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Research report

Overexpression of NMDAR2B in an inflammatory model of Alzheimer's disease: Modulation by NOS inhibitors

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ABSTRACT

Background: Alzheimer's disease (AD) is a common form of age-related dementia, characterized by deposition of amyloid Aβ plaques, neuroinflammation and neurodegeneration. N-methyl-D-aspartate receptors (NMDAR) are postsynaptic glutamate receptors that play a role in memory formation and are targets for memantadine, an anti-AD drug. Nitric oxide (NO) signaling has been involved in both memory development through neuronal NO synthase (nNOS), and neuroinflammation through inducible NO synthase (iNOS) which mediates CNS inflammatory processes.
Aim: To study the expression of the NMDAR2B subunit in an inflammatory model of AD before and after treatment with NO modulators.
Materials and methods: AD was induced in mice by a single dose of lipopolysaccharide (LPS). Behavioral tests for spatial and non-spatial memories and locomotor activity were performed to assess disease severity and progression. The effects of L-NAME (general NOS inhibitor), 1400W (iNOS inhibitor), diflunisal (systemic anti-inflammatory drug that does not cross the blood brain barrier), and L-arginine, the substrate for NOS was determined. Immunohistochemistry was done to confirm AD and brain lysates were tested for Aβ formation, levels of NMDAR2B subunits, and brain NO levels.
Results: Systemic LPS induced AD, as shown by cognitive impairment; increased levels of Aβ and concomitant increase in the brain NO concentrations. This was associated with overexpression of NMDAR2B. All tested drugs improved behavioral dysfunction, prevented Aβ formation and NMDAR overexpression, and lead to decrease in NO concentration in the brain. L-Arginine alone, however, did not produce similar improvements.
Conclusion: NMDAR2B subunits are overexpressed in an inflammatory model of AD and NO inhibitors ameliorate this expression.

1. Introduction

AD is a neurodegenerative disorder affecting regions in the brain responsible for memory and cognition. It is accompanied by prominent neuroinflammation and biochemically is characterized by the presence of amyloid beta (Aβ) plaques and neurofibrillary tangles (NFT) (Medeiros et al., 2011).

LPS induces progressive neurotoxicity through binding to Toll-like receptor-4 (TLR-4) on the microglial cells causing the release of variable neurotoxic inflammagens as TNF-α, IL-6, NO and PGE_2. Thus, LPS is an accepted model for AD suffering from memory impairment and amyloidogenesis and is applicable for the study of neuroinflammation and neuroinflammation-associated pathogenesis of AD (Lee et al., 2012; Liu and Bing, 2011).

NO is a highly diffusible molecule with both neuroprotective and neurotoxic properties. It is directly related to L-glutamate and NMDA receptors in the CNS (Benarroch, 2011) and thus to long-term potentiation (LTP).

NMDAR are tetrameric ionotropic glutamate receptors that allow Ca^{2+} influx. GluN1 makes up two of the four subunits and the other two vary according to the location and the stage of development. GluN2A and GluN2B are the most predominant suggesting

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that they play a role in the synaptic function and plasticity (Paoletti et al., 2013).

The drugs used in this study were L-NG-nitroarginine methyl ester (L-NAME), N-((3-(aminomethyl)phenyl]methyl)-ethanimidamide, dihydrochloride (1400W), diflunisal and L-arginine. L-NAME is a competitive inhibitor of the 3 NOSs with higher potency as an inhibitor of eNOS and nNOS (K_i = 60 nM and 90 nM, respectively) than for the iNOS (K_i = 1.4 μM) (Pfeiffer et al., 1996). 1400W is a selective inhibitor for iNOS (K_i = 2 nM) over the nNOS and eNOS (K_i is 2 μM and 50 μM) (Garvey et al., 1997). Diflunisal is a salicylate anti-inflammatory analgesic that does not pass the blood brain barrier (Nuernberg et al., 1991). L-Arginine is the substrate for the NOS enzymes. Several studies had shown that the level of L-arginine decreases in old age and in AD (Yi et al., 2009) and that its increase is responsible for improving memory (Rayatnia et al., 2011; Yildiz Akar et al., 2009).

The aim of the study was to study the effect of AD on the expression of NMDAR2B and the role of NO modulators on this expression. L-Arginine was reported to decrease in regions in the brain involved with memory formation such as the entorhinal and postrhinal cortices with old age (Rushaidhi et al., 2012), so effect of increasing its serum level was tested, as it is the source of endogenous NO.

2. Experimental procedures

2.1. Animals

Adult male mice weighing 25–30 g between 3 and 4 months old were purchased from the National Research Centre (NRC), Giza Cairo. The mice were kept in a temperature-controlled room 23–24 °C with a 12-h dark/light cycle. Free access to food and water was allowed and all the experiments were carried according to the guidelines of the Animal Care and Use Committee of the German University in Cairo.

2.2. Chemicals

The LPS used for the induction of Alzheimer's was obtained from Sigma–Aldrich Chemie GmbH, Taufkirchen as well as the anti-inflammatory drug diflunisal. The 1400W and L-NAME were obtained from Cayman Chemicals, Germany.

The LPS, 1400W, L-NAME and L-arginine were dissolved in saline and diflunisal was mixed with Tween 80 then dissolved in saline.

2.3. Experimental design

In this study, intraperitoneal (ip) injection of LPS was given to the mice at a dose of 0.8 mg/kg (Sheng et al., 2003) in 100 μl saline for the induction of AD. After 6 days the mice were trained on the behavioral experiments and then tested on the following day. On the 8th day the mice were sacrificed and the brains were immediately frozen in liquid nitrogen then stored at −80 °C. A mouse from each group was sacrificed and its brain preserved in 4% formaldehyde at 4 °C for immunohistochemical staining. The mice were divided into five groups each with n = 10 (Fig. 1).

Another group was named "Arg Group" was used to study the effect of L-arginine on improving the memory after AD had developed (n = 10) (Fig. 2).

2.4. Behavioral tests

2.4.1. The Y-maze

It is a measure of the spatial memory. Mice were trained on the apparatus 6 days after the LPS injection. The mice were put individually in the middle of the apparatus and left to explore the apparatus freely for 8 min to get familiar with the apparatus and

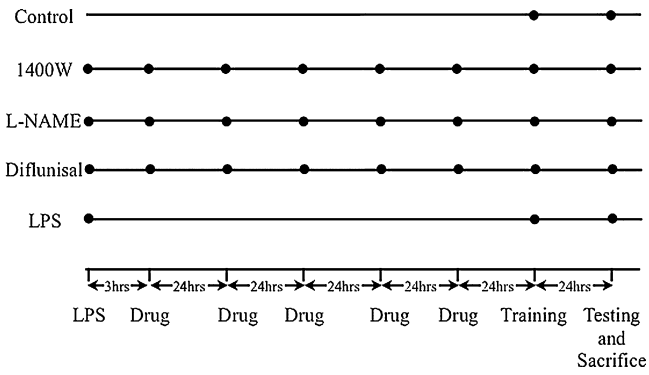

Fig. 1. Working protocol for the control, LPS, 1400W, L-NAME and diflunisal groups. The control group was given 100 μl saline. The LPS dose was 0.8 mg/kg of LPS in 100 μl saline. It was given to the LPS group. The doses of 1400W and L-NAME were 5 mg/kg (Garvey et al., 1997) and 20 mg/kg (Traystman et al., 1995) dissolved in 100 μl saline. The doses were given at 24-h intervals to ensure maximum inhibition of the NOSs (Nemec et al., 2010). The diflunisal dose was 100 mg/kg dissolved in 100 μl of saline (Meunier et al., 1998).

to enhance its memory acquisition (Van der Borght et al., 2007). The following day, the arms were labeled arbitrary A, B and C. The mice were put in the middle and monitored for 8 min and the sequence of arm entry was recorded. The maze was washed with 70% ethanol after each mouse. The experiment was performed under white light in a quiet room and the behavior recorded for analysis later.

The alternation behavior was defined as the successive entries into the three different arms A, B and C in overlapping triplet sets (Tamura et al., 2006). The ratio between the actual and possible alternation was calculated and the percentage alternation was obtained through this equation:

$$\%\,\text{Alternation} = \frac{\text{Number of alternations}}{\text{Total arm entries}-2} \times 100$$

2.4.2. The novel object recognition test

This is a measure for the non-spatial memory of the mouse. After 6 days from the LPS injection two identical objects were put at one end of the box 10 cm away from the sidewalls and the mouse put at the opposite side. The mouse was left to explore the box for 15 min. After 24 h, one of the objects was replaced by a completely different new one and the mouse was left for 4 min while recording the time spent in exploring each of the objects. The box and the objects were washed with 70% ethanol after each mouse. The experiment was performed under white light in a quiet room and the behavior recorded for analysis later.

Exploration is counted if the mouse directs his nose to the object at a distance of 2 cm or less (Ohta et al., 2012). The percentage of explorations to the new object was calculated using this equation:

$$\%\,\text{Exploratory preference} = \frac{\text{Time spent exploring new object}}{\text{Total time of time experiment(4 min)}} \times 100$$

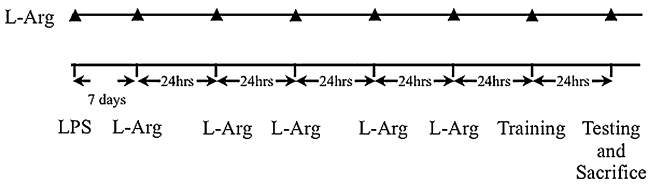

Fig. 2. Working protocol for L-arginine group. L-Arginine was dissolved in saline at a concentration of 50 mg/ml and given at a dose of 500 mg/kg orally.

2.4.3. The open field test

This is used to test the motor abilities of the mouse with regards to the ambulation, rearing and grooming frequencies. After 6 days from the LPS injection, the mouse was put in a specific corner square and left to explore the arena for 3 min. A day later the mouse was put on the same square, left for 3 min and the ambulation, rearing and grooming frequencies were measured. The arena was washed with ethanol 70% after each mouse. The experiment was performed under white light in a quiet room and the behavior recorded for analysis later (Kim et al., 2005).

2.5. Immunohistochemistry

2.5.1. Chemicals

The primary antibody ab10148 (anti-beta amyloid 1–42) was bought from Abcam plc, Germany. Trilogy (20×) (920P-06), the immunohistochemistry tissue fixation kit, was obtained from Cell Marque, USA, and the EconoTek HRP anti-polyvalent (DAB) kit was from Scytek Laboratories, Inc., USA.

2.6. Western blot

Western blot was done for two proteins, the Aβ and the NMDAR N2B subunit (more common at extrasynapse (Petralia et al., 2010)). Tris–tricine 16% gel was used for the Aβ as it has a low molecular weight of 4 kDa and Tris–glycine 12% gel for the NMDAR2B as it has a bigger molecular weight of 166 kDa.

2.6.1. Chemicals

Glycine, tris, tricine (N-tris-(hydroxymethyl)-methyl-glycine) and SDS (sodium dodecyl sulphate) were obtained from Carl Roth, Germany. The primary antibodies ab10148 (anti-beta amyloid 1–42), ab65783 (anti-NMDAR2B), ab8227 (anti-beta actin) and the secondary antibody ab97061 (donkey polyclonal secondary antibody to rabbit IgG-H&L (alkaline phosphatase)) were all bought from Abcam plc, Germany. The nitrocellulose membrane was bought from Whatman GmbH, Germany and had a pore size of 0.2 μm.

Protein concentration was measured using a Qubit fluorometer, Invitrogen, Carlsbad, CA, USA.

2.6.2. Work protocol

2.6.2.1. Sample preparation. Half a hemisphere of mouse brain was cut and weighed then put in RIPA buffer (400 μl for each 100 mg brain). The brain was then homogenized using a tissue homogenizer at 28,000 rpm for 30 s at 0 °C. The homogenate was then centrifuged at 3000 × *g* for 5 min at 4 °C. The supernatant was aliquoted and stored at −80 °C.

2.6.2.2. Tris–tricine gel electrophoresis and transfer. The casting of the Tris–tricine gel was done according to the protocol published by Schagger and von Jagow (1987).

Tricine was chosen as it has a higher electrophoretic mobility and thus increases the ionic strength of the media and thus allows for the separation of small proteins at high acrylamide concentrations (Schagger and von Jagow, 1987). The gel was made of a 4% stacking gel, a 10% spacer gel and a 16.5% resolving gel. The presence of the spacer was reported to sharpen the bands (Schagger, 2006). After gel polymerization, 70 μg of protein was taken from the brain homogenate and an equal volume of loading dye was added to it. This mixture was heated at 99 °C for 5 min then added to one of the wells along with the protein ladder and a standard amyloid protein as a positive control.

The run was performed at a constant voltage of 100 V for approximately 15 min till the bands finish the spacer gel. After that the voltage was raised to 150 V for another 35 min. During the run, a nitrocellulose membrane and six pieces of filter paper all cut the same size as the gel was soaked in Towbin buffer.

After the run was over, the 4% stacking gel was removed and the gel was left in Towbin buffer for 5 min. The gel, membrane and filter papers were then arranged appropriately in the blotter and left for 40 min at 400 mA for the transfer of the proteins from the gel to the membrane.

2.6.2.3. Tris–glycine gel electrophoresis and transfer. Tris–glycine gel was used as the molecular weight of NMDAR was big enough and the Tris–tricine was not needed.

After gel polymerization, 50 μg of protein were taken from the brain homogenate and an equal volume of loading dye was added to it. This mixture was heated at 95 °C for 5 min then loaded into the wells along with the protein ladder and a standard NMDAR protein as a positive control.

The run was performed at a constant voltage of 125 V for approximately 15 min till the bands finish the spacer gel. After that the voltage was raised to 150 V for another 60 min. The proteins were transferred to a nitrocellulose membrane using 400 mA for 40 min.

2.6.2.4. Detection. The membrane was washed with TBST buffer for 15 min to remove remaining Towbin/carhode/anode buffer. The membrane was then left in the blocking buffer (5% non-fat milk in TBST) for one hour to allow binding of the antibody only to the transferred proteins not the whole membrane. After blocking, the membrane was incubated with the primary antibody overnight to allow for complete binding to the target protein. The next day it was washed for 15 min with the blocking buffer then the secondary antibody was added and left for one hour.

After removing the secondary antibody the membrane was washed for 15 min with TBST to remove any traces of the secondary antibody. Ten milliliters of the ALP substrate were mixed with 33 μl of BCIP (50 mg/ml) and 66 μl (50 mg/ml) of NBT directly before adding it to the membrane. Within a couple minutes the protein bands became stained with a purple color.

2.7. NO measurement

The assay was performed according to Griess assay.

2.7.1. Chemicals

VCL_3, N-(1-naphthyl)ethylenediamine dihydrochloride (NED), sulphanilamide, $NaNO_3$ and $ZnSO_4$ were all obtained from Sigma–Aldrich Chemie GmbH, Taufkirchen.

2.7.2. Work protocol

Protein was first precipitated by adding 20 μl $ZnSO_4$ to 200 μl of the homogenate. The samples were vortexed then centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant was collected.

To 100 μl of the supernatant 300 μl of Griess reagent were added followed by 200 μl ddH_2O and 300 μl VCl_3. The mixture was then incubated in a thermomixer for 30 min at 37 °C and 300 rpm. A blank was prepared by adding 300 μl ddH_2O, 300 μl VCl_3 and 300 μl Griess reagent. To 100 μl of each of the standards 200 μl of ddH_2O, 300 μl of Griess reagent and 300 μl VCl_3 were added. The blank and the standards were incubated with the samples.

After 30 min, the absorbance of the samples and the standards were measured at 540 nm. A calibration curve was drawn from the standards and the concentrations of the samples were determined using this curve. The concentration was then calculated as concentration per mg protein.

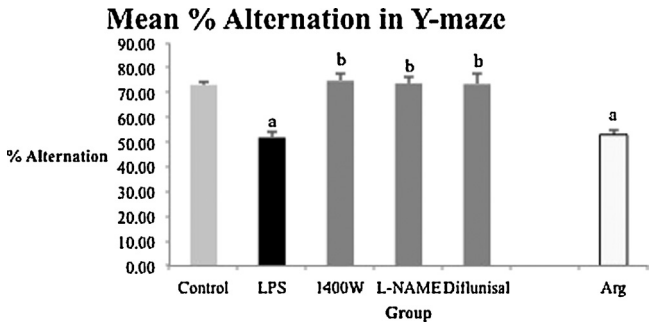

Fig. 3. Mean % alternation in the Y-maze. A significant difference existed between the control and the LPS groups ($p < 0.001$). All the drugs studied showed higher % alternation than the LPS group ($p < 0.001$) while L-arginine showed no improvement. (a) Significantly different from control, (b) significantly different from LPS group.

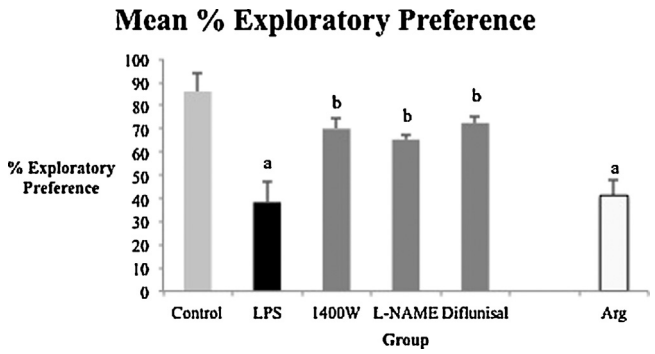

Fig. 4. Effects of LPS and tested drugs on exploratory preference in the Novel Object Recognition Test. A significant difference existed between the control and the LPS groups ($p < 0.05$). All the drugs studied showed higher % exploratory preference than the LPS group ($p < 0.05$) while L-arginine showed no improvement. (a) Significantly different from the control, (b) significantly different from the LPS group.

2.8. Statistical analysis

Statistical significance for the behavioral tests was determined using one-tailed Student *t*-test except for the Arg group where a two-tailed Student *t*-test was used. Immunohistochemistry was done to ensure the precipitation of Aβ in the cortex and was subject for visual examination only.

The bands of the Western blotting were analyzed using Image-J software, which measured the intensity of each band and compared it to the intensity of β-actin obtained from the same sample thus using it as an internal standard. The ratio obtained was used for comparison between the groups using a one-tailed Student *t*-test.

Statistical analysis was done using GraphPad Prism version 5a (GraphPad Software, Inc., USA). A *p* value of 0.05 was considered significant in both tests.

3. Results

3.1. Behavioral tests

3.1.1. The effect of 1400W, L-NAME, diflunisal and arginine on spatial memory in the Y-maze

Spatial memory was tested in a Y-maze, with the percentage alternation taken as a measure for the normal cognitive function. In the LPS group, alternation decreased compared to the control group ($p < 0.001$) (Fig. 3). In all treatment groups, significant increase in the alternation percentage compared to the untreated LPS group was observed ($p < 0.001$), completely reversing the effect of LPS. There was no significant difference between the group receiving the arginine and the LPS group. Arginine had no effect on LPS-induced decrease in alternation frequency.

3.1.2. The effect of 1400W, L-NAME, diflunisal and arginine on the novel object recognition test

Exploratory preference in the LPS group decreased by more than 50% when compared to the control group ($p < 0.05$). In the treatment groups, all drugs elicited a significant increase of exploratory preference compared to the untreated LPS group ($p < 0.05$) (Fig. 4). There was no difference between the group receiving arginine and LPS and that receiving the LPS only.

3.1.3. The effect of 1400W, L-NAME, diflunisal and arginine on the open field test

Ambulation frequency was massively reduced in LPS-treated animals (Fig. 5). All drugs were able to reverse those effects, returning value to control levels. Rearing frequency and grooming were less affected, all tested drugs increased rearing. Arginine had no effect on ambulation, but caused a slight, non-significant increase in rearing frequency.

3.2. Immunohistochemistry

Brain slices treated with Aβ antibody and stained showed that ip injection of LPS indeed induced the deposition of amyloid plaques in the cortex. Animal groups that received treatment with L-NAME, 1400W or diflunisal showed less amyloid plaques by visual observation (Fig. 6).

3.3. Biochemical assay

3.3.1. Detection of Aβ in brain lysates

In brain lysates of mice treated with LPS alone or is combined with the tested drugs, levels of Aβ were estimated using an Aβ-specific antibody (ab10148 from Abcam, Germany), and compared to β-actin as an internal standard. The intensity of the bands was measured using the software Image-J to compare the relative concentration of Aβ. Monomeric Aβ was only detected in the LPS and arginine group (Fig. 7A). Aβ oligomers however, were found in all animal groups (including the control group in some cases) (Fig. 7A). Quantitation relative to β-actin (Fig. 7A) showed a marked increase in Aβ after LPS treatment (≈10 folds), which was partially repressed by 1400W, L-NAME and diflunisal, but not by arginine (Fig. 7B).

3.3.2. NMDAR2B levels

The NMDAR2B bands appeared in all lanes as were expected at the 166 kDa mark. Image-J was used for the comparison of the

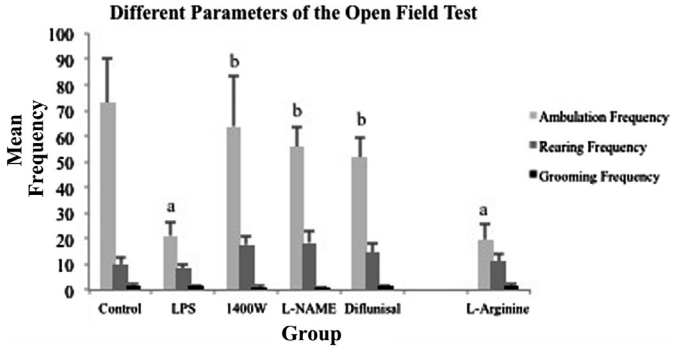

Fig. 5. Effect of LPS and various compounds on the Open Field Test. Significance difference was found between the ambulation in the control group and the LPS group at *p*-value < 0.05. The three groups receiving the drugs showed a marked improvement in the ambulation frequency when compared to the LPS group (*p*-value < 0.05). There was no difference between the group receiving the arginine and the LPS group. The rest of the measured parameters showed no difference from the control group. (a) Significantly different from the control, (b) significantly different from the LPS group.

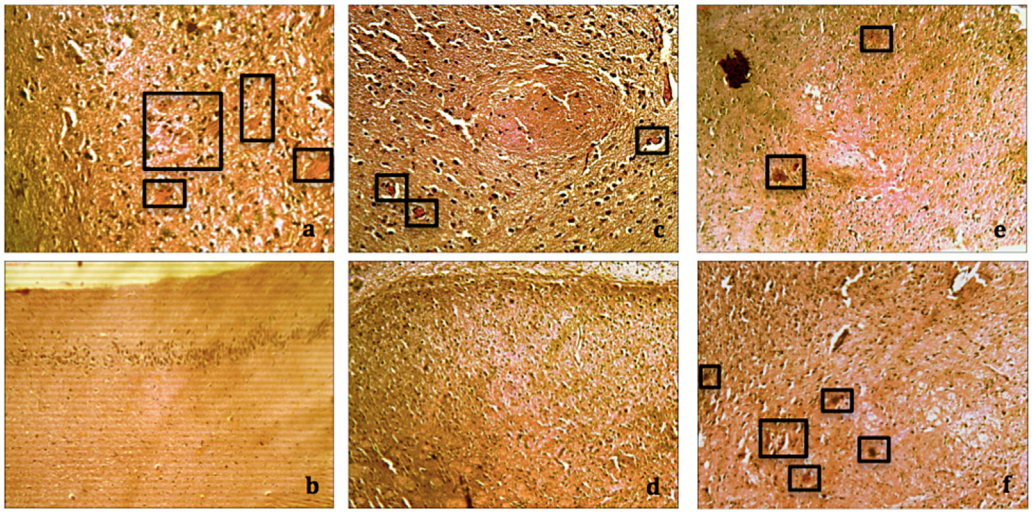

Fig. 6. Immunohistochemistry of Aβ deposition in the cortex of mice brain. (a) LPS group, (b) control group, (c) group receiving 1400W, (d) group receiving L-NAME, (e) group receiving diflunisal and (f) group receiving L-Arg. The amount of Aβ plaques was greater in the LPS group than in the control group. The 3 groups receiving the drugs showed a decrease in the amount of Aβ plaques. The group receiving L-Arg showed no improvement.

bands. The WB signals for NMDAR2B subunits were detected using a specific antibody (ab65783 from Abcam, Germany), showing a distinct band at 166 kDa. Upon LPS treatment, NMDA2B signal increased ≈4 folds (Fig. 7A and C). 1400W, diflunisal and arginine had no or minor effects, but L-NAME was able to reduce the NMDAR2B subunit almost to control level (Fig. 7C).

3.4. NO levels

The concentration of NO was determined using Griess method and calculated as μM/mg protein brain lysate. The concentration was increased ≈1.5 folds ($p < 0.001$) in the LPS group compared to control. Systemic arginine had no effect on NO levels. All other drugs reduced NO to control levels, with L-NAME showing the strongest reduction (Fig. 8).

4. Discussion

Alzheimer's is characterized by the deposition of Aβ plaques in the brain, although the soluble form of Aβ is considered to be responsible for the neuronal damage accompanying the disease, mainly through Ca^{2+} excitotoxicity (Parameshwaran et al., 2008).

LPS is an endotoxin of gram-negative bacteria that acts as a potent inflammagen through stimulation of TLR-4 receptors and inducing the release of proinflammatory cytokines (as IL-1 α/β, TNF-α and IL-6), NO, prostaglandin E_2 (PGE_2) and O_2^-. These changes will result in neuronal death (Liu and Bing, 2011).

4.1. Verifying systemic LPS as a model for AD

In this study, mice were given LPS ip to induce AD. The behavioral experiments showed a decline in both the both spatial and non-spatial memories as well as the motor skills in mice when compared to control animals (Figs. 3–5). Immunohistochemistry showed that LPS treatment induced the deposition of amyloid plaques that were absent in the control group. Although the plaques themselves are not toxic, their formation gives an indication on the degree of polymerization of Aβ (Hardy and Selkoe, 2002) and correlates to AD progression.

Western blot analysis showed a marked increase in Aβ in LPS treated mice. In particular we noticed a band just below the 55 kDa (≈12 × Aβ) in the LPS group that was absent in the control group. This agrees with findings by (Li et al., 2007), that soluble dodecamers of Aβ were responsible for the synaptic dysfunction and cognition decline.

Aβ oligomers cause neuronal toxicity through one of the following pathways as proposed by Berridge (2013), either through induction of inflammation or through the generation of ROS or through the excessive Ca^{2+} influx. Besides the fact that inflammation itself may lead to deposition of Aβ, Aβ oligomers are able to stimulate the TLR-4 on the microglia and cause the release of NF-κB, which in turn increases the transcription of IL-1β and TNFα. Microglia also phagocytize Aβ, which upon entry into the nucleus enhance the transcription of additional inflammatory mediators such as IL-1β, which stimulates the release of more NO (Berridge, 2013).

The high levels of NO observed in brain homogenates of mice receiving LPS can be attributed to the high amount of proinflammatory mediators released due to the presence of Aβ.

4.2. NMDAR2B

4.2.1. Overexpression

Aβ affects memory and LTP through activating the STEP protein responsible for the down-regulation of NMDA receptors thus, causing a sustainable moderate increase in Ca^{2+} resulting in long-term depression and depotentiation instead of LTP. Here we found that the expression of NMDAR2B subunit was markedly increased in the LPS group compared to control. It is believed that Aβ enhances the substitution of the GluN2A subunit in the NMDAR with GluN2B to facilitate it binding to SAP102 (Gladding and Raymond, 2011). SAP102 is a highly mobile protein. As a result, NMDAR2B subunit will laterally diffuse to the extra-synapse rather than being internalized. This agrees with the findings of (Gladding and Raymond, 2011) that showed that up regulated Aβ indeed favors the prevalence of NMDAR2B. It should be noted that NMDAR2A opens briefly but reliably following glutamate release while NMDAR2B have a lower opening probability but activation persists for several 100 ms after glutamate release (Lisman et al., 2012) and that NMDAR2B is more sensitive toward glutamate than NMDAR2A (Vizi et al., 2013). Indeed, a recent report demonstrates the improvement of AD by NitroMemantine, a selective extra-synaptic NMDAR blocker (Talantova et al., 2013).

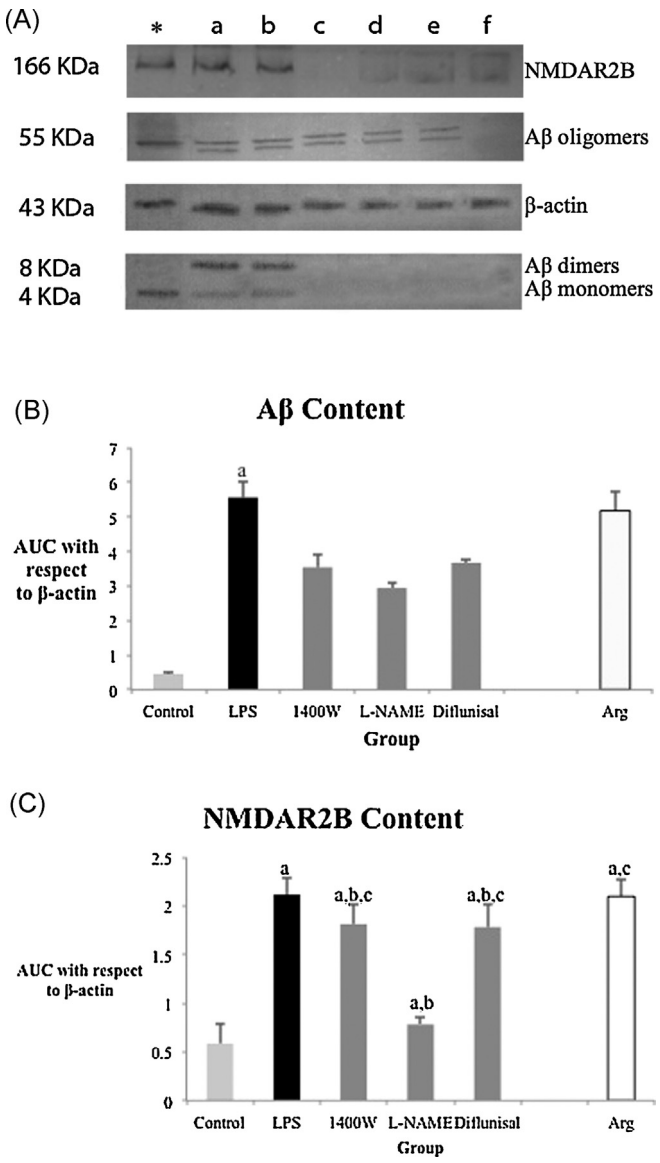

Fig. 7. A (a) LPS group, (b) Arg group, (c) L-NAME (d) 1400W group, (e) diflunisal group and (f) control group (*) +ve control (except in 55 kDa was part of ladder). B. Aβ content measured relative to β-actin using Image-J. All the groups showed a higher Aβ content than the control group ($p < 0.001$). The Aβ content was significantly higher in the LPS and Arg compared to the groups receiving the drugs ($p < 0.001$). The L-NAME group showed a significantly lower Aβ content than the 1400W and the diflunisal groups ($p < 0.05$). No difference existed between the 1400W group and the diflunisal group. (a) Significant difference from the control group ($p < 0.001$), (b) significant difference from LPS group ($p < 0.001$) and (c) significant difference from the L-NAME group. C. NMDAR2B content measured relative to β-actin using Image-J. NMDAR2B content was lower in the control than the rest of the groups ($p < 0.001$). The LPS and arginine groups had a higher NMDAR2B content compared to the groups receiving the drugs. The L-NAME group had lower NMDAR2B than the 1400W and the diflunisal ($p < 0.05$). (a) Significant difference from the control group ($p < 0.001$), (b) significant difference from LPS group, (c) significant difference from L-NAME group.

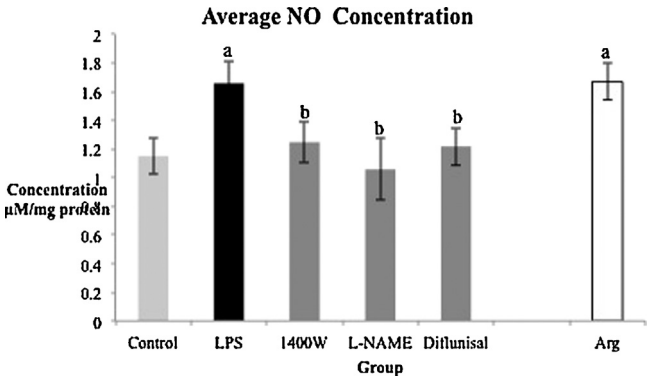

Fig. 8. Average NO concentration. The average NO concentration was higher in the LPS and the Arg group compared to the control ($p < 0.001$) and the groups receiving the drugs. (a) Significant difference from control ($p < 0.001$) and (b) significant difference from LPS.

4.2.2. Correlation with nNOS

NMDARs are attached to post synaptic density protein-95 (PSD-95), which is in contact with nNOS through their PDZ domain (Cao et al., 2005). Glutamate can reach extra-synaptic NMDARs either through glutamate spillover after synaptic excitation (Carter and Regehr, 2000) or through glutamate release from astrocytes that face the extra-synaptic region as proposed by Jourdain et al. (2007). Glutamate allows Ca^{2+} influx through NMDAR and thus, activates the nNOS. The elevated level of NO causes the release of more glutamate from the pre-synaptic neurons and thus, elevates intracellular levels of Ca^{2+}. Also NO causes S-nitrosylation affecting protein folding and reactive nitrogen species (RNS), which are free radicals responsible for DNA destruction (Mukherjee et al., 2014). Nitric oxide was reported to activate stress activated p38 MAP kinase (with and without the aid of glutamate) triggering apoptosis (Cao et al., 2005). Separating the nNOS from NMDAR reduces significantly Ca^{2+} excitotoxicity caused by NMDAR overstimulation (Courtney et al., 2014; Eastwood, 2005).

4.3. Effect of treatment

4.3.1. NOS inhibitors

Inflammation has been previously suggested to play a role in the incidence of AD (Fassbender et al., 2000; Zilka et al., 2012), so reducing the inflammation by NOS inhibitors or NSAIDs will improve the condition.

Behavioral tests showed that treatment with the NOS inhibitors guarded against LPS-induced loss of cognitive abilities in tested mice (Figs. 3–5). Both compounds decreased NO levels compared to the LPS treated group. Both inhibitors reduced levels of Aβ as detected by western blotting thus, highlighting the role played by NO in Aβ formation and oligomerization.

Generally, the non-selective NOS inhibitor L-NAME gave better improvements in all tests. This was surprising as iNOS produces larger amounts of NO, so its inhibition by 1400W was expected to have a stronger impact. Studies showed that prolonged activation of extrasynaptic NMDA receptors in neurons could shift APP expression from APP695 to the KPI-containing APP isoforms, accompanied with increased production of Aβ (Zhang et al., 2011) thus, we suggest that L-NAME results were due to the blocking of the nNOS attached to the overexpressed extra-synaptic NMDARs as it has a higher K_i toward nNOS than to iNOS (Pfeiffer et al., 1996).

4.3.2. Diflunisal

Diflunisal, being an acid, cannot pass the blood brain barrier (BBB) (Nuernberg et al., 1991) unlike other NSAIDs such as sulindac sulphide and Celecoxib, and unlike L-NAME and 1400W. Thus, diflunisal does not act in the CNS but rather is a systemic anti-inflammatory agent. The improvement of the mice conditions and the concomitant decrease in NO concentration suggests that NO is responsible for the neurodegeneration but its release is mediated by other proinflammatory cytokines such as TNFα and IL-1β, in regions outside the brain. This extra-neuronal effect is relevant, as only a small amount of LPS can cross the BBB under normal conditions (Nadeau and Rivest, 1999).

These cytokines act mainly on microglia and astrocytes causing release of NO and other inflammatory mediators, which lead to neurodegeneration and AD (Qin et al., 2007). By inhibiting production of these inflammatory mediators, diflunisal is able to improve the cognition and counteract progression of AD symptoms even without crossing the BBB. Diflunisal excludes the possibility of local action of NOS inhibitors on the expression of NMDARs.

Diflunisal guarded mice against cognition loss and neuronal damage induced by LPS and decreased the Aβ protein. The expression of NMDAR2B was much lower in mice treated with diflunisal confirming the correlation between inflammation and AD that has been reported in the literature (Chintamaneni and Bhaskar, 2012; Luterman et al., 2000) and the correlation between AD and the overexpression of the NMDAR2B.

4.3.3. *Effect of L-arginine*

The role of L-arginine was controversial, with some studies stating that it improves the memory in specific regions in the brain (Jing et al., 2013; Liu et al., 2009; Rushaidhi et al., 2012). In this study L-arginine was only given 2 weeks after LPS administration, because our aim was to study its effect on developed AD rather than its progression. However, given at this late stage of the disease, L-arginine did not improve behavioral or biochemical diagnostic tests for AD, suggesting that beneficial effects of arginine, through its established improvements of circulation, are only apparent in early stages of the disease.

We concluded that systemic inflammation is a potential cause of AD, or contributes to disease progression and this effect is mediated through NO. Both nNOS and iNOS contribute to the progression of AD with nNOS having a stronger effect. We also concluded that NMDAR2B subunits could be used as a measure for the progression of AD. NMDAR2B also plays a major role in AD pathology and targeting this receptor or its bound nNOS holds great potential for future therapies.

References

Benarroch, E.E., 2011. Nitric oxide: a pleiotropic signal in the nervous system. Neurology 77, 1568–1576.

Berridge, M.J., 2013. Calcium regulation of neural rhythms, memory and Alzheimer's disease. J. Physiol. 3.

Cao, J., Viholainen, J.I., Dart, C., Warwick, H.K., Leyland, M.L., Courtney, M.J., 2005. The PSD95-nNOS interface: a target for inhibition of excitotoxic p38 stress-activated protein kinase activation and cell death. J. Cell Biol. 168, 117–126.

Carter, A.G., Regehr, W.G., 2000. Prolonged synaptic currents and glutamate spillover at the parallel fiber to stellate cell synapse. J. Neurosci. 20, 4423–4434.

Chintamaneni, M., Bhaskar, M., 2012. Biomarkers in Alzheimer's disease: a review. ISRN Pharmacol. 2012, 984786.

Courtney, M.J., Li, L.L., Lai, Y.Y., 2014. Mechanisms of NOS1AP action on NMDA receptor-nNOS signaling. Front. Cell. Neurosci. 8, 252.

Eastwood, S.L., 2005. Does the CAPON gene confer susceptibility to schizophrenia? PLoS Med. 2, e348.

Fassbender, K., Masters, C., Beyreuther, K., 2000. Alzheimer's disease: an inflammatory disease? Neurobiol. Aging 21, 433–436, discussion 451–3.

Garvey, E.P., Oplinger, J.A., Furfine, E.S., Kiff, R.J., Laszlo, F., Whittle, B.J., Knowles, R.G., 1997. 1400W is a slow, tight binding, and highly selective inhibitor of inducible nitric-oxide synthase in vitro and in vivo. J. Biol. Chem. 272, 4959–4963.

Gladding, C.M., Raymond, L.A., 2011. Mechanisms underlying NMDA receptor synaptic/extrasynaptic distribution and function. Mol. Cell. Neurosci. 48, 308–320.

Hardy, J., Selkoe, D.J., 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 297, 353–356.

Jing, Y., Fleete, M.S., Collie, N.D., Zhang, H., Liu, P., 2013. Regional variations and age-related changes in arginine metabolism in the rat brain stem and spinal cord. Neuroscience 252, 98–108.

Jourdain, P., Bergersen, L.H., Bhaukaurally, K., Bezzi, P., Santello, M., Domercq, M., Matute, C., Tonello, F., Gundersen, V., Volterra, A., 2007. Glutamate exocytosis from astrocytes controls synaptic strength. Nat. Neurosci. 10, 331–339.

Kim, S.H., Han, J., Seog, D.H., Chung, J.Y., Kim, N., Hong Park, Y., Lee, S.K., 2005. Antidepressant effect of Chaihu–Shugan–San extract and its constituents in rat models of depression. Life Sci. 76, 1297–1306.

Lee, Y.J., Choi, D.Y., Choi, I.S., Kim, K.H., Kim, Y.H., Kim, H.M., Lee, K., Cho, W.G., Jung, J.K., Han, S.B., Han, J.Y., Nam, S.Y., Yun, Y.W., Jeong, J.H., Oh, K.W., Hong, J.T., 2012. Inhibitory effect of 4-O-methylhonokiol on lipopolysaccharide-induced neuroinflammation, amyloidogenesis and memory impairment via inhibition of nuclear factor-kappaB in vitro and in vivo models. J. Neuroinflamm. 9, 35.

Li, M., Chen, L., Lee, D.H., Yu, L.C., Zhang, Y., 2007. The role of intracellular amyloid beta in Alzheimer's disease. Prog. Neurobiol. 83, 131–139.

Lisman, J., Yasuda, R., Raghavachari, S., 2012. Mechanisms of CaMKII action in long-term potentiation. Nat. Rev. Neurosci. 13, 169–182.

Liu, M., Bing, G., 2011. Lipopolysaccharide animal models for Parkinson's disease. Parkinsons Dis. 2011, 327089.

Liu, P., Jing, Y., Zhang, H., 2009. Age-related changes in arginine and its metabolites in memory-associated brain structures. Neuroscience 164, 611–628.

Luterman, J.D., Haroutunian, V., Yemul, S., Ho, L., Purohit, D., Aisen, P.S., Mohs, R., Pasinetti, G.M., 2000. Cytokine gene expression as a function of the clinical progression of Alzheimer disease dementia. Arch. Neurol. 57, 1153–1160.

Medeiros, R., Baglietto-Vargas, D., LaFerla, F.M., 2011. The role of tau in Alzheimer's disease and related disorders. CNS Neurosci. Ther. 17, 514–524.

Meunier, C.J., Burton, J., Cumps, J., Verbeeck, R.K., 1998. Evaluation of the formalin test to assess the analgesic activity of diflunisal in the rat. Eur J Pharm Sci 6, 311–316.

Mukherjee, P., Cinelli, M.A., Kang, S., Silverman, R.B., 2014. Development of nitric oxide synthase inhibitors for neurodegeneration and neuropathic pain. Chem. Soc. Rev. 43, 6814–6838.

Nadeau, S., Rivest, S., 1999. Effects of circulating tumor necrosis factor on the neuronal activity and expression of the genes encoding the tumor necrosis factor receptors (p55 and p75) in the rat brain: a view from the blood–brain barrier. Neuroscience 93, 1449–1464.

Nemec, A., Pavlica, Z., Petelin, M., Crossley, D.A., Sentjurc, M., Jerin, A., Erzen, D., Zdovc, I., Hitti, T., Skaleric, U., 2010. Systemic use of selective iNOS inhibitor 1400W or non-selective NOS inhibitor l-NAME differently affects systemic nitric oxide formation after oral Porphyromonas gingivalis inoculation in mice. Arch Oral Biol 55, 509–514.

Nuernberg, B., Koehler, G., Brune, K., 1991. Pharmacokinetics of diflunisal in patients. Clin. Pharmacokinet. 20, 81–89.

Ohta, H., Arai, S., Akita, K., Ohta, T., Fukuda, S., 2012. Effects of NK-4 in a transgenic mouse model of Alzheimer's disease. PLoS One 7, e30007.

Paoletti, P., Bellone, C., Zhou, Q., 2013. NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. Nat. Rev. Neurosci. 14, 383–400.

Parameshwaran, K., Dhanasekaran, M., Suppiramaniam, V., 2008. Amyloid beta peptides and glutamatergic synaptic dysregulation. Exp. Neurol. 210, 7–13.

Petralia, R.S., Wang, Y.X., Hua, F., Yi, Z., Zhou, A., Ge, L., Stephenson, F.A., Wenthold, R.J., 2010. Organization of NMDA receptors at extrasynaptic locations. Neuroscience 167, 68–87.

Pfeiffer, S., Leopold, E., Schmidt, K., Brunner, F., Mayer, B., 1996. Inhibition of nitric oxide synthesis by NG-nitro-L-arginine methyl ester (L-NAME): requirement for bioactivation to the free acid, NG-nitro-L-arginine. Br. J. Pharmacol. 118, 1433–1440.

Qin, L., Wu, X., Block, M.L., Liu, Y., Breese, G.R., Hong, J.S., Knapp, D.J., Crews, F.T., 2007. Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration. Glia 55, 453–462.

Rayatnia, F., Javadi-Paydar, M., Allami, N., Zakeri, M., Rastegar, H., Norouzi, A., Dehpour, A.R., 2011. Nitric oxide involvement in consolidation, but not retrieval phase of cognitive performance enhanced by atorvastatin in mice. Eur. J. Pharmacol. 666, 122–130.

Rushaidhi, M., Jing, Y., Kennard, J.T., Collie, N.D., Williams, J.M., Zhang, H., Liu, P., 2012. Aging affects L-arginine and its metabolites in memory-associated brain structures at the tissue and synaptoneurosome levels. Neuroscience 209, 21–31.

Schagger, H., 2006. Tricine–SDS-PAGE. Nat. Protoc. 1, 16–22.

Schagger, H., von Jagow, G., 1987. Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166, 368–379.

Sheng, J.G., Bora, S.H., Xu, G., Borchelt, D.R., Price, D.L., Koliatsos, V.E., 2003. Lipopolysaccharide-induced-neuroinflammation increases intracellular accumulation of amyloid precursor protein and amyloid beta peptide in APPswe transgenic mice. Neurobiol. Dis. 14, 133–145.

Talantova, M., Sanz-Blasco, S., Zhang, X., Xia, P., Akhtar, M.W., Okamoto, S., Dziewczapolski, G., Nakamura, T., Cao, G., Pratt, A.E., Kang, Y.J., Tu, S., Molokanova, E., McKercher, S.R., Hires, S.A., Sason, H., Stouffer, D.G., Buczynski, M.W., Solomon, J.P., Michael, S., Powers, E.T., Kelly, J.W., Roberts, A., Tong, G., Fang-Newmeyer, T., Parker, J., Holland, E.A., Zhang, D., Nakanishi, N., Chen, H.S., Wolosker, H., Wang, Y., Parsons, L.H., Ambasudhan, R., Masliah, E., Heinemann, S.F., Pina-Crespo, J.C., Lipton, S.A., 2013. Abeta induces astrocytic glutamate release, extrasynaptic NMDA receptor activation, and synaptic loss. Proc. Natl. Acad. Sci. U. S. A. 110, E2518–E2527.

Tamura, H., Ishikawa, Y., Hino, N., Maeda, M., Yoshida, S., Kaku, S., Shiosaka, S., 2006. Neuropsin is essential for early processes of memory acquisition and Schaffer collateral long-term potentiation in adult mouse hippocampus in vivo. J. Physiol. 570, 541–551.

Traystman, R.J., Moore, L.E., Helfaer, M.A., Davis, S., Banasiak, K., Williams, M., Hurn, P.D., 1995. Nitro-L-arginine analogues. Dose- and time-related nitric oxide synthase inhibition in brain. Stroke 26, 864–869.

Van der Borght, K., Havekes, R., Bos, T., Eggen, B.J., Van der Zee, E.A., 2007. Exercise improves memory acquisition and retrieval in the Y-maze task: relationship with hippocampal neurogenesis. Behav. Neurosci. 121, 324–334.

Vizi, E.S., Kisfali, M., Lorincz, T., 2013. Role of nonsynaptic GluN2B-containing NMDA receptors in excitotoxicity: evidence that fluoxetine selectively inhibits

these receptors and may have neuroprotective effects. Brain Res. Bull. 93, 32–38.
Yi, J., Horky, L.L., Friedlich, A.L., Shi, Y., Rogers, J.T., Huang, X., 2009. L-Arginine and Alzheimer's disease. Int. J. Clin. Exp. Pathol. 2, 211–238.
Yildiz Akar, F., Celikyurt, I.K., Ulak, G., Mutlu, O., 2009. Effects of L-arginine on 7-nitroindazole-induced reference and working memory performance of rats. Pharmacology 84, 211–218.
Zhang, Y.W., Thompson, R., Zhang, H., Xu, H., 2011. APP processing in Alzheimer's disease. Mol. Brain 4, 3.
Zilka, N., Kazmerova, Z., Jadhav, S., Neradil, P., Madari, A., Obetkova, D., Bugos, O., Novak, M., 2012. Who fans the flames of Alzheimer's disease brains? Misfolded tau on the crossroad of neurodegenerative and inflammatory pathways. J. Neuroinflamm. 9, 47.