Amyloid β induces microglia to phagocytose neurons via activation of protein kinase Cs and NADPH oxidase

Urte Neniskyte[1,2], Michael Fricker[1,3], Guy C. Brown[1]

[1]Department of Biochemistry, University of Cambridge, UK

[2]Current affiliation: Mouse Biology Unit, European Molecular Biology Laboratory, Via E. Ramarini 32, 00015 Monterotondo, Italy

[3]Current affiliation: Hunter Medical Research Institute, University of Newcastle, Kookaburra Circuit, New Lambton Heights, 2305, New South Wales, Australia.

Corresponding author: Guy C. Brown, Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, United Kingdom; Tel: +44 1223 766055; Fax: +44 1223 766002; E-mail: gcb3@cam.ac.uk.

Abstract

Alzheimer's disease is characterized by brain plaques of amyloid beta and by neuronal loss, but it is unclear how amyloid beta causes neuronal loss and how to prevent this loss. We have previously shown that amyloid beta causes neuronal loss by inducing microglia to phagocytose neurons, and here we investigated whether protein kinase Cs and NADPH oxidase were involved in this. The loss of neurons induced by amyloid beta in co-cultures of primary glia and neurons was completely prevented by inhibiting protein kinase Cs with Gö6976 or Gö6983. Directly activating protein kinase Cs with phorbol myristate acetate stimulated microglial phagocytosis, and induced neuronal loss mediated by MFG-E8/vitronectin receptor pathway of microglial phagocytosis. Blocking phagocytosis by MFG-E8 knockout or receptor inhibition left live neurons, indicating microglial phagocytosis was the cause of neuronal death. Phorbol myristate acetate stimulated the microglial NADPH oxidase, and inhibiting the oxidase prevented neuronal loss. A physiological activator of NADPH oxidase, fMLP, also induced neuronal loss dependent on microglia. Amyloid beta-induced neuronal loss was blocked by NADPH oxidase inhibitors, superoxide dismutase or Toll-like receptor function-blocking antibodies.

The results indicate that amyloid beta induces microglial phagocytosis of neurons via activating protein kinase Cs and NADPH oxidase, and that activating the kinases or oxidase is sufficient to induce neuronal loss by microglial phagocytosis. Thus inhibiting protein kinase Cs or NADPH oxidase might be beneficial in Alzheimer's disease or other brain pathologies involving inflammatory neuronal loss mediated by microglia.

Keywords

Neuroinflammation, phagoptosis, phagocytosis, microglia, neurodegeneration.

List of abbreviations

Aβ, amyloid beta; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride; cRGD, cyclic peptide arginine-glycine-aspartate-D-phenylalanine-valine; fMLP, N-formylmethionyl-leucyl-phenylalanine; iNOS, inducible NO synthase; LME, L-leucine methyl ester; LPS, lipopolysaccharide; MFG-E8, milk fat globule EGF factor-8; NADPH, nicotinamide adenine dinucleotide phosphate; NBT, nitro blue tetrazolium; PHOX, phagocyte NADPH oxidase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; SOD1, superoxide dismutase; TLR, toll-like receptor; TNFα, tumour necrosis factor alpha; VNR, vitronectin receptor.

1. Introduction

Brain damage, infection and neurodegeneration result in brain inflammation, often accompanied by loss of neurons and/or synapses (Glass et al., 2010, Heneka et al., 2014, Heneka et al., 2015). Behind an intact blood-brain barrier, neuroinflammation is mediated by microglia, the brain's main, resident macrophages (Ransohoff and Perry, 2009, Gomez-Nicola and Perry, 2015). In the healthy brain, microglia are 'resting', but if microglia detect damage, infection or protein aggregates they become 'activated'. Microglial activation is mediated by pattern recognition receptors such as the Toll-like receptors (TLRs), which activate transcription factors such as NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which cause expression of pro-inflammatory cytokines. The activated microglia become amoeboid, motile and highly phagocytic (Ransohoff and Perry, 2009, Gomez-Nicola and Perry, 2015), and may protect neurons by killing pathogens, removing debris and aiding repair, but they may also damage or kill neurons (Brown and Neher, 2010). Understanding how

microglia damage neurons and how this is regulated is important to understanding how to prevent neuronal damage in brain pathology.

We have found that inflammatory activation of microglia can cause delayed neuronal loss in culture and *in vivo* via activating microglial phagocytosis of stressed-but-viable neurons (Neher et al., 2011). Cell death by phagocytosis (called 'phagoptosis' for short) has the defining characteristic that inhibition of phagocytosis or phagocytic signalling prevents death of the cell (Brown and Neher, 2012). Inflammatory stimuli found to activate microglial phagocytosis of neurons include lipopolysaccharide (LPS) and lipoteichoic acid that activate microglia via TLR4 or TLR2 respectively (Neher et al., 2011), tumour necrosis factor α (TNFα) that activates via TNF receptors (Neniskyte et al., 2014) and rotenone that activates via inhibition of mitochondrial complex I (Emmrich et al., 2013). Activation of microglia causes: i) increased phagocytic capacity of microglia, ii) release of oxidants from microglia that induce neurons to reversibly expose the 'eat-me' signal phosphatidylserine, and iii) release of the opsonin milk fat globule EGF factor-8 (MFG-E8) from microglia and astrocytes that binds exposed phosphatidylserine on neurons and promotes their phagocytosis by also binding and activating the microglial vitronectin receptor (VNR) (Neher et al., 2011, Neher et al., 2013, Brown and Neher, 2014). Alzheimer's disease is characterised by tau tangles and amyloid plaques, the latter consisting mainly of aggregated peptide amyloid beta (Aβ). High concentrations of Aβ can kill neurons directly, but lower concentrations kill neurons indirectly by activating microglia (Neniskyte et al., 2011). We have shown that Aβ can induce a delayed loss of neurons and synapses mediated by neuronal exposure of phosphatidylserine, binding the opsonin MFG-E8 that then drives phagocytosis of the neurons via the vitronectin receptor (Neniskyte et al., Neniskyte and Brown). However, we do not know what regulates this process, and therefore how it might be prevented.

Aβ binds to TLR-2 and TLR-4 and activates the phagocyte NADPH oxidase (PHOX) in microglial cells (Reed-Geaghan et al., 2009), which in turn upregulates production of pro-inflammatory cytokines, such as tumour necrosis factor-α, and expression of inducible nitric oxide synthase (Combs et al., 2001, Jekabsone et al., 2006). Microglia highly express PHOX, which when activated produces superoxide that can either dismutate to hydrogen peroxide, or react with NO to produce peroxynitrite (Bal-Price et al., 2002). Superoxide and NO synergise to induce neuronal death (Mander and Brown, 2005) and their reaction product peroxynitrite can stress neurons to expose phosphatidylserine and thereby induce phagoptosis (Neher et al., 2011).

PHOX is regulated by protein kinases C (PKCs), which phosphorylate the cytosolic components of PHOX to cause assembly on the membrane (Gao et al., 2012). Classical PKCs (α, β or γ) are implicated in Aβ-induced activation of microglia (Combs et al., 1999). We recently found that inflammatory stimuli induce multinucleation of microglia via PKCs, and that PKC activator phorbol 12-myristate 13-acetate (PMA) induces microglia to form giant multinucleated cells that are highly phagocytic (Hornik et al., 2014).

Therefore, in the presented work, we investigated whether NADPH oxidase or PKC activation are necessary for the microglial phagocytosis of neurons induced by Aβ, and whether activation of PKCs by PMA is sufficient to induce microglial phagocytosis of neurons.

2. Methods

2.1 Materials

Aβ1-42 peptide (EZBiolab), Alexa Fluor 488 conjugate of isolectin B_4 from *Griffonia simplicifolia* (Invitrogen), carboxylate-modified fluorescent microspheres (Invitrogen), cyclo(RGDfV) and cyclo(RADfV) peptides (Bachem), apocynin, Gö6976 and Gö6983 (Calbiochem), TNFα Quantikine Elisa Kit (R&D Systems), function blocking antibodies to TLR4 (HTA-125), TLR2 (T2.5) and isotype control antibodies IgG1, IgG2a (eBioscience). Cell culture reagents were from PAA. Other reagents were from Sigma.

Monomeric amyloid β peptide 1–42 was prepared as described previously (Demuro et al., 2005, Cizas et al., 2010): 1.0 mg of lyophilised peptide was dissolved in 400 µL 1,1,1,3,3,3-hexafluorisopropanol and solution was incubated for 30–60 minutes at room temperature to ensure complete removal of any pre-existing secondary structure. To prepare monomeric $A\beta_{1-42}$ HFIP was removed by evaporation and the peptide was resuspended in DMSO at a concentration of 5 mM.

2.2 Primary cell culture and treatment

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986) and approved by the Cambridge University local ethical committee. Mixed neuronal/glial cultures were prepared from the cerebella of postnatal day 5-7 rats or mice as previously described (Kinsner et al., 2005). Mixed neuronal/glial cells were plated at a density of 5×10^5 cells/well on poly-l-lysine coated

24-well plates in DMEM supplemented with 5 % foetal calf serum, 5 % horse serum, 20 mM KCl, 13 mM D-glucose, 5 mM HEPES, 2 mM L-glutamine and antibiotics penicillin/streptomycin (100 U/ml and 100 µg/ml respectively). The cultures were allowed to mature *in vitro* for at least 7 days prior to treatment, when they contained 85±5% neurons, 7±3% astrocytes, and 5±3% microglia. Some cultures were prepared from *Mfge8*$^{-/-}$ mice from Clotilde Théry (Silvestre et al.) and *Bax*$^{-/-}$ mice (originally from Stanley Korsmeyer, crossed into a CD1 background by Aviva Tolkovsky (Wyttenbach and Tolkovsky)). Microglia were selectively eliminated from cultures (where indicated) by addition of L-leucine methyl ester (LME, Sigma) as previously described (Neher et al., 2011).

The cultures were allowed to mature for 7–9 days *in vitro* and were then stimulated for three days with Aβ (250 nM), phorbol 12-myristate 13-acetate (PMA, 30 nM), or formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP, 10 – 100 µM), with or without cyclo(RGDfV) peptide (50 µM), superoxide dismutase (SOD, 100 U/ml), 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, 20 µM), apocynin (100 µM), Gö6976 (0.1 – 1 µM) or Gö6983 (0.1 – 1 µM) in regular cell culture medium. Cell densities and cell death three days after treatment were evaluated as described previously (Neniskyte et al., 2011). Neurons with regular soma shape and normal nuclear Hoechst 33342 staining (10 µg/ml) were counted as alive, neurons with condensed chromatin were considered as apoptotic, whereas neurons staining with propidium iodide staining (1 µg/ml) were defined as necrotic. For some treatments, TNFα was measured in culture media at 3 days after stimulation using Quantikine Elisa Kit (R&D Systems) following manufacturer's instructions.

2.3 Phagocytosis assay

Phagocytic capacity of microglial cells was evaluated as described previously (Neniskyte et al., 2011). In short, pure microglial culture was treated with 30 nM PMA for 24 hours before 3 µl of 1:10 dilution of 1 µm fluorescently labelled carboxylate-modified microspheres were added, and cells were incubated for 1 hour at 37 °C, 5% CO_2. The medium was removed, and the culture was washed several times to remove excess beads. Microglia cells were then labelled with Alexa Fluor 488-tagged isolectin B_4 (2 µg/ml) and bead number per cell was evaluated in >50 cells per condition.

2.4 Nitro-blue tetrazolium (NBT) assay to measure superoxide production

After stimulation, mixed neuronal/glial cultures were incubated with 1 mg/ml of NBT for 30 min at 37 °C. Then cells were washed to remove remaining NBT and cells containing dark formazan precipitate were identified under a transmission light microscope.

2.5 Measurement of H_2O_2 production by microglial cells

H_2O_2 production by microglia was measured in a fluorometric Amplex Red hydrogen peroxide/peroxidase assay. Isolated microglia were suspended in HBSS supplemented with 50 mM D-glucose. The reaction mixture of a control sample contained 6×10^4 microglia cells in black 96-well plate, 15 μM Amplex Red and 30 μg/ml horseradish peroxidase. Production of reactive oxygen species in microglial cells was induced by the treatment with PMA (30 nM). PKC inhibitors Gö6976 (1 μM) and Gö6983 (1 μM) were added together with the stimulant. The rate of H_2O_2 production was assessed for two hours by continuously measuring fluorescence development in a Fluorstar Optima plate reader (BMG Labtechnologies) with excitation at 550 ± 10 nm and emission at 590 ± 10 nm.

2.6 Assessment of NO levels in cell culture medium

Nitric oxide production was evaluated by the accumulation of nitrite, which is a stable break down product of NO. Levels of nitrite were assessed using the Griess reaction, which quantifies nitrite through the formation of a red azo dye. Cell culture supernatants (50 μl) were added to a 96-well plate and mixed with 50 μl of ice-cold sulfanilamide (2 mM) in hydrochloric acid (1.2 M). The mixture was incubated for 10 minutes at room temperature, protected from light. Then N-1-(1-naphtyl)ethylenediamine (3 mM, 50 μl) was added followed by another incubation for 10 minutes at room temperature, protected from light. The absorbance of the resulting reaction solution was measured at 550 ± 10 nm wavelength in a Fluorstar Optima plate reader (BMG Labtechnologies) with wavelength correction at 380 ± 10 nm.

2.7 Statistical analysis

For all experiments, each treatment condition was repeated at least in duplicate for each culture, and then repeated in at least three independent cultures, for statistical analysis means of each independent culture were used as repeats (*n*). Statistical analysis was performed using IBM SPSS Statistics v20 software. Normality of data was verified by Shapiro-Wilk test. Means were compared by

one-way ANOVA and *post-hoc* Bonferroni test. p values < 0.05 were considered as significant. Numbers of alive, apoptotic and necrotic neurons were compared separately. All data presented are expressed as mean ± standard error of the mean (s.e.m.).

3. Results

3.1 Active PKC is necessary and sufficient for Aβ-induced neuronal loss

We have previously shown that nanomolar levels of Aβ can induce delayed neuronal loss in mixed neuronal-glial culture mediated by microglial phagocytosis of stressed-but-viable neurons (Neniskyte et al., 2011). Cultures were treated with 250 nM monomeric Aβ for three days, resulting in loss of about 30% of the neurons without any increase in neuronal apoptosis or necrosis (Fig. 1). These conditions were chosen as we had previously found that: monomeric, oligomeric and fibrillar Aβ caused similar neuronal loss (different forms are likely to equilibrate over 3 days), 10-250 nM Aβ caused similar neuronal loss but loss was more reproducible at 250 nM, and the neuronal loss was delayed until 1-2 days after Aβ addition (Neniskyte et al., Neniskyte and Brown).

We tested here whether this neuronal loss could be prevented by PKC inhibitors Gö6976 (an inhibitor of PKCα, PKCβI and PKCμ (Martiny-Baron et al.) as well as Trk A an Trk B) or Gö6983 (an inhibitor of PKCα, PKCβ, PKCγ, PKCδ and PKCζ (Gschwendt et al.)). Both inhibitors completely prevented Aβ-induced neuronal loss, leaving live neurons (Fig. 1), indicating that one or more PKCs (possibly PKCα or PKCβ, based on the overlap of the specificity of the two inhibitors used) mediate Aβ-induced neuronal loss and death.

As PKC activity was necessary for Aβ-induced neuronal loss, we tested whether activating PKCs with PMA was sufficient to induce neuronal loss, and if so by what means. We found that 30 nM PMA induced a similar amount of neuronal loss as Aβ over three days in culture, without significantly increasing the level of neuronal apoptosis or necrosis (Fig. 2*A*,*B*). The PMA-induced neuronal loss was completely prevented by pre-treating the cultures with L-leucine methyl ester (LME) to eliminate microglia, leaving live neurons (Fig. 2*B*), indicating that PMA was not directly toxic to neurons and the neuronal loss/death was mediated by microglia.

PMA can activate a variety of PKCs, but also other kinases such as protein kinase D (PKD), so we tested whether the PMA-induced neuronal loss could be blocked by the PKC inhibitors Gö6976

and Gö6983. We found that PMA-induced neuronal loss could be completely blocked by either Gö6976 or Gö6983 (Fig. 2*C*,*D*), indicating that PMA-induces neuronal loss by activating PKCs (possibly PKCα or PKCβ).

To further test whether apoptosis was involved in the neuronal loss, mixed neuronal-glial cultures were prepared from *Bax* knockout mice. Due to availability we used *Bax* knockout mice rather than rats. Post-mitotic neurons are wholly reliant on the expression of BAX for induction of apoptosis via the mitochondrial pathway, as they do not express full length Bak (Deckwerth et al., Sun et al.). However, PMA-induced neuronal loss was the same in these cultures as in wild-type cultures (Fig. 3), indicating that PMA-induced neuronal loss did not involve mitochondrial apoptosis.

Inflammatory activation of microglia can cause TNFα expression and release, and we have shown that high, sustained levels of TNFα (100 ng/ml) can cause neuronal loss mediated by microglial phagocytosis (Neniskyte et al.). However, we found that PMA caused little or no TNFα release in the cultures. TNFα levels detected by ELISA in medium of glial-neuronal cultures 3 days after treatment were, untreated: 0.2 ± 0.3 pg/ml, PMA: 1.8 ± 0.2 pg/ml (c.f. positive control LPS gave 570 ± 32 pg/ml). This makes it unlikely that the PMA-induced neuronal loss was mediated by TNFα. Furthermore this also indicated that PMA was not causing a classical activation of microglia.

3.2 Microglial phagocytosis via MFG-E8 and the vitronectin receptor mediates neuronal loss

As we had previously found that neuronal loss induced by Aβ and other inflammatory stimuli is mediated by microglial phagocytosis (Neher et al., Neniskyte et al.), we tested whether PMA increased microglial phagocytosis. PMA stimulated microglial phagocytosis of negatively-charged beads (Fig. 4*A*), indicating that PMA increases the phagocytic capacity of microglia. Microglial phagocytic activity was diminished by pre-treatment with PKC inhibitors Gö6976 or Gö6983 and PHOX inhibitor apocynin (Fig. 4A), indicating that PKC and subsequent PHOX activation is required for microglial phagocytosis.

We had previously found that neuronal loss induced by Aβ and other inflammatory stimuli is mediated by the phosphatidylserine/MFG-E8/vitronectin receptor pathway of phagocytosis [7, 19], so we tested whether MFG-E8 was required for PMA-induced neuronal loss. Due to availability *Mfge8* knockout mice rather than rats were used to evaluate the role of MFG-E8. We compared PMA-induced neuronal loss in cultures from *Mfge8* knockout and wild-type mice. We found that cultures

from *Mfge8* knockout mice completely lacked PMA-induced neuronal loss or death (Fig. 3), indicating that such loss and death requires MFG-E8. This also indicates that PMA does not directly kill neurons, and that the neuronal death is caused by phagocytosis.

MFG-E8 activates phagocytosis by binding phosphatidylserine on target cells and the vitronectin receptor on phagocytes. In order to test whether the vitronectin receptor is involved in PMA-induced neuronal loss, we inhibited the vitronectin receptor with a specific inhibitor, cRGDfV (50 μM). This completely prevented neuronal loss, without increasing neuronal death (Fig. 4*B*), again indicating microglial phagocytosis was the cause of neuronal death induced by PMA. The structurally similar cyclic peptide cRADfV, which does not block VNR did not prevent neuronal loss (Fig. 4*B*), indicating the neuroprotective effect of cRGDfV is due to VNR blocking.

3.3 PHOX, superoxide and TLRs mediate neuronal loss

We had previously found that LPS-induced neuronal loss is mediated by activation of the PHOX (Neher et al.), and as PMA is known to activate PHOX via PKCs, we tested here whether PMA activated microglial oxidant production via PKCs. We found that PMA acutely activated H_2O_2 production by microglia, and this was inhibited by PKC inhibitors Gö6976 or Gö6983 (1 μM, Fig. 5*C*). To test whether PMA and Aβ induce oxidant production in the neuronal-glial cultures, we added PMA or Aβ to such cultures and 3 days later assayed oxidant production by nitro blue tetrazolium staining. This staining was primarily restricted to microglia (Fig. 5*A*), and PMA and Aβ increased the number of microglia producing oxidants (Fig. 5*B*). In order to test whether Aβ- or PMA-induced neuronal loss was mediated by PHOX activity, we added two different inhibitors of PHOX activation: apocynin (Stolk et al.) and AEBSF (Diatchuk et al.). Apocynin prevents the assembly of NADPH oxidase subunits – cytoplasmic $p47^{phox}$ and plasmalemmal $gp91^{phox}$ – thus arresting its activation (Stolk et al.), while AEBSF is a serine protease inhibitor, which abrogates ROS production by NADPH oxidase by interfering with the binding of its subunits $p47^{phox}$ and/or $p67^{phox}$ to cytochrome b558 ($gp91^{phox}$) (Diatchuk et al.). We found that inhibition of PHOX by either 100 μM apocynin or 20 μM AEBSF prevented neuronal loss induced by PMA or Aβ (Fig. 6), indicating that PHOX activity is required for Aβ- and PMA-induced neuronal loss, and that both apocynin and AEBSF are neuroprotective in inflammatory conditions.

PHOX generates extracellular superoxide that either dismutates to hydrogen peroxide (which promotes microglial activation (Mander et al.)) or reacts with NO to produce peroxynitrite (which promotes phagoptosis (Neher et al.)). In order to test whether superoxide mediates Aβ-induced neuronal loss, we added to the cultures superoxide dismutase (SOD1), which rapidly dismutates extracellular superoxide to hydrogen peroxide. We found that addition of SOD1 prevented Aβ-induced neuronal loss (Fig. 5*D*), indicating that extracellular superoxide mediates Aβ-induced neuronal loss. As superoxide can react with NO to produce peroxynitrite that promotes phagoptosis, we tested whether NO was produced in the conditions used, and found that treatment with Aβ stimulated nitric oxide production (4.8 ± 0.4 μM compared to control levels of 1.2 ± 0.6 μM).

Aβ has been reported to activate microglia via a variety of different receptors, including TLR2 and TLR4 (Reed-Geaghan et al.). Thus we tested whether blocking TLR2 or TLR4 with function-blocking antibodies could prevent Aβ-induced neuronal loss. We found that function-blocking antibodies to TLR2 and TLR4 (as previously published (Reed-Geaghan et al., 2009)) each partially blocked Aβ-induced neuronal loss (Fig. 7*A*,*B*). Combining the antibodies gave neuronal survival not significantly different from the untreated control (Fig. 7*C*). Thus the data are consistent with Aβ inducing neuronal loss via activating TLR2 and/or TLR4, which are known to activate PKCs and/or PHOX in microglia (Nakajima et al., Reed-Geaghan et al., Wen et al.).

Although PMA and Aβ activate PHOX, they are not present physiologically, so we were interested in whether a physiological activator of PHOX, N-formylmethionyl-leucyl-phenylalanine (fMLP), could cause neuronal loss mediated by microglia. We found that activation of PHOX with 10 μM fMLP caused neuronal loss without neuronal apoptosis or necrosis, and this neuronal loss was prevented by eliminating microglia with LME (Fig 8). Thus physiological activation of PHOX appears sufficient to induce microglia-mediated neuronal loss.

4. Discussion

Aβ is known to activate PKCs in microglia, and we have shown here that inhibition of PKCs with Gö6976 or Gö6983 completely prevents Aβ-induced neuronal loss. At the concentrations used here, Gö6976 is reported to inhibit PKCα, β and μ (Martiny-Baron et al. 1993), whereas Gö6983 is reported to inhibit PKCα, β, γ and δ (Gschwendt et al. 1996). Thus if one isoform of PKC is required for inflammatory neuronal loss it is likely to be PKCα or β. However, more than one isoform of PKC might

be involved. As PKC was required for Aβ-induced neuronal loss, we tested whether PKC activation was sufficient to induce neuronal loss by adding PMA, which is known to directly activate classical and novel forms of PKC. We found that PMA did induce a similar level of neuronal loss to Aβ, and this was blocked by Gö6976 or Gö6983, indicating that the neuronal loss was mediated by classical PKCs. The PMA-induced neuronal loss was completely prevented by prior elimination of microglia, indicating that PMA was not directly toxic to neurons, but rather that the neuronal loss required microglia. PMA did not increase the number of apoptotic or necrotic neurons in the mixed cultures, and using cultures from *Bax* knockout mice, where neurons lack mitochondrial apoptosis, had no effect on the neuronal loss.

PMA-induced neuronal loss was completely prevented in cultures from *Mfge8* knockout mice, indicating that MFG-E8-mediated phagocytosis was required for neuronal loss. PMA increased the phagocytic capacity of microglia, and blocking the vitronectin receptor (VNR) required for MFG-E8-mediated phagocytosis, prevented PMA-induced neuronal loss. The neurons saved from phagocytosis by blocking MFG-E8 or VNR were viable. If the microglia had killed neurons first before phagocytosing them, then inhibition of phagocytosis would have left dead neurons. Thus PMA-induced neuronal death was caused by phagocytosis of the neurons by microglia – a form of neuronal death that we and other have found in a variety of inflammatory conditions in culture and in vivo (reviewed in (Brown and Neher).

How does PKC activation lead to neuronal loss? There are probably a variety of processes involved, but we find that PMA activates the NADPH oxidase in a PKC-dependent manner, and inhibiting the NADPH oxidase with AEBSF or apocynin prevents PMA- or Aβ-induced neuronal loss. A physiological activator of the NADPH oxidase fMLP also induced neuronal loss via microglia. Thus PMA/PKC may induce neuronal loss via activation of NADPH oxidase. The NADPH oxidase produces superoxide, which may either dismutate to hydrogen peroxide, which promotes activation of microglia (Mander et al., Jekabsone et al.), or may react with NO to give peroxynitrite (Bal-Price et al.), which can stress neurons to expose phosphatidylserine that promotes their phagocytosis by microglia (Neher et al.). We found that addition of superoxide dismutase to the culture medium prevented Aβ-induced neuronal loss. This suggests that the neuronal loss is mediated by extracellular superoxide reacting with NO to produce peroxynitrite that stresses neurons.

The NADPH oxidase has previously been implicated in AD and other neurodegenerative pathologies (Sorce and Krause, 2009, Block, 2008). The findings reported here support this idea, but also suggest a novel mechanism by which this occurs i.e. by driving microglial phagocytosis of stressed-but-viable neurons.

Aβ-induced neuronal loss was at least partially prevented by blocking TLR2 and TLR4, indicating that TLR2/4 may mediate the activation of microglia by Aβ. And this may explain why the neuronal loss induced Aβ, LPS (a TLR4 agonist) and lipoteichoic acid (a TLR2 agonist) are similar. Alternatively, they may be similar because they all activate PKCs to drive neuronal loss.

Alzheimer's disease is characterised by tau tangles and amyloid plaques (containing Aβ). The disease is also accompanied by neuroinflammation and extensive loss of neurons and synapses, which probably cause the dementia. However, there is little or no evidence of excess neuronal death in AD patient brains compared with age-matched controls (Perry et al., 1998, Raina et al., 2000, Calissano et al., 2009). One possible explanation for this is that neuronal death rates are low, and the dead neurons are removed almost immediately after they die (Perry et al., 1998). An alternative explanation is that the neurons are removed by phagocytosis before they die, and this explanation is supported by the results reported here, at least for Aβ-induced neuronal loss in vitro.

Others have shown that phagocytosis of otherwise-viable neurons by activated microglia mediates neuronal loss and death in models of AIDS dementia (Marker et al., 2012), Parkinson's disease (Barcia et al., 2012, Bodea et al., 2014) and Motor Neuron Disease (Liu et al., 2012), as well as developmental loss of neuronal precursors that may be related to autism spectrum disorders or schizophrenia (Cunningham et al., 2013) and loss of photoreceptor during retinal degeneration (Zhao et al., 2015). Thus inflammatory neuronal loss during a variety of brain pathologies may involve microglial phagocytosis of stressed-but-viable neurons.

We have identified in this work a variety of targets and/or agents that prevent Aβ-induced neuronal loss, including: TLR2 and TLR4 (blocked by antibodies), classical PKCs (blocked by Gö6976 and Gö6983), PHOX (blocked by AEBSF and apocynin), superoxide (removed by SOD), MFG-E8 (genetic knockout) and VNR (blocked by cRGDfV) (Fig. 9). Thus it may be worth testing whether blocking these targets is neuroprotective in models of Alzheimer's disease of other neuropathologies with inflammatory neuronal loss. Gö6976 has previously been found to be neuroprotective in an animal model of inflammatory neuronal loss (Dvoriantchikova et al., 2014), and thus might have

potential as a treatment at least for acute pathologies. Apocynin is neuroprotective in a variety of animal models of brain pathology, but it is not known whether it prevents inflammatory neuronal loss in Alzheimer's disease (t Hart et al.).

5. Conclusion

We have found that microglial activation by Aβ and PMA causes neuronal cell death by phagocytosis (phagoptosis) via PKC and PHOX activation. A possible mechanism, compatible with our data, by which Aβ and PMA induce this neuronal loss is as follows (see Fig. 9). Aβ, as well as bacterial wall components such as LPS, stimulate microglia by binding to TLR2 and TLR4, which activate classical PKCs. PKCs can also be activated directly either by PMA or by diacylglycerol (DAG) released by phospholipase C (PLC) after stimulation with fMLP. PKCs phosphorylate $p47^{phox}$ and other subunits of NADPH oxidase, causing assembly and activation of the oxidase. Superoxide from NADPH oxidase reacts with NO, produced by iNOS induced by TLR2/4 activation, to form peroxynitrite that triggers phosphatidylserine exposure on neurons. The phosphatidylserine on the external surface of neurons is bound by opsonin MFG-E8, released by activated glia, and the bound MFG-E8 is in turn recognized by the microglial vitronectin receptor (VNR), leading to the engulfment of the tethered neuron.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

UN participated in the design of the study, performed experiments on rat cultures and drafted the manuscript. MF participated in the design of the study and carried out experiments on mice cultures. GCB conceived of the study, participated in its design and coordination, and drafted the manuscript. All authors read and approved the final manuscript.

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Figures

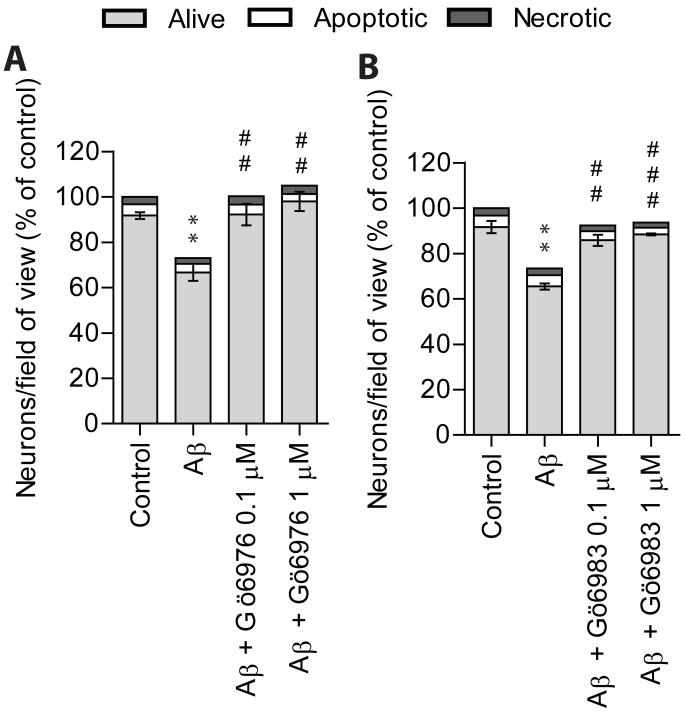

Figure 1. Aβ induce neuronal loss that is blocked by PKC inhibitors Gö6976 or Gö6983. Mixed neuronal-glial cultures from rat cerebellum were untreated (*Control*) or treated with Aβ (250 nM) with and without PKC inhibitors *A*: Gö6976 (0.1 or 1 µM) or *B*: Gö6983 (0.1 or 1 µM), and after 3 days the density of neurons that were necrotic (staining with propidium iodide), apoptotic (chromatin condensed, but not necrotic) or alive (neither apoptotic nor necrotic) was determined. Data are presented as means ± s.e.m. for ≥ 3 independent experiments; **/*** $p < 0.01/0.001$ *versus* untreated *Control*, ##/### $p < 0.01/0.001$ *versus* Aβ alone.

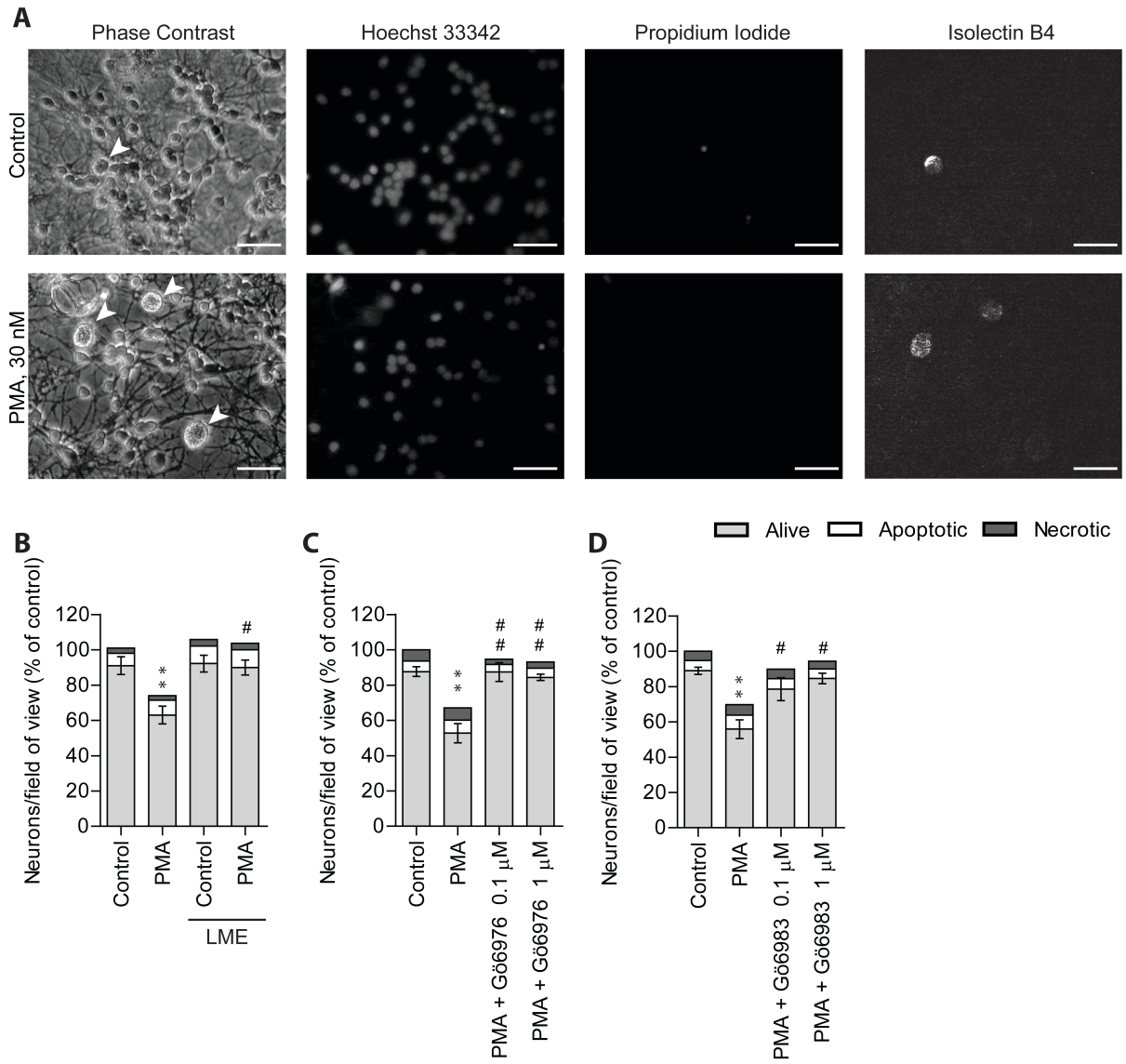

Figure 2. PMA induces neuronal loss mediated by microglia and blocked by PKC inhibitors. Rat neuronal-glial cultures were untreated (*Control*) or treated for three days with PMA (30 nM). *A*: Cell nuclei were labelled with Hoechst 33342, necrotic cells with propidium iodide, microglia cells with isolectin B_4; *scale bars*, 25 µm; in phase contrast images, microglia are indicated with white arrowheads. *B:* Cultures were treated with or without L-leucine-methyl ester (*LME*) to eliminate microglia. *C*: Cultures were treated with or without PKC inhibitor Gö6976 (0.1-1 µM). *D*: Cultures were treated with or without PKC inhibitor Gö6983 (0.1-1 µM). Data are presented as means ± s.e.m. for ≥ 3 independent experiments; ** p <0.01 *versus* untreated Control, #/## p < 0.05/0.01 *versus* PMA alone.

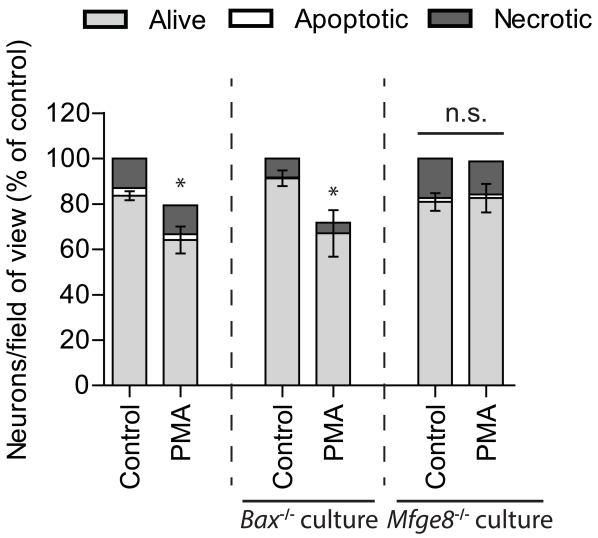

Figure 3. PMA-induced neuronal loss is unchanged in *Bax*$^{-/-}$ cultures, but is prevented by genetic deletion of MFG-E8. Neuronal-glial cultures from wild-type, *Bax* knockout, or *Mfge8* knockout mice were untreated (*Control*) or treated with PMA (30 nM), and after 3 days the density of necrotic, apoptotic and alive neurons was determined. Data are presented as means ± s.e.m. for ≥ 3 independent experiments; * $p < 0.05$ *versus* Control.

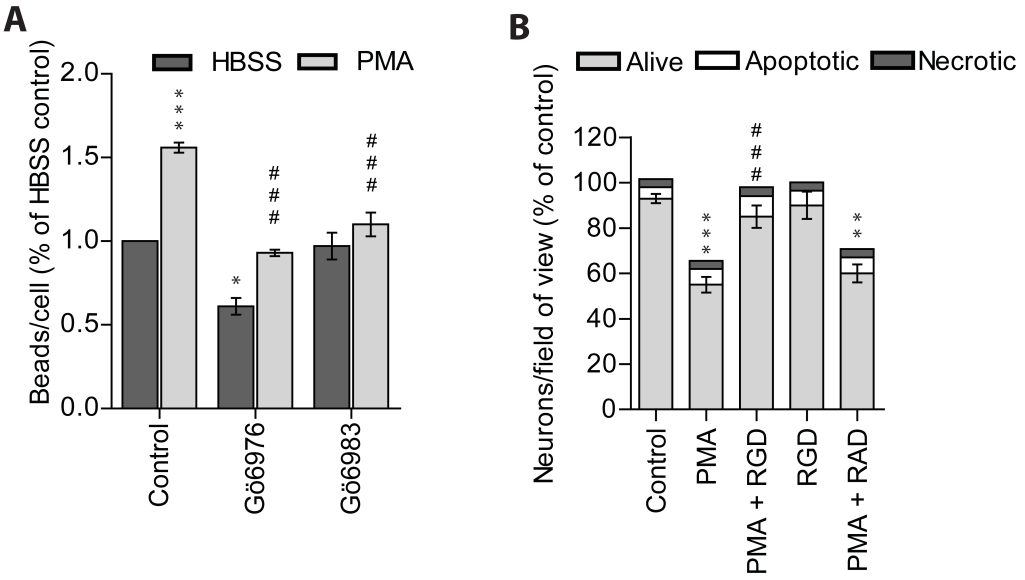

Figure 4. PMA increases PKC/PHOX-dependent microglial phagocytosis, and PMA-induced neuronal loss is prevented by blocking the phagocytic vitronectin receptor with cRGDfV. *A*: Isolated rat microglia were untreated (*Control*) or stimulated with PMA (30 nM) for 24 hours and then treated with or without Gö6976 (1 μM) or Gö6983 (1 μM) for 30 minutes. After adding fluorescent, negatively-charged, 1 μm beads for 1 hour the number of beads within the microglia was determined. *B*: Rat neuronal-glial cultures were untreated (*Control*) or treated with PMA (30 nM), PMA + cRGDfV (50 μM), cRGDfV (50 μM) or PMA + cRADfV (50 μM), and after 3 days the density of necrotic, apoptotic and alive neurons was determined. Data are presented as means ± s.e.m. for ≥ 3 independent experiments; */**/*** $p < 0.05/0.01/0.001$ *versus* HBSS Control (*A*) or unteated Control (*B*), ### $p < 0.001$ *versus* PMA alone Control (*A*) or PMA alone (*B*).

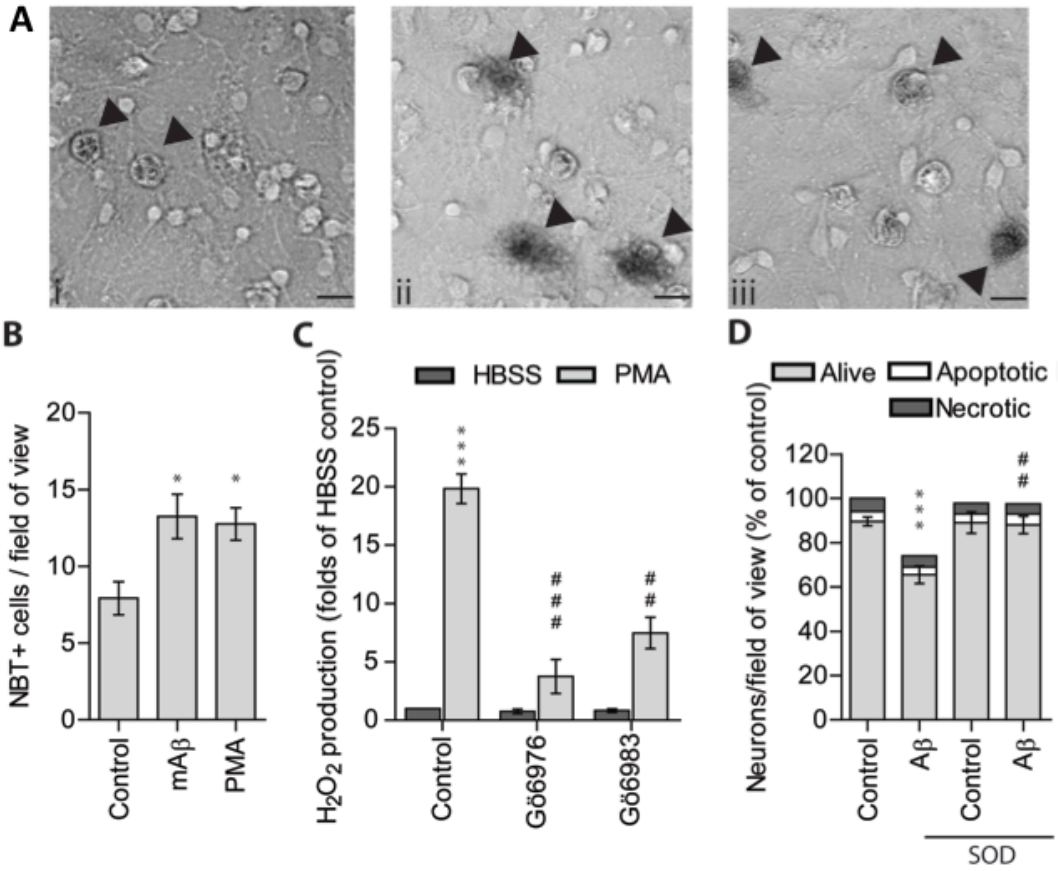

Figure 5. Aβ and PMA induce microglial oxidant production, and superoxide dismutase prevents Aβ-induced neuronal loss. *A & B*: Microglia were untreated (*Control, Ai*), treated with Aβ (250 nM, *Aii*), or PMA (30 nM, *Aiii*) for three days, and then ROS-producing cells were identified by NBT assay, cells were imaged (*A*) and NBT^+ cells counted (*B*). *C*: PMA (30 nM) acutely increased hydrogen peroxide production by isolated rat microglia, as measured with Amplex Red, and this was inhibited by co-treatment with Gö6976 (1 μM) or Gö6983 (1 μM). *D*: Neuronal-glial cultures from rats were untreated (*Control*) or treated with Aβ (250 nM) with or without superoxide dismutase (*SOD*, 100 U/ml), and after 3 days neuronal densities were determined. Data are presented as means ± s.e.m. for ≥ 3 independent experiments; */*** p <0.05/ 0.001 *versus* untreated Control, ##/### p < 0.01/0.001 *versus* PMA alone (*C*) or Aβ alone (*D*).

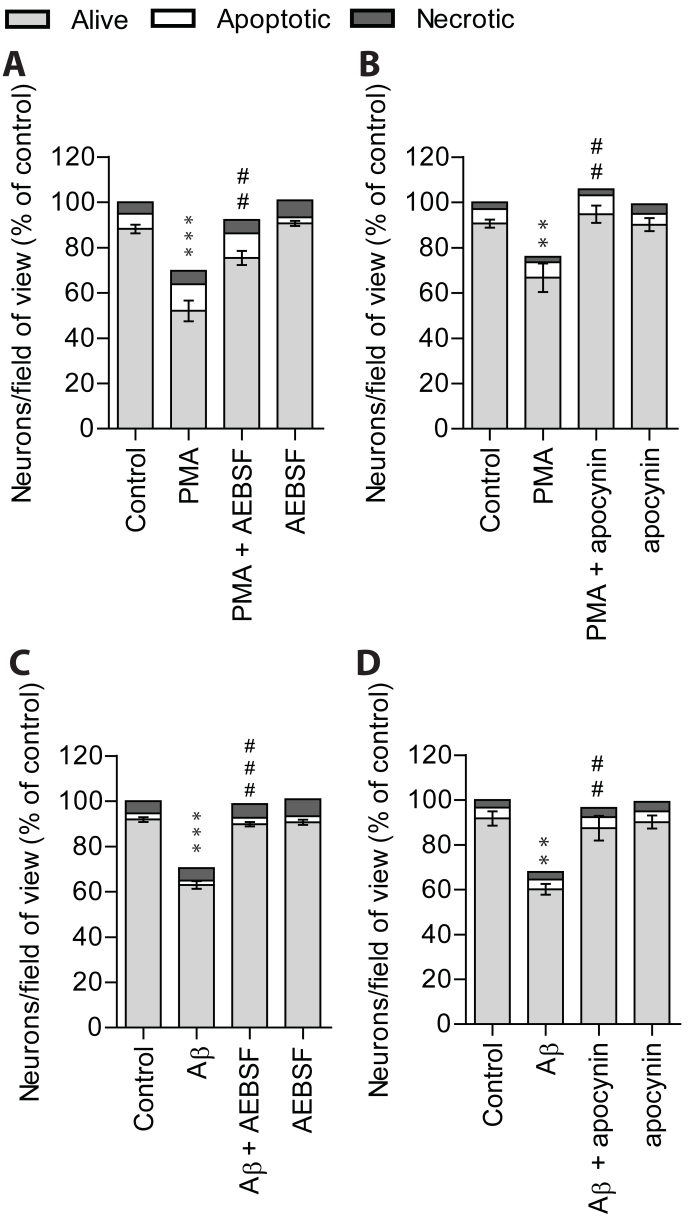

Figure 6. PHOX inhibitors, AEBSF and apocynin, prevent PMA- or Aβ-induced neuronal loss. Neuronal-glial cultures from rats were untreated (*Control*) or treated with PMA (30 nM, *A* and *B*) or Aβ (250 nM, *C* and *D*) with or without AEBSF (20 µM, *A* and *C*) or apocynin (100 µM, *B* and *D*), and after 3 days neuronal densities were determined. Data are presented as means ± s.e.m. for ≥ 3 independent experiments; **/*** $p < 0.01/0.001$ *versus* unteated Control, ##/### $p < 0.01/0.001$ *versus* PMA alone (*A*, *B*) or Aβ alone (*C*, *D*).

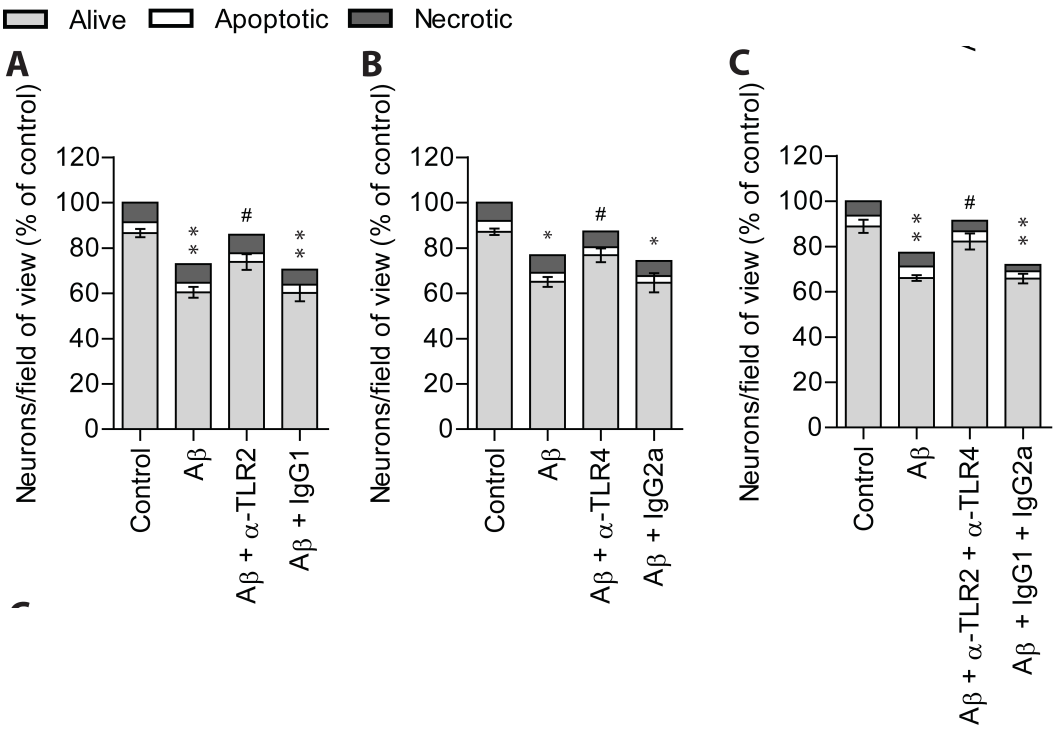

Figure 7. Aβ-induced neuronal loss is prevented by blocking Toll-like receptors-2 and -4. Neuronal-glial cultures from rats were either untreated or pre-treated for 30 minutes with *A*: anti-Toll-like receptor-2 antibody (*α-TLR2*, 10 µg/ml) or control immunoglobulin (*IgG1*, 10 µg/ml); *B:* anti-Toll-like receptor-4 antibody (*α-TLR4*, 10 µg/ml) or control immunoglobulin IgG2a (*IgG2a*, 10 µg/ml); *C*: both α-TLR2 and α-TLR4 or IgGs together (10 µg/ml each). Then cultures were either untreated (Control) or treated with Aβ (250 nM) for three days, whereafter neuronal densities were determined. Data are presented as means ± s.e.m. for ≥ 3 independent experiments; */** $p < 0.05/0.01$ *versus* untreated Control, # $p < 0.05$ *versus* Aβ alone.

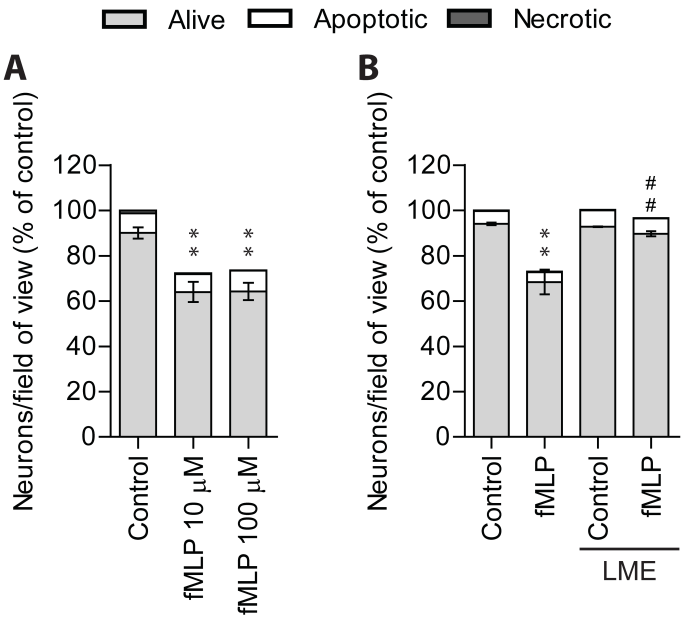

Figure 8. fMLP induces neuronal loss mediated by microglia. *A*: Neuronal-glial cultures from rats were untreated (*Control*) or treated with fMLP (10-100 µM), and after 3 days neuronal densities were determined. *B*: Cultures were pre-treated with LME to remove microglia and then treated with or without fMLP (10 µM) for three days. Data are presented as means ± s.e.m. for ≥ 3 independent experiments; ** $p < 0.01$ *versus* untreated Control, ## $p < 0.01$ *versus* fMLP alone.

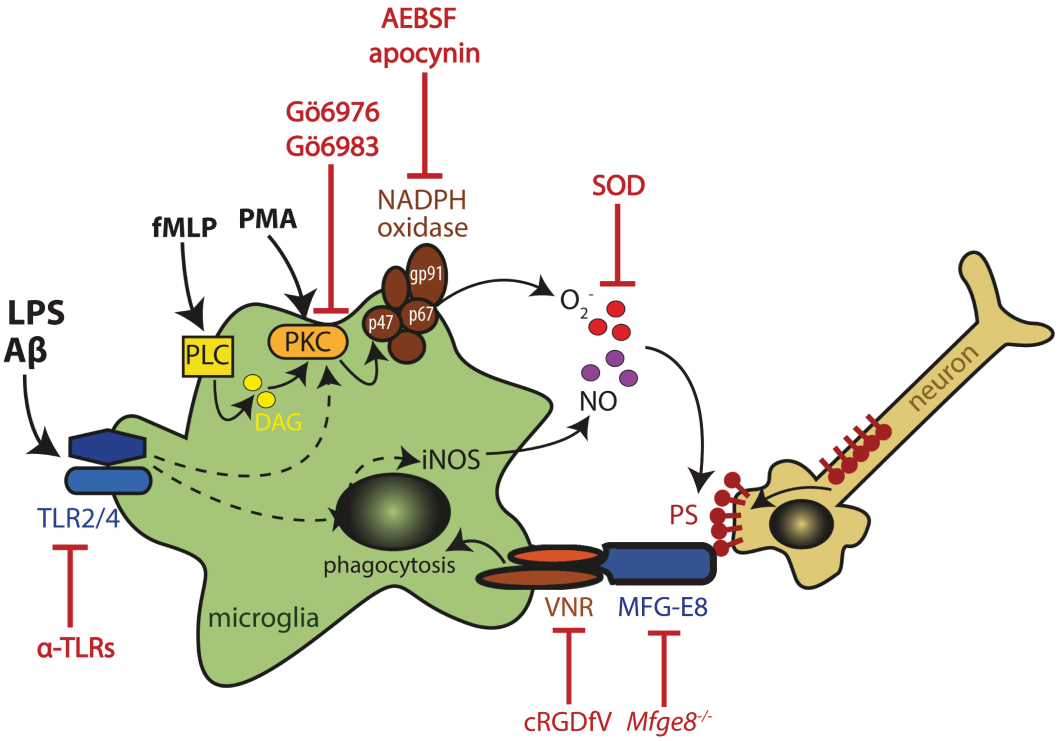

Fig 9. Suggested mechanism by which Aβ and PMA induce neuronal loss by successively activating microglial TLR2/4, PKC, NADPH oxidase, inducing neuronal phosphatidylserine (PS) exposure and triggering microglial phagocytosis of these neurons via MFG-E8 and the vitronectin receptor (VNR). Activation of TLR2/4 also causes iNOS expression, producing NO that reacts with superoxide from NADPH oxidase to form peroxynitrite that induces PS exposure. fMLP can also activate PKCs via phospholipase C (PLC) and its product diacylglycerol (DAG). Agents blocking neuronal loss, and where they act, are also indicated.