immunobiology 221 (12) (2016 Dec) 1355–1361, http://dx.doi.org/10.1016/j.imbio.2016.07.012.
[43] M.V. Skok, L.P. Voitenko, S.V. Voitenko, E.Y. Lykhmus, E.N. Kalashnik, T. Litvin, et al., Study of α subunit composition of nicotinic acetylcholine receptor in the neurons of autonomic ganglia of the rat with subunit-specific anti-α(181–192) peptide antibodies, Neuroscience 93 (4) (1999) 1437–1446.
[44] O. Lykhmus, L. Koval, S. Pavlovych, M. Zouridakis, P. Zisimopoulou, S. Tzartos, et al., Functional effects of antibodies against non-neuronal nicotinic acetylcholine receptors, Immunol. Lett. 128 (2010) 68–73.
[45] G. Gergalova, O. Lykhmus, S. Komisarenko, M. Skok, α7 Nicotinic acetylcholine receptors control cytochrome c release from isolated mitochondria through kinase-mediated pathways, Int. J. Biochem. Cell Biol. 49 (2014) 26–31.
[46] E. Harlow, D. Lane, Antibodies. A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988, pp. 341–342.
[47] G. Gergalova, O. Lykhmus, O. Kalashnyk, L. Koval, V. Chernyshov, E. Kryukova, et al., Mitochondria express α7 nicotinic acetylcholine receptors to regulate Ca^{2+} accumulation and cytochrome c release: study on isolated mitochondria, PLoS One 7 (2) (2012) e31361.
[48] G. Paxinos, K.B.J. Franklin, The Mouse Brain in Stereotaxic Coordinates, Academic Press, New York, 2001.
[49] M. Antunes, G. Biala, The novel object recognition memory: neurobiology, test procedure, and its modifications, Cogn. Process. 13 (2012) 93–110.
[50] O. Lykhmus, N. Mishra, L. Koval, O. Kalashnyk, G. Gergalova, K. Uspenska, S. Komisarenko, H. Soreq, M. Skok, Molecular mechanisms regulating LPS-induced inflammation in the brain, Front. Mol. Neurosci. 9 (2016) 19.
[51] O.M. Kalashnyk, O.Yu. Lykhmus, O.A. Oliinyk, S.V. Komisarenko, M.V. Skok, α7 nAChR-specific antibodies stimulate pro-inflammatory reaction in human astrocytes through p38-dependent pathway, Int. Immunopharmacol. 23 (2) (2014) 475–479.
[52] W. Jongbloed, K.A. Bruggink, M.I. Kester, et al., Amyloid-β oligomers relate to cognitive decline in Alzheimer's disease, Alzheimer Dis. 45 (1) (2015) 35–43.
[53] A. Garcia-Osta, C.M. Alberini, Amyloid beta mediates memory formation, Learn. Mem. 16 (4) (2009) 267–272.
[54] G.K. Gouras, C.G. Almeida, R.H. Takahashi, Intraneuronal Abeta accumulation and origin of plaques in Alzheimer's disease, Neurobiol. Aging 26 (9) (2005) 1235–1244.
[55] M. Faizi, E. Seydi, S. Abarghuyi, A. Salimi, S. Nasoohi, J. Pourahmad, A search for mitochondrial damage in Alzheimer's disease using isolated rat brain mitochondria, Iran. J. Pharm. Res. 15 (2016) 185–195 (Suppl.).
[56] P.M. Schaefer, B. von Einem, P. Walther, E. Calzia, C.A. von Arnim, Metabolic characterization of intact cells reveals intracellular amyloid beta but not its precursor protein to reduce mitochondrial respiration, PLoS One 11 (12) (2016) e0168157.
[57] N. Mamada, D. Tanokashira, K. Ishii, A. Tamaoka, W. Araki, Mitochondria are devoid of amyloid β-protein (Aβ)-producing secretases: Evidence for unlikely occurrence within mitochondria of Aβ generation from amyloid precursor protein, Biochem. Biophys. Res. Commun. 486 (2) (2017) 321–328.
[58] M.S. Arrázola, E. Ramos-Fernández, P. Cisternas, D. Ordenes, N.C. Inestrosa, Wnt signaling prevents the aβ oligomer-induced mitochondrial permeability transition pore opening preserving mitochondrial structure in hippocampal neurons, PLoS One 12 (1) (2017) e0168840.
[59] W.A. Devane, L. Hanus, A. Breuer, R.G. Pertwee, L.A. Stevenson, G. Griffin, et al., Isolation and structure of a brain constituent that binds to the cannabinoid receptor, Science 258 (1992) 1946–1949.
[60] P. Movahed, B.A. Jцnsson, B. Birnir, J.A. Wingstrand, T.D. Jørgensen, A. Ermund, et al., Endogenous unsaturated C18 N-acylethanolamines are vanilloid receptor (TRPV1) agonists, J. Biol. Chem. 280 (46) (2005) 38496–38504.
[61] S.E. O'Sullivan, Cannabinoids go nuclear: evidence for activation of peroxisome proliferator-activated receptors, Br. J. Pharmacol. 152 (2007) 576–582.
[62] J. Lindstrom, Neuronal nicotinic acetylcholine receptors, in: T. Narahashi (Ed.), Ion Channels, vol. 4, Plenum Press, New York, 1996, pp. 377–449.
[63] S.E. O'Sullivan, D.A. Kendall, Cannabinoid activation of peroxisome proliferator-activated receptors: potential for modulation of inflammatory disease, Immunobiology 215 (8) (2010) 611–616.
[64] G. Watt, T. Karl, In vivo evidence for therapeutic properties of cannabidiol (CBD) for Alzheimer's disease, Front. Pharmacol. 8 (2017) 20.
[65] J.R. Piro, D.I. Benjamin, J.M. Duerr, Y. Pi, C. Gonzales, K.M. Wood, J.W. Schwartz, D.K. Nomura, T.A. Samad, A dysregulated endocannabinoid-eicosanoid network supports pathogenesis in a mouse model of Alzheimer's disease, Cell Rep. 1 (6) (2012) 617–623.