

## Chronic amyloid $\beta$ oligomer infusion evokes sustained inflammation and microglial changes in the rat hippocampus via NLRP3

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Statement: The authors have no conflict of interest to declare.

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## **Abbreviations**

A $\beta$ : amyloid beta; A $\beta$ O: A $\beta$ <sub>1-42</sub> oligomers; AD: Alzheimer's disease; CSF: cerebrospinal fluid; icv.: intracerebroventricular; IL-10: interleukin-10; mRNA: messenger RNA; MWM: Morris water maze; **NLRP3**: Nod-like receptor family, pyrin domain-containing 3

## Abstract

Microglia are instrumental for recognition and elimination of amyloid  $\beta_{1-42}$  oligomers (A $\beta$ O), but the long-term consequences of A $\beta$ O-induced inflammatory changes in the brain are unclear. Here, we explored microglial responses and transcriptome-level inflammatory signatures in the rat hippocampus after chronic A $\beta$ O challenge. Middle-aged Long Evans rats received intracerebroventricular infusion of A $\beta$ O or vehicle for 4 weeks, followed by treatment with artificial CSF or MCC950 for the subsequent 4 weeks. A $\beta$ O infusion evoked a sustained inflammatory response including activation of NF- $\kappa$ B, triggered microglia activation and increased the expression of pattern recognition and phagocytic receptors. A $\beta_{1-42}$  plaques were not detectable likely due to microglial elimination of infused oligomers. In addition, we found upregulation of neuronal inhibitory ligands and their cognate microglial receptors, whilst downregulation of *Esr1* and *Scn1a*, encoding estrogen receptor alpha and voltage-gated sodium-channel Na(v)1.1, respectively, was observed. These changes were associated with impaired hippocampus-dependent spatial memory and resembled early neurological changes seen in Alzheimer's disease. To investigate the role of inflammatory actions in memory deterioration, we performed MCC950 infusion, which specifically blocks the NLRP3 inflammasome. MCC950 attenuated A $\beta$ O-evoked microglia reactivity, restored expression of neuronal inhibitory ligands, reversed downregulation of ER $\alpha$  and Na(v)1.1, and abolished memory impairments. Furthermore, MCC950 abrogated A $\beta$ O-invoked reduction of serum IL-10. These findings provide evidence that in response to A $\beta$ O infusion microglia change their phenotype, but the resulting inflammatory changes are sustained for at least one month after the end of A $\beta$ O challenge. Lasting NLRP3-driven inflammatory alterations and altered hippocampal gene expression contribute to spatial memory decline.

## Keywords

amyloid  $\beta_{1-42}$  oligomer, hippocampus, microglia, NLRP3 inflammasome, estrogen receptors, spatial memory

## Introduction

Microglia constantly survey the brain parenchyma (Nimmerjahn, Kirchhoff, & Helmchen, 2005) and react promptly to disturbances to maintain homeostasis (Hanisch & Kettenmann, 2007). Microglia are equipped with receptors to monitor neurotransmission, neuronal damage, and to recognize any foreign or harmful substances (Lucin & Wyss-Coray, 2009). Microglial cells are instrumental in the recognition and elimination of amyloid beta ( $A\beta$ ). Cell surface receptors including scavenger receptors (El Khoury et al., 1996), CD14, TLR2 and TLR4 (Reed-Geaghan, Savage, Hise, & Landreth, 2009) bind  $A\beta$ , resulting in activation of NF- $\kappa$ B (Li, Long, He, Belshaw, & Scott, 2015). Activation of NF- $\kappa$ B triggers transcription of a set of proinflammatory genes (Muruve et al., 2008) including *IL1B* and *NLRP3*. Thereafter, encounter of  $A\beta$  lysosomal fragments and NLRP3 facilitates formation and activation of the NLRP3 inflammasome complex which promotes the release of IL-1 $\beta$  and IL-18, modulates eicosanoid synthesis, autophagy, metabolism and gene transcription (Rathinam & Fitzgerald, 2016). Activation of the NLRP3 inflammasome has been demonstrated in Alzheimer's disease (AD), and suggested that it plays a causative role in disease pathogenesis (Halle et al., 2008; Heneka et al., 2015). Recent preclinical studies have supported this notion. Genetic ablation of *Nlrp3* improves memory in APP/PS1 mice (Heneka et al., 2013), whilst pharmacological inhibition of the NLRP3 inflammasome reduces genotype-related microglia activation and enhances memory in transgenic mouse models (Daniels et al., 2016; Dempsey et al., 2016).

$A\beta$  peptides form soluble oligomers of various sizes, and aggregate into amyloid fibrils that gradually accumulate as insoluble senile plaques in the AD brain (Ahmed et al., 2010; Burdick et al., 1992; Halverson, Fraser, Kirschner, & Lansbury, 1990; Kirschner et al., 1987; Kuo et al., 1996). The presence of activated microglia, many of which express IL-1 (Griffin et al., 1989), has been demonstrated at the plaques throughout the cerebral cortex (Itagaki, McGeer, Akiyama, Zhu, & Selkoe, 1989; McGeer, Itagaki, Tago, & McGeer, 1987). While  $A\beta_{1-42}$  fibrils are unable to evoke a strong microglial response, soluble  $A\beta_{1-42}$  oligomers ( $A\beta$ O) activate microglia at the low nanomolar concentrations (Maezawa, Zimin, Wulff, & Jin, 2011). Soluble  $A\beta$ O has been implicated in the pathogenesis of AD (Bilousova et al., 2016; Lesne et al., 2006; Muller-Schiffmann et al., 2016; Shankar et al., 2008; Wang et al., 2017). The correlation between the degree of dementia in AD and the level of soluble  $A\beta$ O (Lue et al., 1999) supports this notion.

Infusion of  $A\beta_{1-42}$  into the cerebral ventricles of young adult rats is an established approach to mimic the neurological phenotype of late-onset AD (Forny-Germano et al., 2014; Garcia-Matas

et al., 2010; He et al., 2012; Lecanu, Greeson, & Papadopoulos, 2006). Early studies suggest that A $\beta$  fibrils alone are not effective to induce memory loss, but pro-oxidant agents potentiate the effect of A $\beta_{1-42}$ , and the mixture of A $\beta_{1-42}$  and pro-oxidants causes microglia activation and memory disturbances (Lecanu et al., 2006). Recent studies have demonstrated that A $\beta$ O diffuse into the brain parenchyma (Forny-Germano et al., 2014) and evoke a more robust microglial response and memory loss than do A $\beta_{1-42}$  fibrils (He et al., 2012). Aging remains the major risk for the development of dementia. As age-related changes of the rat hippocampal transcriptome already occur at 6 month of age (Kadish et al., 2009), middle-aged rats have also been used in A $\beta$  infusion experiments (Frautschy et al., 2001; Frautschy, Yang, Calderon, & Cole, 1996). However, it is currently unclear whether A $\beta$ O itself induces long-lasting inflammatory changes in the brain and memory deficits in an NLRP3-dependent manner.

In this study, we examined the impact of a 4-week A $\beta$ O infusion into the lateral cerebral ventricle of middle-aged Long Evans rats on microglia phenotype, inflammatory changes and transcriptomic fingerprints involved in neuron-microglia communication, estrogen signaling, inhibitory interneuron function and spatial memory. We also explored putative mechanisms that may link the microglial and inflammatory responses to A $\beta$ O infusion and spatial memory impairments.

## Experimental procedures

### Reagents

A $\beta$ <sub>1-42</sub> peptide was purchased from Bachem (Bubendorf, Switzerland). Buthionine sulfoximine and iron sulfate heptahydrate were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Sigma (St. Louis, MO, USA), respectively. Rabbit polyclonal antiserum to Iba1 was ordered from Wako Chemicals GmbH, (Neuss, Germany). Monoclonal anti-A $\beta$  antibody was obtained from Abcam (Cambridge, UK). Artificial CSF and MCC950 (Coll et al., 2015) were purchased from Tocris (Bristol, UK) and Sigma, respectively.

### Preparation of A $\beta$ <sub>1-42</sub> oligomers

A $\beta$ <sub>1-42</sub> peptide (Bachem, H-1368) was dissolved in 10% (w/v) NH<sub>4</sub>OH (Ryan et al., 2013). Aliquots were prepared, freeze dried, and stored at -20°C. Prior to use, 1 mg of A $\beta$ <sub>1-42</sub> was dissolved by adding 20  $\mu$ l of 60 mM NaOH and immediately diluted with 80  $\mu$ l water. Then, the alkaline solution was neutralized with 1.011 ml of low salt buffer (10 mM HEPES pH7, 10 mM NaCl) to make a 0.2 mM A $\beta$ <sub>1-42</sub> solution. The solution was incubated for 4 hours on ice to promote oligomer formation (Ahmed et al., 2010). As pro-oxidant agents potentiate the effect of A $\beta$  (Garcia-Matas et al., 2010; Lecanu et al., 2006), we added 2.5 ml low salt buffer containing 10 mg FeSO<sub>4</sub>·7H<sub>2</sub>O and 96 mg buthionine sulfoximine to the A $\beta$ O solution. The resulting solution consisted of 61.5  $\mu$ M A $\beta$ O, 10 mM FeSO<sub>4</sub> and 0.12 M buthionine sulfoximine. In parallel, the same solution without A $\beta$ <sub>1-42</sub> was prepared and used for the treatment of control animals. Alzet 2004 minipumps (Durect, Cupertino, CA, USA), filled with these solutions, were incubated at room temperature for 16 hours in saline before use.

### Experimental animals

Young male Long Evans rats were obtained from Charles River Laboratories (Calco, Italy). Animals were housed in the animal care facility of Institute of Experimental Medicine for the subsequent months. Eight month old rats were used in the study. APP<sup>NL-F/NL-F</sup> mice were generated by Takaomi Saido's laboratory (Nilsson, Saito, & Saido, 2014; Saito et al., 2014). For generation of the transgenic animal, the targeting vector was the mouse APP gene which included the Swedish and the Iberian human APP mutations (Mullan et al., 1992). The A $\beta$  sequence within the mouse APP was humanized. These mice have normal levels of full length APP, but show increased A $\beta$ <sub>1-42</sub>:A $\beta$ <sub>1-40</sub> ratio characteristic for AD pathology (Hardy, 1997). Animals were kept under normal conditions, with 12/12 hour light cycles, food and water was available *ad libitum*. Unlike in any other existing AD mouse models, the APP<sup>NL-F/NL-F</sup> mice reproduce human AD pathology with the highest fidelity. All studies were carried out with

permission from the Animal Welfare Committee of Institute of Experimental Medicine (Permission Number: A5769-01), and in accordance with the legal requirements of the European Community (Directive 2010/63/EU). Animal experimentation described was conducted in accord with accepted standards of animal care.

### **Surgery and treatment**

Surgery was carried out under isoflurane anesthesia, and with buprenorphine pain relief. Stainless steel infusion guide cannula (22G, Plastics One, Roanoke, VA, USA) was implanted into the right lateral cerebral ventricle (D=5.3 mm, L=1.4 mm, AP=0.8 mm). Alzet 2004 minipumps filled with either 0.2 ml of pro-oxidant-containing 61.5  $\mu$ M A $\beta$ O solution or vehicle, were connected to the cannula via PE50 tubing (Plastics One) and placed under the skin in the interscapular region. Pumps released 1.67  $\mu$ g A $\beta$ O daily for 4 weeks. Dosage was selected based on the work of Frautschy et al. (Frautschy et al., 2001). After A $\beta$ O infusion, minipumps were replaced with new ones filled with either 0.2 ml of 0.167  $\mu$ g/ml MCC950 in artificial CSF (aCSF), or 0.2 ml of aCSF. Dose of MCC950 was calculated based on published data on IC<sub>50</sub> for NLRP3 (Coll et al., 2015). Pumps released 1.0  $\mu$ g MCC950 daily for 4 weeks. Consecutive treatments generated four experimental groups including control group (vehicle + aCSF, n=6), drug control group (vehicle + DRUG, n=3), A $\beta$ O challenged group (A $\beta$ O + aCSF, n=6) and MCC950 treatment group (A $\beta$ O + DRUG, n=8) were examined.

### **Morris water-maze task**

On the fourth week of the second treatment, we started to perform Morris water maze (MWM). We tested spatial acquisition for 4 days, reversal on the next day and spatial learning for 2 days. The maze used in this study was a black circular pool (180 cm in diameter) filled to a 40 cm-depth with 24°C water. The black platform (10 cm in diameter) was hidden 2 cm below the water surface in the SW quadrant. On each day, rats were given four successive trials with a maximum duration of 90 sec, the inter-trial interval was 1 minute. Starting points (N, E, NW, SE) were randomized (Vorhees & Williams, 2006). Multiple color distal cues were placed on the wall and remained there throughout the experiment. Performance was recorded and analyzed by the EthoVision software (Noldus, Wageningen, Netherlands).

### **Isolation of total RNA from the hippocampus**

At the completion of the experiment, animals were deeply anaesthetized with a mixture of ketamine and xylazine (50 mg/kg ketamine, 10 mg/kg xylazine). After collection of CSF from the *cisterna magna*, animals for gene expression analysis were transcardially perfused with ice-cold PBS containing 10% RNAlater (Invitrogen, Carlsbad, CA, USA). Brains were quickly removed from the skull and placed into an ice-cold RBM-4000C rat brain matrix (ASI

Instruments, Warren, MI, USA). A 4 mm thick coronal slice was dissected with two blades positioned at bregma -2.2 and -6.2. Hippocampal formations were dissected from this slice. Samples were collected in Eppendorf tubes containing 1 ml of ice-cold *RNAlater*, incubated on ice for 1 hour, frozen on dry ice and stored at -80°C. Total RNA was isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). RNA analytics included capillary electrophoresis using Nano RNA Chips on 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions (Applied Biosystems, Foster City, CA, USA).

### **Real-time PCR**

TaqMan inventoried gene expression assays (Applied Biosystems) were used to study transcriptional regulation of 75 selected genes by real-time PCR. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), hypoxanthine phosphoribosyl-transferase (*Hprt1*) and peptidyl-prolyl isomerase A (*Ppia*) were used as house-keeping genes. Each assay consisted of a FAM dye-labeled TaqMan MGB probe and two PCR primers. Real-time PCR was performed on a ViiA7 Real-Time PCR System (Applied Biosystems) as described earlier (Sarvari, Kallo, Hrabovszky, Solymosi, & Liposits, 2014). The ViiA7 RUO software and relative quantification against calibrator samples ( $\Delta\Delta Ct$ ) were used for data analysis. A computed internal control corresponding to the geometric mean of Ct values of selected house-keeping genes was used for  $\Delta Ct$  calculation. Relative quantity ( $RQ = 2^{-\Delta\Delta Ct}$ ) was used to characterize gene expression in the various experimental groups. PCR experiments conformed to minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin et al., 2009).

### **Immunohistochemistry**

After behavioural tests, animals were deeply anaesthetized with a mixture of ketamine and xylazine, and CSF was collected from the *cisterna magna*. Three rats per group were perfused transcardially with PBS followed by 120 ml of 4% paraformaldehyde (PFA) in 0.1 M PBS. Brains were quickly removed from the skull and post-fixed overnight in 4% PFA. Brains were cryoprotected overnight in 30% sucrose and frozen on powdered dry ice. Coronal, 30  $\mu m$  thick sections were prepared using a freezing microtome (SMR2000R, Leica Biosystems, Nussloch, Germany). Sections were collected into a series of six wells, thus each well contained consecutive sections that were 180  $\mu m$  apart rostro-caudally from one another.

For Iba1 immunohistochemistry (IHC), after pretreatment with a solution containing 0.5% Triton X-100 and 0.5%  $H_2O_2$  in PBS for 20 minutes, sections were immersed in a rabbit polyclonal antiserum against Iba1 (1:2,000 dilution, #019-19741, Wako Chemicals) for 72 hours at 4°C, followed by 1-hour incubations in biotin conjugated anti-rabbit IgG (1:500



dilution, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and ABC elite reagent (1:1,000 dilution, Vector Laboratories, Burlingame, CA, USA). The immunoreaction product was visualized with nickel-enhanced diaminobenzidine (0.15% NiNH<sub>4</sub>SO<sub>4</sub>, 0.005% H<sub>2</sub>O<sub>2</sub>, 0.05% diaminobenzidine in Tris pH7.6). Sections were mounted on glass slides and coverslipped with DPX mounting medium (VWR, Radnor, PA, USA). Slides were analyzed using a Zeiss Axioskop-2 (Carl-Zeiss Microscopy, Jena, Germany) microscope. Sections were photographed with an AxioCam HRc digital camera controlled by AxioVision 4.6 software (Carl-Zeiss). Digital pictures were taken using 20x and 40x objectives.

For immunofluorescent detection of A $\beta$ <sub>1-42</sub>, sections were pretreated with a solution containing 1% human serum albumin, 0.1% Triton X-100 and 1mg/ml digitonin for 1 hour and incubated with mouse monoclonal antibody against A $\beta$ <sub>1-42</sub> (#126649, Abcam) at the dilution of 1:200 for 72 hours at 4°C. Cy3-conjugated anti-mouse IgG (1:500 dilution, Jackson ImmunoResearch Laboratories) was diluted in PBS containing 2% normal horse serum and applied on the sections for 2 hours. Similarly prepared sections from the cerebral cortex of APP<sup>NL-F/NL-F</sup> mice (Nilsson, Saito, & Saido, 2014) were used as positive controls. Sections were mounted on glass slides and coverslipped with VectaShield mounting medium (Vector Laboratories). Slides were investigated using a Zeiss Axioskop-2 microscope under epifluorescent illumination using a filter set for Cy3 dye. Specificity of the A $\beta$ <sub>1-42</sub> antibody was confirmed by the lack of staining on wild-type mouse tissue, and the characteristic staining in APP<sup>NL-F/NL-F</sup> mice with A $\beta$  plaques.

### **Cytometric bead array**

The measurement of rat IFN $\gamma$ , IL-1 $\alpha$ , IL-10 and TNF $\alpha$  was performed by cytometric bead array (CBA) as earlier (Denes et al., 2015), using BD<sup>TM</sup> CBA Flex Sets (BD Biosciences, Franklin Lakes, NJ, USA) according to manufacturer's protocol. Samples were acquired on a BD FACSVerse<sup>TM</sup> flow cytometer and data analyzed using FCAP Array v3 software (BD Bioscience).

### **Statistics**

In PCR data evaluation, group data were expressed as relative quantity: RQ(mean) $\pm$ standard deviation. Statistical significance of the changes in gene expression was analyzed using one-way ANOVA followed by Newman-Keuls *post-hoc* test (Statistica 13.1, Dell Software, Round Rock, TX, USA). In the assessment of MWM data, we used one-way ANOVA with Newman-Keuls *post-hoc* test to determine probability of the changes. Differences were considered significant at p<0.05.

## Results

### A $\beta$ O infusion evoked lasting microglial activation and inflammation in the hippocampus

We characterized changes of the microglia phenotype in the hippocampus after intracerebroventricular (icv.) infusion A $\beta$ O and artificial CSF by measuring the expression of *Iba1*, *Cd11b*, *Cd68*, *Cd80*, *Cd86* and *RT1-EC2* (Fig. 1A-F). All selected microglia reactivity markers were upregulated in the A $\beta$ O challenged group compared to controls. IHC and quantitative evaluation of Iba1 immunoreactivity were performed to verify the presence of activated microglia (**Fig. 1G**). In accordance with elevated expression of reactivity marker genes, Iba1 integrated density was significantly higher in the CA1 region of animals challenged with A $\beta$ O compared to controls. To characterize further the microglia phenotype, we investigated mRNA expression of a set of microglia related genes encoding receptors for recognition, recruitment and phagocytosis (**Table 1**). Expression of all selected recognition (*Tlr2*, *Tlr3*, *Tlr4*, *Tlr9*, co-receptor *Cd14*), recruitment (*C3ar1*, *C5ar1*, *Ccr2*, *Cxcr4*), phagocytic and scavenger receptor genes (*Cd11b*, *Cd11c*, *Cd163*, *Cd18*, *Cd68*, *Crry*, *Fcgr1a*, *Fcgr2a*, *Fcgr2b*, *Fcgr3a*, *Mfge8*, *Mrc1*) was enhanced in the A $\beta$ O challenged group compared to controls.

MCC950 treatment after A $\beta$ O infusion reversed mRNA expression of all selected reactivity marker genes (**Fig. 1A-F**). MCC950 also decreased Iba1 integrated density (**Fig. 1G**) supporting the results of gene expression analysis described above. MCC950 fully reversed upregulation of recognition, recruitment and phagocytosis receptor genes (**Table 1**).

We studied expression of selected chemokine, complement and cytokine genes, some of them related to the status of the transcription factor NF- $\kappa$ B (**Table 2**). Infusion of A $\beta$ O and CSF robustly enhanced chemokine (*Ccl2*, *Cxcl10*) and complement (*C3*, *Cfb*) expression in the hippocampus. A $\beta$ O also increased *Nlrp3* and *Il1b* expression indicating activation of NF- $\kappa$ B. Upregulation of additional proinflammatory cytokines (*Tnf*, *Il12b*) and *Nos2* supported the notion that microglia remained activated after icv. infusion of A $\beta$ O and CSF. On the other hand, icv. infusion of MCC950 immediately after A $\beta$ O infusion reduced expression of all pro-inflammatory genes to control levels (**Table 2**) indicating that the NLRP3 inhibitor abolished NF- $\kappa$ B-dependent transcriptional activation.

We couldn't detect extracellular A $\beta$ <sub>1-42</sub> plaques in the hippocampus after A $\beta$ O and CSF infusion (**Fig. 2**), although we detected A $\beta$ <sub>1-42</sub> immunoreactivity in the cerebral cortex of APP<sup>NL-F/NL-F</sup> mice (Nilsson et al., 2014). This finding indicated that microglia may have contributed to the elimination of A $\beta$ O or prevented its accumulation in the brain, but remained activated after A $\beta$ O challenge.

### **A $\beta$ O infusion activated transcription of genes related to neuronal control of microglia**

Neurons control microglia reactivity by neuronal inhibitory ligands via their cognate microglial receptors (Hanisch & Kettenmann, 2007; Tian, Rauvala, & Gahmberg, 2009). After A $\beta$ O and CSF infusion, expression of *Cx3cl1* (**Fig. 3A**), *Cd200* (**Fig. 3B**), and *Cd22* (**Fig. 3C**) increased moderately, while their cognate microglial receptors, *Cx3cr1* (**Fig. 3D**), *Cd200r1* (**Fig. 3E**), and *Cd45* (**Fig. 3E**) enhanced robustly compared to controls. Expression of other neuronal inhibitory ligands such as *Cd47* (RQ=1.67 $\pm$ 0.33, p=0.01) and *Sema3a* (RQ=1.63 $\pm$ 0.15, p=0.001) elevated moderately. Transcription of *Cd86*, which is a key player in the modulation of the microglia phenotype (Louveau et al., 2015), also enhanced significantly (**Fig. 1E**). MCC950 treatment restored expression of both neuronal ligands and their microglial receptors (**Fig. 3**).

### **A $\beta$ O infusion altered expression of estrogen receptor genes**

Estradiol is synthesized from testosterone by aromatase in the hippocampus. Its effects are mediated by estrogen receptors, mainly via estrogen receptor alpha (ER $\alpha$ ) and beta (ER $\beta$ ), which are encoded by *Esr1* and *Esr2*, respectively. A $\beta$ O challenge inversely regulated the expression of estrogen receptors, decreased *Esr1* (**Fig. 4A**) and increased *Esr2* (**Fig. 4B**) expression. Inhibition of NLRP3 inflammasome activation restored *Esr1* mRNA expression and attenuated upregulation of *Esr2*.

### **A $\beta$ O infusion suppressed *Scn1a* mRNA expression**

Interneuron-specific and parvalbumin-positive basket cell predominant voltage-gated sodium channel subunit Nav1.1 shows decreased expression in AD (Verret et al., 2012). As the process takes place at early phase of the disease, we followed mRNA expression *Scn1a* encoding the Na(v)1.1 subunit in the hippocampus after icv. infusion A $\beta$ O and CSF. *Scn1a* expression was decreased (RQ=0.79 $\pm$ 0.02, p=0.032), but MCC950 restored *Scn1a* (RQ=0.95 $\pm$ 0.06) expression, although the change did not reach statistical significance (p=0.09).

### **A $\beta$ O infusion leads to impaired spatial memory**

Hippocampus-dependent spatial memory was tested in MWM tasks. During spatial acquisition, decreasing escape latency times indicated fast spatial learning in the case of the control groups from day 2 (**Fig. 5A**). Vehicle and drug control groups showed indistinguishable parameters, therefore only latency times of the vehicle control group were shown. The A $\beta$ O challenged group showed higher latency times than controls from day 2, and the difference became statistically significant on day 4 (**Fig. 5A**).

On day 5, the hidden platform was moved to the opposite quadrant (reversal) and spatial acquisition was followed for three days (**Fig. 5B**). Control animals learned the new position of

the platform fast, while the A $\beta$ O challenged group showed impaired learning. The difference between latency times reached statistical significance on day 3 (**Fig. 5B**).

In the MCC950 treatment group, we measured decreased latency times compared to the A $\beta$ O challenged group, similar to control groups (**Fig. 5A**). After reversal, the MCC950 treatment group learned the new position of the platform fast, while the A $\beta$ O challenged group showed impaired learning (**Fig. 5B**). The performance of control groups and the MCC950 treatment group was similar (and was not different statistically) indicating that MCC950 completely reversed A $\beta$ O-evoked memory impairments.

#### **Blockade of the NLRP3 inflammasome reversed A $\beta$ O-induced decreases in serum IL-10**

Activation of microglia and the NLRP3 inflammasome may influence immune-related cells in the circulation. Therefore, we investigated serum cytokine profiles by measuring concentrations of IL-1 $\alpha$ , TNF $\alpha$ , IL-10 and IFN $\gamma$ . TNF $\alpha$  and IFN $\gamma$  levels were below the detection limit of the CBA assay. In contrast to the low serum levels of proinflammatory cytokines, concentrations of IL-10 (**Fig. 6A**) were 105 $\pm$ 1.7pg/ml in control animals, which decreased to 83 $\pm$ 14.0pg/ml in A $\beta$ O challenged animals, **but the change did not reach statistical significance (p=0.064)**. MCC950 treatment normalized serum IL-10. Serum levels of IL-1 $\alpha$  (**Fig. 6B**) showed no difference among treatment groups.

## Discussion

The microglia driven innate immune response against A $\beta$ O in the hippocampus is implicated in the pathogenesis of late-onset AD (Akiyama et al., 2000; Heneka et al., 2015; Lucin & Wyss-Coray, 2009). To explore the A $\beta$ O-induced microglia response and putative downstream mechanisms acting in the aging hippocampus, we induced an early AD-like neurological phenotype in middle-aged male rats by A $\beta$ O (and CSF) infusion into the lateral cerebral ventricle. Then, we studied spatial memory, as well as selected molecular elements of the innate immune response and interneuron-related processes. To clarify the impact of the microglia driven innate immune response to A $\beta$ O on spatial memory impairment in our middle-aged infusion model, we blocked activation of the NLRP3 inflammasome by icv. infusion of MCC950.

Infusion of A $\beta_{1-40}$  and A $\beta_{1-42}$  into the cerebral ventricles of middle-aged rats is an established approach to mimic the neurological phenotype of late-onset AD (Frautschy et al., 2001; Frautschy et al., 1996). As familial AD mutations lead to increased A $\beta_{1-42}$ : A $\beta_{1-40}$  ratio (Hardy, 1997), and A $\beta_{1-40}$  and A $\beta_{1-42}$  are the predominant species in neuritic plaques of AD patients (Masters et al., 1985; Mori, Takio, Ogawara, & Selkoe, 1992), we chose A $\beta_{1-42}$  to form oligomers. Of note, structure and composition of A $\beta$ O are markedly different from the neurotoxic oligomers of A $\beta_{1-40}$  (Ahmed et al., 2010). This structural difference goes with distinct prefibrillar to fibrillar oligomer transitions, and while A $\beta$ O are stable at low temperature and low ionic strength, A $\beta_{1-40}$  oligomers slowly form protofibrils and fibrils under the same condition (Ahmed et al., 2010; Glabe, 2008). Our oligomer preparation under low temperature-low salt condition results in mainly pentamers (and decamers), but we cannot exclude the possibility that the structure and composition of A $\beta$ O may change during infusion, although we found no sign of fibril formation. Using expression analysis and evaluation of Iba1 immunoreactivity, we provided evidence that 4 weeks after A $\beta$ O infusion still there is microglia activation in the hippocampus (**Fig. 1**). Although extracellular A $\beta_{1-42}$  plaques were not present (**Fig. 2**), we found evidence for upregulation of chemokines, complement and proinflammatory cytokines indicating the activation of NF- $\kappa$ B (Table 2). Robust transcriptional activation of genes encoding C3-Cd11b/Cd18 ligand-receptor pairs indicated increased microglial phagocytosis. In accordance, upregulated production and activation of complement have been demonstrated in the AD brain (Eikelenboom & Stam, 1982; Yasojima, Schwab, McGeer, & McGeer, 1999). Early activation of complement in AD has been proved recently (Hong et al., 2016; Zanjani et al., 2005). Upregulation of complement genes in the hippocampus after icv.

infusion A $\beta$ O (**Table 2**) lends further support for the role of complement in early AD. Although we observed enhanced expression of some microglia receptors and their neuronal inhibitory ligands (**Fig. 3**), regulatory mechanisms were unable to prevent microglia activation after the encounter with A $\beta$ O. Uncontrolled microglia activation coincided with downregulation of *Esr1* (**Fig. 4**) and *Scn1a* expression, and impairment of spatial memory (**Fig. 5**).

Estradiol is synthesized from testosterone by aromatase in the hippocampus (Hojo et al., 2004). Its effects are mediated by estrogen receptors, mainly via ER $\alpha$  and ER $\beta$ , both of them are expressed in the rat hippocampus (Shughrue & Merchenthaler, 2000). In microglia, the expression of ER $\alpha$  and ER $\beta$  is still controversial. While both have been observed *in vitro*, only ER $\alpha$  has been observed *in vivo* (Sierra, Gottfried-Blackmore, Milner, McEwen, & Bulloch, 2008). We found downregulation of *Esr1* indicating possible impairment of powerful microglia-dependent anti-inflammatory mechanisms (Vegeto et al., 2003). The regulatory role of ER $\alpha$ -mediated E2 action in neuroinflammation and NLRP3 inflammasome activation remains largely unexplored in AD. On the other hand, reduced ER $\alpha$  expression may also have an impact on neuromodulation (B. McEwen et al., 2001), as ER $\alpha$  is localized in dendrites, dendritic spines, and presynaptic terminals (B. S. McEwen, Akama, Spencer-Segal, Milner, & Waters, 2012). ER $\alpha$  mediates translational regulation of synaptogenesis via activating protein synthesis for dendritic function (Akama & McEwen, 2003). In addition, ER $\alpha$ , associated with clusters of vesicles in perisomatic inhibitory boutons, regulates vesicle trafficking at inhibitory synapses (Hart, Snyder, Smejkalova, & Woolley, 2007). These effects make E2 a powerful regulator of the hippocampus. Cessation of ovarian E2 synthesis renders the hippocampus vulnerable to A $\beta$ O and may contribute to the increased risk of AD in postmenopausal women (Henderson & Buckwalter, 1994). Decreased mRNA expression of ER $\alpha$  isoforms (Ishunina, Fischer, & Swaab, 2007) and reduced number of both cytoplasmic and nuclear ER $\alpha$  positive neurons (Hu et al., 2003) have been reported in the AD hippocampus. We found downregulation of *Esr1* indicating impairment of powerful anti-inflammatory mechanisms (Vegeto et al., 2003) and neuromodulation (B. McEwen et al., 2001). ER $\alpha$  is localized at both nuclear and extranuclear sites of the hippocampus and expressed in dendrites, dendritic spines, presynaptic terminals and glial processes (B. S. McEwen, Akama, Spencer-Segal, Milner, & Waters, 2012). ER $\alpha$  mediates translational regulation of synaptogenesis via activating protein synthesis for dendritic function (Akama & McEwen, 2003). In addition, ER $\alpha$ , associated with clusters of vesicles in perisomatic inhibitory boutons, regulates vesicle trafficking at inhibitory synapses (Hart, Snyder, Smejkalova, & Woolley, 2007). Importantly, decreased mRNA expression of

~~ER $\alpha$  isoforms (Ishunina, Fischer, & Swaab, 2007) and reduced number of both cytoplasmic and nuclear ER $\alpha$  positive neurons (Hu et al., 2003) has been reported in the AD hippocampus.~~

*Scn1a*, which encodes the voltage-dependent sodium channel alpha subunit Na(v)1.1 expressed predominantly in parvalbumin-positive basket cells, is downregulated in AD brains at both mRNA and protein levels (Verret et al., 2012). The relationship between reduced Na(v)1.1 expression, hypersynchrony and memory impairment has been established in AD (Verret et al., 2012). A $\beta$ O-induced downregulation of *Esr1* and *Scn1a* may interfere with the function of inhibitory interneurons, which represent a link between NLRP3 inflammasome activation and memory decline. A $\beta$ O-mediated memory decline became significant on the 4<sup>th</sup> day of spatial acquisition in MWM (**Fig. 5**), in accord with other studies using the middle-aged rat infusion model (Frautschy et al., 2001).

We found that inhibition of the NLRP3 inflammasome by icv. infusion of MCC950 after A $\beta$ O challenge attenuated A $\beta$ O-evoked microglia reactivity (**Fig. 1**) and NF- $\kappa$ B activation, and abolished spatial memory impairment (**Fig. 5**). These observations agree with the results of recent pharmacological studies in APP transgenic mouse models (Daniels et al., 2016; Dempsey et al., 2016). An important novel observation that adds to these earlier studies is that A $\beta$ O is sufficient to induce long-lasting inflammation and memory deficits in an NLRP3-dependent manner, even in the absence of major amyloid deposits in the brain parenchyma. In addition, we found that MCC950 restored transcriptomic changes of genes involved in neuron-microglia communication, and reversed downregulation of *Esr1* and *Scn1a*, **although in the latter case the effect did not reach statistical significance.** As MCC950 infusion leads to the resolution of inflammation and restoration of spatial memory, it is likely that A $\beta$ O-evoked inflammatory responses play causative role in the suppression of estrogen signaling, alteration of basket cell specific sodium transport and impairment of spatial memory. Based on these findings, we propose that A $\beta$ O activates microglia, which persists even after the elimination of the pathogenic substance. Enduring activation of microglia interferes with ER $\alpha$  signaling and subunit composition of voltage-gated sodium channels in basket cells among others, resulting in reversible impairment of spatial learning and memory.

Central inflammatory reactions may have an impact on the peripheral immune system, and *vice versa*. Cognitively impaired patients with amyloidosis have shown higher serum levels of proinflammatory cytokines and lower levels of IL-10 compared to patients without brain amyloidosis (Cattaneo et al., 2017). Therefore, activation of microglia in the hippocampus may influence immune-related cells in the circulation and alter the serum cytokine profiles. **Although statistical significance was not achieved in our study, serum levels of IL-10 tended to**

decrease in middle-aged rats following A $\beta$ O challenge (Fig. 6). We demonstrated that serum levels of IL-10 were decreased in middle aged rats following A $\beta$ O challenge (Fig. 6). Importantly, serum IL-10 levels are affected in individuals with AD (Asselineau et al., 2015; Remarque et al., 2001; Swardfager et al., 2010). In peripheral blood mononuclear cells, IL-10 synthesis is almost ceased in fast cognitive decliners, whereas its level is slightly increased in slow cognitive decliners with AD (Asselineau et al., 2015). In our middle-aged rat infusion model, MCC950 restored normal serum IL-10 levels (Fig. 6). This finding gives support to the idea that A $\beta$ O-induced microglia activation results in a decrease in serum IL-10 levels. Emerging evidence indicates that IL-10 acts in a negative feedback loop to regulate the NLRP3 inflammasome during chronic stimulations (Gurung et al., 2015; Ip, Hoshi, Shouval, Snapper, & Medzhitov, 2017; Yao et al., 2015). A very recent paper has demonstrated that IL-10 promotes mitophagy that eliminates dysfunctional mitochondria in macrophages (Ip et al., 2017). However, the functional contribution of IL-10 to the transcriptomic, inflammatory and behavioral changes observed has not been tested in this study. This finding gives support to the idea that A $\beta$ O induced microglia activation results in a decrease in serum IL-10 levels. Proinflammatory cytokine TNF $\alpha$  were undetectable in the serum, while IL-1 $\alpha$  levels showed no difference among the experimental groups.

In conclusion, these results demonstrate that icv. infusion of A $\beta$ O evokes lasting microglial and inflammatory responses in the hippocampus of middle-aged rats leading to reversible impairment of spatial memory. The underlying mechanisms might include interference with *Esr1* and *Scn1a* expression in the hippocampus, and peripheral IL-10-mediated action could potentially contribute to the changes observed, which will need to be tested in further studies.



## **Acknowledgements**

The authors thank Dr. Gábor Mező (Department of Organic Chemistry, Eötvös University, Budapest, Hungary) for his help in the preparation of A $\beta$ <sub>1-42</sub> oligomers, and members of the Behavioral Study Unit of Institute of Experimental Medicine for support in MWM experiments. This work was supported by the National Brain Research Program for CF (KTIA\_13\_NAP\_A\_1/3), the ‘Momentum’ Program of Hungarian Academy of Sciences for ÁD (LP2016-4/2016), the Hungarian Scientific Research Fund for EH and ZL (OTKA 112669, OTKA K115984), the National Research, Development & Innovation Office for GN (VKSZ 14-1-2015\_0155) and the Theme Program of Gedeon Richter Plc. for MS (TP8-005).

## **Authors’ contributions**

MS designed the study with CF and ZL. CF performed surgeries, CF, CV, EH, IK and MS collected tissues and samples. MS run and analyzed MWM and real-time PCR. CV performed and analyzed Iba1 IHC in conjunction with EH. CV performed A $\beta$ <sub>1-42</sub> IHC together with GN. ÁD run and analyzed CBA and provided conceptual support for these studies. MS wrote the manuscript with editing from all other authors. All authors read and approved the final version of the manuscript.

## Tables

**Table 1** Expression of recognition, recruitment and phagocytosis receptors associated with microglia in the rat hippocampus after A $\beta$ O, artificial CSF and MCC950 infusions.

| Gene                | A $\beta$ O+aCSF |      |       | A $\beta$ O+MCC950 |      |       |
|---------------------|------------------|------|-------|--------------------|------|-------|
|                     | RQ               | SD   | p     | RQ                 | SD   | p     |
| <b>Recognition</b>  |                  |      |       |                    |      |       |
| <i>Cd14</i>         | 5.32             | 1.99 | 0.005 | 1.77               | 0.35 | 0.399 |
| <i>Tlr2</i>         | 2.48             | 0.80 | 0.004 | 0.77               | 0.23 | 0.651 |
| <i>Tlr3</i>         | 3.20             | 1.09 | 0.003 | 0.88               | 0.18 | 0.741 |
| <i>Tlr4</i>         | 1.80             | 0.33 | 0.002 | 0.84               | 0.22 | 0.548 |
| <i>Tlr9</i>         | 1.76             | 0.38 | 0.020 | 0.87               | 0.05 | 0.586 |
| <b>Recruitment</b>  |                  |      |       |                    |      |       |
| <i>C3ar1</i>        | 1.77             | 0.39 | 0.005 | 0.76               | 0.21 | 0.321 |
| <i>C5ar1</i>        | 3.00             | 1.48 | 0.027 | 1.02               | 0.32 | 0.965 |
| <i>Ccr2</i>         | 5.09             | 1.55 | 0.002 | 0.56               | 0.28 | 0.625 |
| <i>Cxcr4</i>        | 1.73             | 0.28 | 0.003 | 0.84               | 0.17 | 0.288 |
| <b>Phagocytosis</b> |                  |      |       |                    |      |       |
| <i>Cd11b</i>        | 4.12             | 1.29 | 0.000 | 0.79               | 0.21 | 0.494 |
| <i>Cd11c</i>        | 14.37            | 6.80 | 0.033 | 1.99               | 0.75 | 0.850 |
| <i>Cd163</i>        | 1.63             | 0.22 | 0.012 | 0.97               | 0.03 | 0.893 |
| <i>Cd18</i>         | 2.80             | 0.49 | 0.001 | 0.83               | 0.14 | 0.579 |
| <i>Cd68</i>         | 3.52             | 1.17 | 0.005 | 0.89               | 0.33 | 0.867 |
| <i>Crry</i>         | 1.31             | 0.09 | 0.006 | 0.83               | 0.13 | 0.043 |
| <i>Fcgr1a</i>       | 3.81             | 0.83 | 0.001 | 1.01               | 0.28 | 0.993 |
| <i>Fcgr2a</i>       | 1.89             | 0.26 | 0.001 | 1.17               | 0.18 | 0.222 |
| <i>Fcgr2b</i>       | 4.14             | 1.31 | 0.006 | 1.17               | 0.28 | 0.827 |
| <i>Fcgr3a</i>       | 5.85             | 1.60 | 0.001 | 0.90               | 0.19 | 0.900 |
| <i>Mfge8</i>        | 1.24             | 0.06 | 0.043 | 0.99               | 0.14 | 0.716 |
| <i>Mrc1</i>         | 2.09             | 0.18 | 0.001 | 0.90               | 0.22 | 0.519 |

Twenty-one genes, encoding microglia receptors involved in recognition, recruitment and phagocytosis, were selected and studied by quantitative real-time PCR. A $\beta$ O- and MCC950-evoked changes were compared to controls. One-way ANOVA with Newman-Keuls *post-hoc* test was used for statistical analysis. In the case of A $\beta$ O+MCC950, p values greater than 0.05 show that mRNA expression of genes in drug treated animals did not differ from controls. RQ, relative quantity; SD, standard deviation; p, probability.

**Table 2** Expression of selected chemokine, complement and cytokine genes in the hippocampus after A $\beta$ O, artificial CSF and MCC950 infusions.

| Gene              | A $\beta$ O+aCSF |      |       | A $\beta$ O+MCC950 |      |       |
|-------------------|------------------|------|-------|--------------------|------|-------|
|                   | RQ               | SD   | p     | RQ                 | SD   | p     |
| <b>Chemokines</b> |                  |      |       |                    |      |       |
| Ccl2              | 10.88            | 1.91 | 0.000 | 1.58               | 0.32 | 0.667 |
| Cxcl10            | 4.22             | 0.69 | 0.001 | 0.92               | 0.26 | 0.806 |
| Cxcl12            | 1.09             | 0.20 |       | 1.05               | 0.16 |       |
| <b>Complement</b> |                  |      |       |                    |      |       |
| C3                | 7.62             | 4.37 | 0.011 | 0.86               | 0.54 | 0.979 |
| Cfb               | 10.84            | 3.08 | 0.001 | 0.93               | 0.22 | 0.996 |
| Cfd               | 2.13             | 0.09 | 0.000 | 0.96               | 0.04 | 0.609 |
| Cfh               | 1.87             | 0.24 | 0.000 | 0.98               | 0.15 | 0.960 |
| Cfp               | 1.58             | 0.11 | 0.000 | 1.02               | 0.13 | 0.944 |
| Clu               | 1.22             | 0.09 | 0.104 | 0.78               | 0.18 | 0.110 |
| Serping1          | 3.70             | 0.90 | 0.000 | 0.84               | 0.17 | 0.715 |
| <b>Cytokines</b>  |                  |      |       |                    |      |       |
| Il1b              | 1.99             | 0.58 | 0.004 | 0.84               | 0.42 | 0.584 |
| Il10              | 1.32             | 0.18 |       | 0.63               | 0.19 |       |
| Il12b             | 4.32             | 0.65 | 0.008 | 1.21               | 0.86 | 0.887 |
| Il6               | 1.22             | 0.19 |       | 0.91               | 0.04 |       |
| Tgfb1             | 2.04             | 0.10 | 0.000 | 0.96               | 0.25 | 0.648 |
| Tnf               | 3.62             | 0.28 | 0.000 | 0.96               | 0.18 | 0.696 |
| <b>Other</b>      |                  |      |       |                    |      |       |
| Nlrp3             | 1.87             | 0.12 | 0.000 | 0.83               | 0.10 | 0.143 |
| Nos2              | 12.00            | 1.24 | 0.000 | 0.57               | 0.57 | 0.532 |

Eighteen genes encoding chemokines, complement and cytokines were selected and studied by quantitative real-time PCR. A $\beta$ O- and MCC950-induced changes were compared to controls. One-way ANOVA with Newman-Keuls *post-hoc* test was used for statistical analysis. RQ, relative quantity; SD, standard deviation; p, probability.

## Legends to figures

**Figure 1** Infusion of A $\beta$ O and CSF enhanced microglia reactivity in the hippocampus. A $\beta$ O (A+CSF, n=6) increased mRNA expression of microglial reactivity marker genes (A) *Aif1/Iba1*, (B) *Cd11b/Itgam*, (C) *Cd68*, (D) *Cd80*, (E) *Cd86* and (F) *RT1-EC2* compared to controls (CTRL). MCC950 treatment (A+DRUG, n=8) attenuated A $\beta$ O-induced upregulation of all marker genes. Quantitative real-time PCR with inventoried gene expression assays were applied to determine mRNA levels in A $\beta$ O plus aCSF (A+CSF), A $\beta$ O plus MCC950 (A+DRUG) and control groups (CTRL). One-way ANOVA with Newman-Keuls *post-hoc* test was used to determine statistical significance (asterisks). (G) Iba1 IHC in the CA1 region of the rat hippocampus. Using Iba1 immunostaining we demonstrated A $\beta$ O-evoked changes of the microglia phenotype. The alteration manifested primarily in the enhanced Iba1 integrated density in microglia.

**Figure 2** A $\beta_{1-42}$  immunohistochemistry. Detection of A $\beta_{1-42}$  immunoreactivity in the cerebral cortex of (A) middle-aged control rats, (B) A $\beta$ O challenged middle-aged rats, (C) 11 months old wild-type mice and (D) APP<sup>NL-F/NL-F</sup> mice. Arrows mark strong A $\beta_{1-42}$  immunoreactivity indicating the presence of extracellular A $\beta$  plaques. Scale bar is 50  $\mu$ m.

**Figure 3** A $\beta$ O infusion enhanced mRNA expression of genes involved in neuronal control of microglia reactivity. A $\beta$ O challenge (A+CSF, n=6, black column) activated transcription of (A) *Cx3cl1*, (B) *Cd200*, (C) *Cd22*, (D) *Cx3cr1*, (E) *Cd200r* and (F) *Cd45* compared to controls (CTRL, white column). MCC950 treatment (A+DRUG, n=8, striped column) abated transcriptional activation of all genes encoding neuronal inhibitory ligands and their cognate microglial receptors. Gene expression was determined by quantitative real-time PCR. One-way ANOVA with Newman-Keuls *post-hoc* test was used to evaluate statistical significance.

**Figure 4** A $\beta$ O challenge altered the expression of estrogen receptor genes. (A) A $\beta$ O (A+CSF, black column) decreased mRNA expression of *Esr1* compared to controls (CTRL, white column). MCC950 (A+DRUG, striped column) restored *Esr1* expression. (B) A $\beta$ O increased mRNA expression of *Esr2* compared to controls. Inhibition of NLRP3 inflammasome attenuated upregulation of *Esr2*.

**Figure 5** Morris water maze tasks, (A) spatial acquisition and (B) reversal, revealed impairments of spatial memory. A $\beta$ O challenged (A+CSF, black solid line, n=6), controls (CTRL, grey, n=6) and A $\beta$ O plus MCC950 (A+DRUG, black dashed line, n=8) groups were examined, and displayed mean of the latencies in relation to the trial number. On each day, rats were given four consecutive trials of a maximum duration of 90 sec. Control (CTRL) and drug

control (VEH+DRUG) groups performed identically, therefore only data of the control group were shown. One-way ANOVA with Newman-Keuls *post-hoc* test was used for statistical analysis.

**Figure 6** Infusion of A $\beta$ O had an impact on serum cytokine profile. **(A)** A $\beta$ O challenge (A+CSF, n=6, black column) decreased serum IL-10 levels compared to controls (CTRL, n=6, white column), although the change did not reach statistical significance (p=0.064). MCC950 after A $\beta$ O infusion (A+DRUG, n=8, striped column) reversed declining serum levels of IL-10. **(B)** There was no change in IL-1 $\alpha$  levels among treatment groups. Serum IL-10 levels were determined by cytometric bead technology. One-way ANOVA with Newman-Keuls *post-hoc* test was used to analyze statistical significance.

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