Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2013 | 126 | 312–317 doi: 10.1111/jnc.12288

SHORT COMMUNICATION

Lactadherin/MFG-E8 is essential for microglia-mediated neuronal loss and phagoptosis induced by amyloid β

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Abstract

Nanomolar β-amyloid peptide (Aβ) can induce neuronal loss in culture by activating microglia to phagocytose neurons. We report here that this neuronal loss is mediated by the bridging protein lactadherin/milk-fat globule epidermal growth factor-like factor 8 (MFG-E8), which is released by Aβ-activated microglia, binds to co-cultured neurons and opsonizes neurons for phagocytosis by microglia. Aβ stimulated microglial phagocytosis, but did not opsonize neurons for phagocytosis. Aβ (250 nM) induced delayed neuronal loss in mixed glial-neuronal mouse cultures that required microglia and occurred without increasing neuronal apoptosis or necrosis. This neuronal death/loss was prevented by antibodies to MFG-E8 and was absent in cultures from *Mfge8* knockout mice (leaving viable neurons), but was reconstituted by addition of recombinant MFG-E8. Thus, nanomolar Aβ caused neuronal death by inducing microglia to phagocytose otherwise viable neurons via MFG-E8. The direct neurotoxicity of micromolar Aβ was not affected by MFG-E8. The essential role of MFG-E8 in Aβ-induced phagoptosis, suggests this bridging protein as a potential therapeutic target to prevent neuronal loss in Alzheimer's disease.

Keywords: Alzheimer's disease, lactadherin, neuroinflammation, phosphatidylserine, SED1.

J. Neurochem. (2013) **126**, 312–317.

Alzheimer's disease (AD) is characterized by aggregated β-amyloid peptide (Aβ) plaques and tau tangles, accompanied by microglial activation (Heneka *et al.* 2010) and progressive loss of neurons and synapses, which are thought to cause progressive dementia (Takata and Kitamura 2012). The mechanisms of Aβ-induced neuronal loss are not clear. Micromolar levels of Aβ can induce direct neurotoxicity (Liao *et al.* 2007), but may not occur in AD brains (Steinerman *et al.* 2008). In contrast, nanomolar levels of Aβ induce neuronal loss mediated by microglia (Maezawa *et al.* 2011; Neniskyte *et al.* 2011). Microglia are resident brain macrophages that become highly phagocytic when activated by pathogens, damaged cells or Aβ (Kettenmann *et al.* 2011).

We have recently reported that neuronal and synaptic loss induced by nanomolar levels of Aβ required microglia and was prevented by blocking neuronal 'eat-me' signal phosphatidylserine (PS) or phagocytic microglial receptor, the $\alpha_v\beta_{3/5}$ integrin (Neniskyte *et al.* 2011). Inhibition of microglial phagocytosis did not increase neuronal apoptosis or necrosis but rather provided sustained protection of neurons.

Amyloid β can activate microglia via Toll-like receptors-2 and 4 (Reed-Geaghan *et al.* 2009), and we have found that agonists of these receptors can also cause progressive loss of neurons in culture or *in vivo* that is prevented by inhibition of phagocytosis (Neher *et al.* 2011). We recently reported that phagocytic neuronal loss was mediated by the bridging protein milk-fat globule epidermal growth factor factor-8 (MFG-E8, also known as lactadherin or SED1) (Fricker *et al.* 2012). MFG-E8 is an extracellular adaptor protein that binds the vitronectin receptor (the integrin $\alpha_v\beta_{3/5}$) found on phagocytes, thereby activating phagocytosis (Hanayama *et al.* 2002). MFG-E8 may mediate microglial phagocytosis of apoptotic neurons by binding exposed PS (Fuller and Van Eldik 2008). However, we and others have shown that viable but stressed neurons can reversibly expose PS, and thus MFG-E8 could potentially mediate the microglial phagocytosis of viable neurons (Kim *et al.* 2010; Neher *et al.* 2011).

Received February 5, 2013; revised manuscript received March 27, 2013; accepted April 21, 2013.

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Abbreviations used: AD, Alzheimer's disease; Aβ, β-amyloid peptide; MFG-E8, milk-fat globule-EGF-like factor 8; PS, phosphatidylserine.

Cell death caused by engulfment and phagocytosis of an otherwise viable cells is called 'phagoptosis', and can be increased by inflammation in body or brain (Brown and Neher 2012). We have previously shown that Aβ-induced neuronal loss is mediated by PS exposure on neurons (Neniskyte *et al.* 2011), thus it is possible that MFG-E8 functions to couple this PS exposure to microglial phagocytosis of neurons.

In this study we set out to test: (i) whether Aβ can induce MFG-E8 expression and release to potentially bridge between neurons and microglia, (ii) whether MFG-E8 mediates Aβ-induced neuronal loss, and (iii) whether preventing microglial phagocytosis by removing MFG-E8 leaves live or dead neurons, and thus whether such interference is potentially beneficial or detrimental. We find that MFG-E8 is essential for Aβ-induced neuronal loss, and the removal of MFG-E8 leaves viable neurons, indicating that MFG-E8 mediates phagoptosis of neurons.

Materials and methods

Reagents

All materials were purchased from Sigma (St Louis, MO, USA) except cell culture reagents (PAA, Piscataway, NJ, USA), amyloid β_{1-42} peptide (EZBiolab, Carmel, IN, USA), authentic peroxynitrite (Cayman Chemical, Ann Arbor, MI, USA), mouse recombinant MFG-E8 (R & D Systems, Minneapolis, MN, USA), MFG-E8 antibody and goat control IgG (Santa Cruz Biotechnology, Dallas, TX, USA), glial fibrillary acidic protein antibody (Dako, Carpinteria, CA, USA), Alexa Fluor 488-labeled *Griffonia simplicifolia* isolectin B_4 and goat anti-rabbit-Alexa Fluor 488 antibody (Invitrogen, Carlsbad, CA, USA), donkey anti-goat-Cy3 antibody and Fc region-specific anti-goat $F(ab)_2$ fragment (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and fluorescent microspheres (Spherotech, Lake Forest, IL, USA).

Primary cell culture

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act (1986) and approved by the Cambridge University local ethical committee. Primary mixed neuronal/glial cultures were prepared from cerebella of postnatal day 57 Wistar rat, C57Bl/6 or *Mfge8* knockout (Silvestre *et al.* 2005) mice pups as previously described (Kinsner *et al.* 2005). Glial cultures and pure microglial cultures were prepared as described previously (Bal-Price and Brown 2001). Microglia-depleted cultures were obtained by adding 50 μM L-leucine-methyl ester for 4 h (Neher *et al.* 2011). Wistar rats were sourced from Harlan Laboratories (Indianapolis, IN, USA). Mfge8 knockout mice, backcrossed onto a C57BL/6 genetic background, were from Clothilde Thery in the Institute Pasteur in Paris, C57BL/6 wild type mice were sourced from Harlan Laboratories. Cell cultures were prepared from both male and female pups.

Cell treatment

Cells were treated with 250 nM–5 μM of amyloid β_{1-42} prepared as previously described (Neniskyte *et al.* 2011). MFG-E8 antibody or control IgG (5 mg/mL) was added together with Aβ treatment or 48 h after stimulation, as specified; antibodies were pre-blocked with fivefold molar excess of a Fc region-specific F(ab′) fragment. *Mfge8* knockout cultures were reconstituted by adding 0.4 μg/mL of mouse recombinant MFG-E8. PS exposure on neurons was induced by treating cultures with 100 μM glutamate for 1 h or 10 μM peroxynitrite for 24 h. Cell densities were evaluated as previously described (Neniskyte *et al.* 2011). Nitric oxide release was evaluated by assessing nitrite levels with Griess reaction as described previously (Kinsner *et al.* 2005).

Immunocytochemistry

For immunofluorescent labelling, cells were grown on poly-L-lysine coated glass cover slips and processed as described previously (Neniskyte *et al.* 2011). Extracellular MFG-E8 was labelled by adding antibodies to live cultures and incubating them for 30 min at 37°C before fixation. Intracellular MFG-E8 was labelled in fixed and permeabilized cultures. Imaging was performed under a Leica (Wetzlan, Germany) DMI6000 CS microscope or a confocal Olympus (Tokyo, Japan) Fluoview 300 microscope.

Phagocytosis assays

Pure microglial cultures were treated with $A\beta_{1-42}$ (250 nM) for 24 h, stained with calcein AM, and then added to either 5 μm carboxylate-modified latex microspheres (phagocyte to bead ratio 1 : 10) or neuronal debris labelled with tetramethylrhodamine (TAMRA) (prepared from neuronal/glial cultures labelled with 10 μM TAMRA for 15 min at 37°C, collected from the plate and disrupted by passing cells 10 times through 27G needle), or TAMRA-labelled neuronal-glial cultures treated with 300 μM glutamate (± pre-coating with 10 μg/mL mouse recombinant MFG-E8 for 30 min at 37°C). Bead and neuronal debris uptake was evaluated after 30 min incubation at 37°C (intact neurons 60 min).

Statistics

For all experiments, all conditions were repeated in duplicate. Experiments were replicated in at least three independent cultures. All data presented are expressed as means ± SEM. Means were compared by one-way analysis of variance and *post hoc* Bonferroni test.

Results and discussion

To test whether Aβ can induce MFG-E8 expression in neuronal-glial co-cultures and trigger its release to potentially bridge between neurons and microglia during Aβ-induced neuronal loss, we imaged extracellular MFG-E8 by adding an MFG-E8 antibody to the non-permeabilized co-cultures 2 days after addition of Aβ. Stimulation with Aβ resulted in an increased number of both neurons and microglia with MFG-E8 bound to them (Fig. 1a and b) and an increase in total extracellular MFG-E8 (Fig. 1c and d). As neurons themselves cannot produce MFG-E8 (Kranich *et al.* 2010; Fricker *et al.* 2012), increased number of MFG-E8-positive neurons indicated that stimulation with Aβ triggered glial MFG-E8 release. Analysis of MFG-E8 expression in glial cultures revealed microglia as the main source of MFG-E8

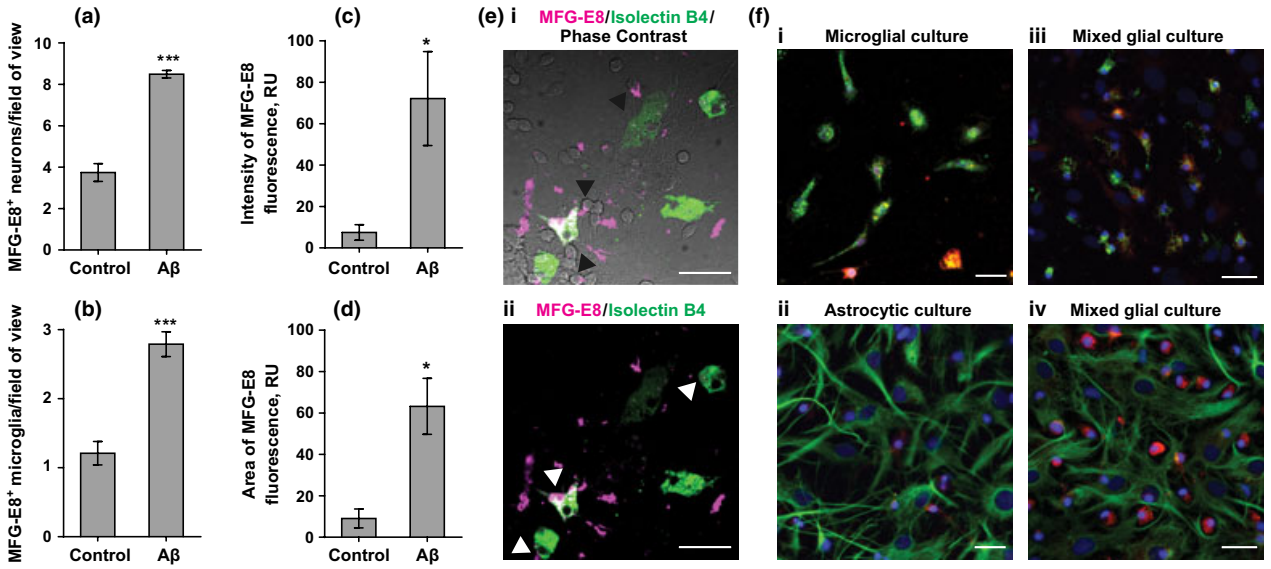

Fig. 1 Amyloid β $(Aβ)_{1–42}$ induces the release of bridging protein milk-fat globule-EGF-like factor 8 (MFG-E8). Extracellular MFG-E8 labelling reveals that $Aβ_{1–42}$ treatment (250 nM for 48 h) induces MFG-E8 binding to neurons (a), microglia association with MFG-E8-bound cells (b), the increase in the amount of deposited MFG-E8 (c) and the size of the MFG-E8-bound structures (d). MFG-E8 is deposited on neurons (round cerebellar granule cells; e(i), closed arrows), which are contacted by microglia (isolectin B_4 labelling; e(ii), open arrows). (f), Intracellular MFG-E8 labelling (*red*) is only observed in permeabilized pure microglial (i) and mixed glial cultures (iii, iv), but not astrocyte-enriched cultures (ii). MFG-E8 is observed in microglia labelled with isolectin B_4 (*green*, i, iii), while astrocytes labelled with glial fibrillary acidic protein antibody (*green*, ii, iv) do not have MFG-E8. Data are presented as means ± SEM for ≥ 3 independent experiments; */***$p < 0.05/0.001$ versus control. *Scale bars*, 10 μm (e), 25 μm (f).

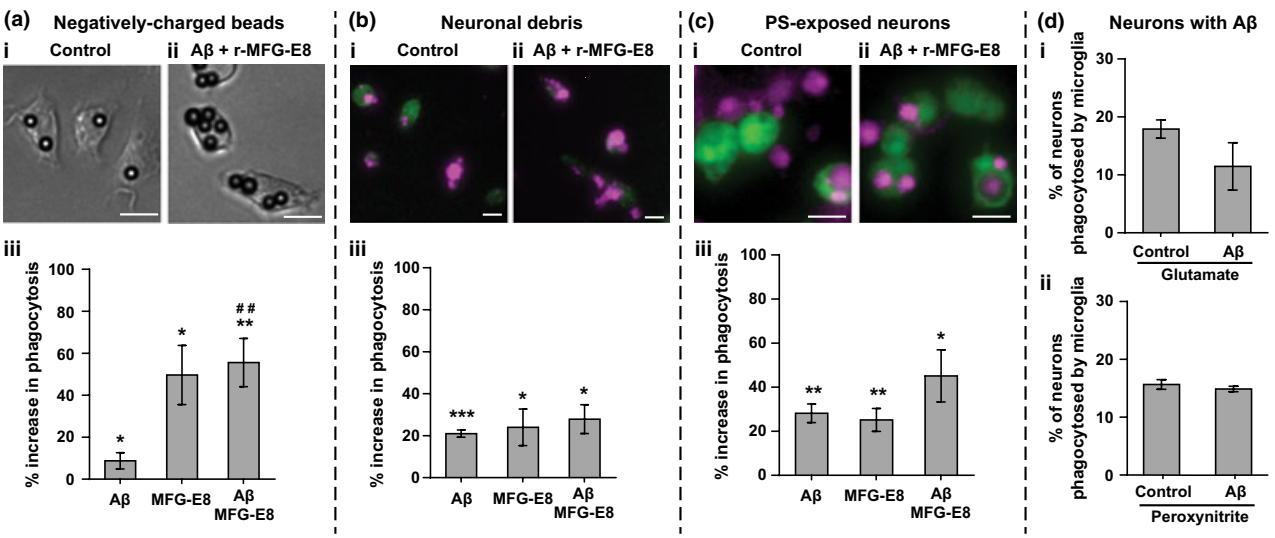

Fig. 2 Milk-fat globule-EGF-like factor 8 (MFG-E8) promotes amyloid β (Aβ)-induced phagocytosis of microspheres, neuronal debris and phosphatidylserine (PS)-exposing neurons. (a), Opsonization with recombinant MFG-E8 (r-MFG-E8) facilitates the uptake of beads by $Aβ_{1–42}$-activated microglia. (b), $Aβ_{1–42}$-stimulated microglia (isolectin B_4 labelling, *green*) more readily phagocytoze neuronal debris [tetramethylrhodamine (TAMRA) labelling, *magenta*] that are coated with r-MFG-E8. (c), Phagocytosis of PS-exposed neurons (treated with 300 μM glutamate for 1 h, TAMRA labelling, *magenta*) by $Aβ_{1–42}$-activated microglia (calcein AM labelling, *green*) is stimulated by pre-incubation with r-MFG-E8. (d), Pre-incubation with $Aβ_{1–42}$ (for 1 h) does not promote the elimination of PS-exposed neurons treated with (i) glutamate (100 μM for 1 h) or (ii) peroxynitrite (10 μM for 24 h) by LPS-activated microglia. Data are presented as means ± SEM for ≥ 3 independent experiments; */**/***$p < 0.05/0.01/0.001$ versus control, $^{\#\#}p < 0.01$ versus $Aβ_{1–42}$. *Scale bars* 25 μm.

(Fig. 1f). Thus, Aβ induced microglial release of MFG-E8 that could potentially act to mediate Aβ-induced microglial phagocytosis of neurons.

To test whether MFG-E8 stimulates microglial phagocytosis, we assayed whether the addition of MFG-E8 increased the uptake into microglia of negatively charged beads (Fig. 2a), neuronal debris (Fig. 2b) or PS-exposed neurons (Fig. 2c). In each case, the addition of MFG-E8 to these targets increased their uptake into microglia. Before addition to PS-exposed neurons, Aβ pre-treated (24 h) microglia were washed to remove Aβ, but this did not interfere with the Aβ–induced stimulation of microglial phagocytosis (Fig. 2c). As all the relevant Aβ might not have been removed by washing, Aβ was added directly to the PS-exposed neurons (rather than used to activate the microglia). However, this did not stimulate microglial phagocytosis (Fig. 2d). Thus, MFG-E8 can act as an opsonin for PS-exposed neurons (i.e. its binding to neurons increases their phagocytosis by microglia), while Aβ does not opsonize neurons but rather activates the phagocytic capacity of microglia and causes MFG-E8 release.

To test whether Aβ can induce delayed neuronal loss in mouse neuronal-glial co-cultures as occurs in rat cultures (Neniskyte *et al.* 2011), we isolated co-cultures from post-natal mouse cerebellum and treated them with 250 nM $A\beta_{1-42}$. After 3 days, Aβ had induced loss of about 25% of the neurons in culture without any increase the number of apoptotic or necrotic neurons (Fig. 3a). To test whether this neuronal loss required microglia, we pre-treated the culture with L-leucine-methyl ester to eliminate microglia (Fig. 3f) without affecting astrocytes or neurons (Neher *et al.* 2011). In the absence of microglia, 250 nM Aβ induced no neuronal loss or death (Fig. 3a), thus at nanomolar levels Aβ-induced neuronal loss requires microglia.

To determine whether MFG-E8 is required for Aβ-induced neuronal loss, we tested whether Aβ could induce neuronal

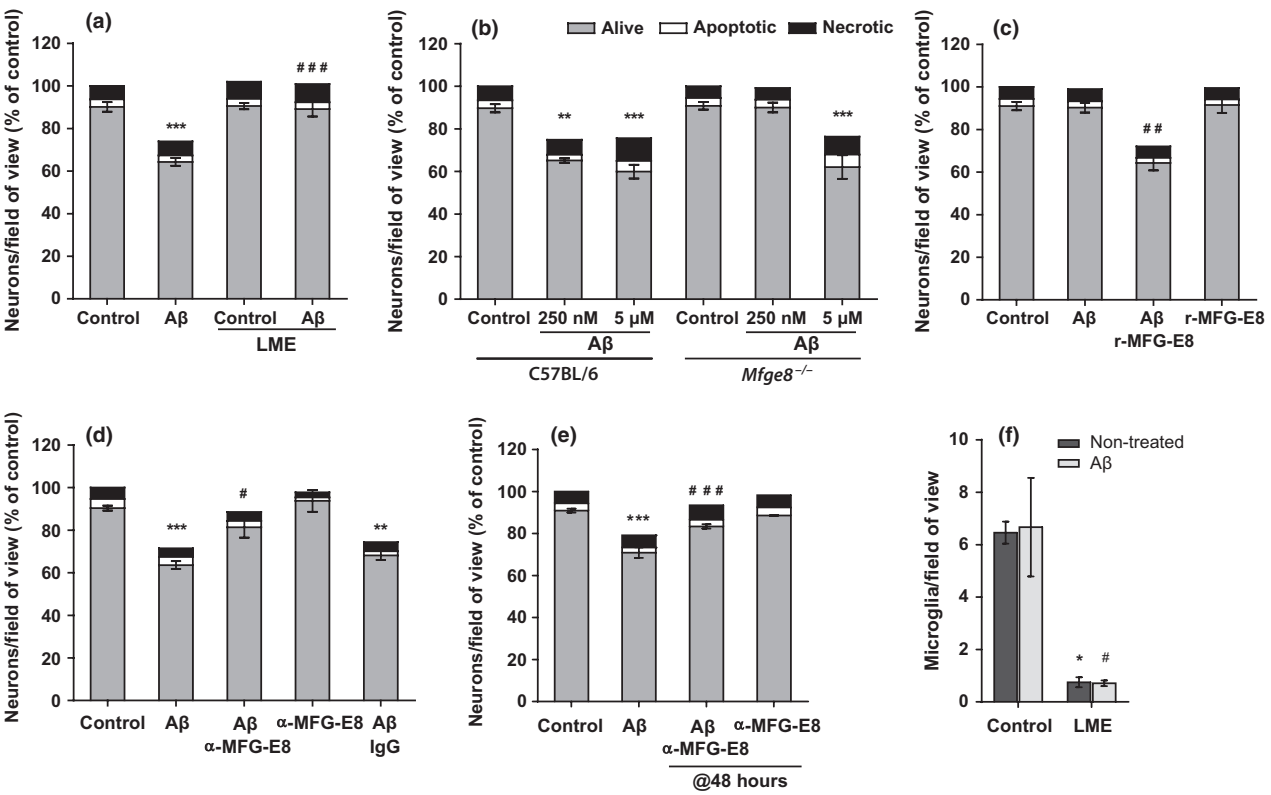

Fig. 3 Amyloid β $(A\beta)_{1-42}$-induced phagoptosis requires the presence of adaptor protein milk-fat globule-EGF-like factor 8 (MFG-E8). (a) In wild type culture $A\beta_{1-42}$ (250 nM for 72 h) induces neuronal loss that is prevented by microglia depletion with L-leucine-methyl ester (LME). (b) Nanomolar $A\beta_{1-42}$ (250 nM) induces neuronal loss in wild type cultures only, whereas 5 μM $A\beta_{1-42}$ is neurotoxic in both wild type (C57Bl/6) and *Mfge8* knockout (*Mfge8*$^{-/-}$) cultures. (c) In *Mfge8*$^{-/-}$ culture neuronal loss induced by 250 nM $A\beta_{1-42}$ is restored by adding recombinant murine MFG-E8 (*r-MFG-E8*, 0.4 μg/mL). (d, e) In rat culture MFG-E8 antibody (*α-MFG-E8*, 5 μg/mL) prevents neuronal loss induced by $A\beta_{1-42}$ (250 nM for 72 h) when added together with Aβ (d) or after 48 h incubation with Aβ (e); goat control IgG (5 μg/mL) do not have an effect (d). (f), In neuronal/glial culture treated with LME and subsequently stimulated with $A\beta_{1-42}$ (250 nM for 72 h), microglia is effectively removed as assessed by isolectin B_4 labelling. Data are presented as means ± SEM for ≥ 3 independent experiments; */**/*** $p < 0.05/0.01/0.001$ versus control, #/##/### $p < 0.05/0.01/0.001$ versus $A\beta_{1-42}$.

loss in neuronal-glial co-cultures from *Mfge8* knockout mice. At 250 nM concentration, Aβ induced no neuronal loss or death in cultures from *Mfge8* knockout mice, whereas it induced 25% neuronal loss in sister cultures from wild type mice (Fig. 3b). Lack of MFG-E8 in knockout cultures did not attenuate microglial activation ± Aβ as measured by nitric oxide release: Aβ stimulation increased nitrite from 2.3 ± 0.5 μM to 4.3 ± 0.4 μM in wild type cultures and from 2.8 ± 0.6 μM to 6.1 ± 1.7 μM in knockout cultures. Addition of 10 nM recombinant murine MFG-E8 to the *Mfge8* knockout cultures reconstituted neuronal loss to the Aβ-treated cultures, but had no effect on neuronal death and loss in the absence of Aβ (Fig. 3c). Thus, MFG-E8 is necessary for neuronal loss induced by nanomolar Aβ, but is not sufficient to induce neuronal loss on its own.

Micromolar levels of Aβ are known to cause direct toxicity to neurons, not mediated by microglia (Liao *et al.* 2007), so we tested whether this neuronal death and loss was also mediated by MFG-E8. We found that 5 μM Aβ induced both neuronal necrosis, apoptosis and loss measured at 3 days, and this Aβ-induced neuronal death and loss was not affected by the absence of MFG-E8 (Fig. 3b). Thus, the direct neurotoxicity of high levels of Aβ in mixed neuronal-glial cultures was not affected by MFG-E8. Note that some studies have reported that high nanomolar Aβ induces neuronal apoptosis directly, but such studies tend to use purified Aβ oligomers (Lambert *et al.* 1998).

We tested whether function-blocking MFG-E8 antibodies (Miksa *et al.* 2007), added at the same time as Aβ, would prevent Aβ-induced neuronal loss, and found that they did so (Fig. 3d). As MFG-E8 has been reported to increase macrophage phagocytosis of Aβ (Boddaert *et al.* 2007) and inhibit macrophage activation (Brissette *et al.* 2012), we tested whether delaying the addition of the MFG-E8 antibodies until after Aβ phagocytosis and microglial activation that occurs within 24 h (Jekabsone *et al.* 2006; Reed-Geaghan *et al.* 2009) would still prevent neuronal loss. We found that antibodies to MFG-E8 added 2 days after the addition of 250 nM Aβ prevented the neuronal loss measured at 3 days without changing the amount of neuronal apoptosis or necrosis (Fig. 3e). Thus, extracellular MFG-E8 is required between 2 and 3 days after Aβ addition to mediate neuronal loss, consistent with our previous finding of neuronal loss at this time (Neniskyte *et al.* 2011).

In conclusion, we have found that neuronal loss induced by nanomolar Aβ (but not micromolar Aβ) requires extracellular MFG-E8, released from activated glia and binding to neurons 2 days after Aβ addition, and inducing subsequent microglial phagocytosis of those neurons. Removal of MFG-E8, genetically or by antibodies, leaves live rather than dead neurons in the presence of nanomolar Aβ, indicating that MFG-E8-mediated microglial phagocytosis is removing live rather than dead neurons. Thus, the neuronal death induced by nanomolar Aβ is caused by phagocytosis, that is the cell death is by phagoptosis (Brown and Neher 2012). That the neuronal loss and death is mediated by the MFG-E8 pathway of phagocytosis is consistent with our previous findings that: (i) nanomolar Aβ induces PS exposure by the neurons, (ii) blocking exposed PS with annexin V or antibodies to PS prevents Aβ-induced neuronal loss and death, and (iii) Aβ-induced neuronal loss is prevented by cyclic arginine-glycine-aspartate-D-phenylalanine-valine peptide, a specific antagonist of the vitronectin receptor, which is known to mediate MFG-E8-induced phagocytosis (Neniskyte *et al.* 2011). Our finding that the MFG-E8 pathway of phagocytosis mediates neuronal loss induced by pathophysiologically relevant levels of Aβ suggests that this protein and pathway may be potential therapeutic targets to prevent neuronal loss in AD.

Acknowledgements

This study was supported by the Wellcome Trust [RG50995]. The authors thank Dr. Clotilde Théry (INSERM, France) for providing *Mfge8* knockout mice and for critically reading the manuscript. The authors have no conflict of interest.

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