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ORIGINAL PAPER

The Diabetes Drug Liraglutide Ameliorates Aberrant Insulin Receptor Localisation and Signalling in Parallel with Decreasing Both Amyloid-β Plaque and Glial Pathology in a Mouse Model of Alzheimer's Disease

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Abstract Alzheimer's disease (AD) has been shown to involve desensitised insulin receptor (IR) signalling. Liraglutide, a novel glucagon-like peptide 1 (GLP-1) analogue that facilitates insulin signalling, is currently approved for use in type 2 diabetes mellitus. In the present study, we show that distinctive alterations in the localisation and distribution of the IR and increased levels of insulin receptor substrate (IRS)-1 phosphorylated at serine 616 (IRS-1 pS^{616}), a key marker of insulin resistance, are associated with amyloid-β plaque pathology in the frontal cortex of a mouse model of AD, APP_{SWE}/PS1dE9. Altered IR status in APP_{SWE}/PS1dE9 is most evident in extracellular deposits with the appearance of dystrophic neurites, with significantly increased IRS-1 pS^{616} levels detected within neurons and neurites. The IR and IRS-1 pS^{616} changes occur in the vicinity of all plaques in the APP_{SWE}/PS1dE9 brain, and a significant upregulation of astrocytes and microglia surround this pathology. We show that liraglutide treatment for 8 weeks at 25 nmol/kg body weight i.p. once daily in 7-month-old mice significantly decreases IR aberrations in conjunction with a concomitant decrease in amyloid plaque load and levels of IRS-1 pS^{616}. Liraglutide also induces a highly significant reduction in astrocytosis and microglial number associated with both plaques and IR pathology. The amelioration of IR aberrations and attenuation of IRS-1 pS^{616} upregulation, plaque and glial activation in APP_{SWE}/PS1dE9 mice treated with liraglutide support the investigation of the therapeutic potential of liraglutide and long-lasting GLP-1 agonists in patients with AD.

Keywords Alzheimer's disease · Diabetes · GLP-1 · Liraglutide · Insulin receptor · Inflammation

Introduction

Type 2 diabetes mellitus (T2DM) is a risk factor for Alzheimer's disease (AD) (Hoyer 2004; Craft 2005). In addition, non-responsive insulin receptors (IR) have been described in the brains of patients with AD (Craft 2007; Moloney et al. 2010). This impairment of insulin function in the brain is believed to be mechanistically important in the processes of synaptic loss and cognitive decline in AD, leading to the hypothesis that AD may be a brain-specific 'Type 3 Diabetes' (Hoyer 1998; Steen et al. 2005; de la Monte and Wands 2008).

Investigation of IR status in the AD brain initially described decreased IR protein and mRNA levels (Steen et al. 2005). More recent studies show a clear loss of dendritic IR in AD temporal cortex and internalisation of the receptor in the soma of these AD neurons with no alteration in the absolute levels of IR subunit levels (Moloney et al. 2010). In addition, markedly reduced responses to insulin signalling have been described in the

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AD brain (Talbot et al. 2012). Related studies demonstrated that soluble Aβ oligomers cause a loss of dendritic IRs in aged primary hippocampal neurons in vitro, with increased expression of IR within the cell soma but no changes in absolute IR subunit levels, rendering IR non-responsive (Zhao et al. 2008; De Felice et al. 2009) and reflecting what is observed in the AD brain (Moloney et al. 2010). A role for insulin resistance in AD has been strengthened by findings that the levels of insulin receptor substrate (IRS)-1 phosphorylated on serine residues, S^{616}, S^{636} and S^{312}, which causes IR to be non-responsive, are increased in neurons in AD brain and in AD animal models (Ma et al. 2009; Moloney et al. 2010; Bomfim et al. 2012; Talbot et al. 2012). Increased levels of phosphorylation at these sites are strongly associated with a downregulation of insulin signalling and are a classic profile for insulin desensitisation as seen in T2DM (see Moloney et al. 2010; Talbot et al. 2012 for discussion). Notably, increased levels of IRS-1 pS^{616} and IRS-1 $pS^{636/639}$ and their activated kinases were found to correlate positively with those of oligomeric Aβ plaques in AD brain and were negatively associated with episodic and working memory in patients with AD, and this effect was particularly striking for IRS-1 pS^{616} levels (Talbot et al. 2012).

Attention has been drawn to the possibility that drugs used therapeutically in the treatment of T2DM may also be beneficial in the treatment of AD. The incretin hormone glucagon-like peptide-1 (GLP-1) facilitates insulin signalling, and novel long-lasting GLP-1 receptor agonists exendin-4 (Exenatide, Byetta®) and liraglutide (Victoza®) are approved for treatment for T2DM (Lovshin and Drucker 2009). GLP-1 also acts as a growth factor in the brain (Perry et al. 2007), and expression of GLP-1 receptors in the hippocampus positively regulates neurite outgrowth, learning and long-term potentiation (LTP) (During et al. 2003; Greenberg and Jin 2006; Abbas et al. 2009). Furthermore, GLP-1 and exendin-4 can reduce levels of Aβ in the brain (Perry et al. 2003) and prevent Aβ-induced neurotoxic effects (Oka et al. 1999, 2000). Inhibition of the enzyme responsible for the rapid metabolism of GLP-1, dipeptidyl peptidase-4 (DPP-4), improved memory impairment as well as decreasing plaque load and markers of inflammation in the APP_{SWE}/PS1dE9 mouse model of AD (D'Amico et al. 2010). Moreover, intraperitoneal injection of the long-lasting GLP-1 analogue, Val(8)GLP-1, which can cross the blood brain barrier (BBB), normalised LTP defects in the hippocampus of the APP_{SWE}/PS1dE9 mouse (Gengler et al. 2012).

Recent research has shown that peripherally administered liraglutide, like GLP-1, can cross the BBB, and intraperitoneal administration of liraglutide to 7-month-old APP_{SWE}/PS1dE9 mice (Jankowsky et al. 2001) significantly decreased Aβ plaques and soluble Aβ oligomers (McClean et al. 2011). In addition, this treatment prevented memory impairment in object recognition and water maze tasks, LTP defects, synapse loss and the number of activated microglia (McClean et al. 2011). Notably, very recent studies show intraperitoneal injection of exendin-4 in APP_{SWE}/PS1dE9 mice can decrease heightened levels of IRS-1 pS^{636} and IRS-1 pS^{312}, at the level of western immunoblot measures of these phospho-epitopes in hippocampal lysates, which correlated with improvements in behavioural tasks of cognition in these mice (Bomfim et al. 2012).

In the light of the above results, the present study sought to determine firstly whether IR status and measures of resistant insulin signalling, with focus on IRS-1 pS^{616}, are altered in vivo, where they localise, and how they relate to Aβ pathology and inflammation as indicated by glial and microglial activation in the APP_{SWE}/PS1dE9 mouse model, and secondly, if altered, whether intraperitoneal administration of liraglutide modulates IR and IRS-1 pS^{616} expression levels, and the relationship of this to Aβ pathogenesis, glial and microglial activation in this AD mouse model.

Materials and Methods

Animals

APPswe/PS1dE9 mice with a C57BL/6 background were obtained from The Jackson Laboratory (http://research.jax.org/repository/alzheimers.html). Heterozygous males were bred with wild-type C57BL/6 females bought locally (Harlan). Offspring were ear-punched and genotyped using PCR with primers specific for the APP sequence (forward: GAA TTCCGACATGACTCAGG, reverse: GTTCTGCTGCAT CTTGGACA), for details see Gengler et al. (2010). Mice not expressing the transgene were used as wild-type controls. Female animals were used in all studies. Animals were caged individually and maintained on a 12-h light/dark cycle (lights on at 8:00 am, off at 8:00 pm) in a temperature-controlled room (21.5 $\pm$ 1 °C). Food and water were available ad libitum. Mice were 7 months of age when treatment began. Mice were injected daily with liraglutide (25 nM/kg bw) or saline (0.9 % w/v) for 8 weeks. All experiments were licensed by the UK Home Office in accordance with the animals (Scientific Procedures) Act of 1986.

Peptides

Liraglutide was purchased from GL Biochem (Shanghai) Ltd. The purity of the peptide was analysed by reversed-phase HPLC and characterised using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Antibodies

Antibodies used in immunofluorescence were as follows: hAPP/Aβ (6E10) (1:50, Signet Laboratories, Dedham, Massachusetts USA); IR β-subunit (1:50, Santa Cruz Biotechnology, Heidelberg, Germany); IRS-1 pS^{616} (1:50, BioSource, Invitrogen, Dublin, Ireland); glial fibrillary acidic protein (GFAP) (1:500, Sigma-Aldrich, Poole, UK); GFAP (1:100, Dako Diagnostics Ireland, Dublin, Ireland); Iba1 (1:100, Wako Chemical Gmbh, Neuss, Germany); secondary antibodies: Cy2-conjugated anti-mouse and Cy3-conjugated anti-rabbit (1:100, Jackson Immunoresearch Laboratories, Inc., West Grove, Pennsylvania, USA); and DyLight 488-conjugated anti-mouse (1:400, Jackson Immunoresearch Laboratories Inc., West Grove, Pennsylvania, USA).

Brain Tissue Fixation and Processing

Animals were perfused transcardially with phosphate-buffered saline (PBS) buffer followed by ice-cold 4 % paraformaldehyde in PBS (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland). Brains were quickly removed and were fixed in 10 volumes of 10 % neutral-buffered formalin (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland) at RT for at least 48 h. Brains were processed in a Histokinnet tissue processor, briefly, brains were dehydrated in a series of increasing EtOH gradients in dH_2O (50 % EtOH for 30 min, 70 % EtOH for 2 h, 95 % EtOH for 2 h, 100 % EtOH for 2 h repeated twice), cleared using Histoclear (50 % Histoclear :50 % EtOH for 1 h, 100 % Histoclear for 2 and 3 h) (National Diagnostics, Atlanta, Georgia, USA) and embedded in paraffin wax (Sakura, Syntec Scientific, Dublin, Ireland). Paraffin blocks were subsequently sectioned to 5 μm thickness and mounted onto SuperFrost Plus slides (VWR International, Leuven, Belgium). Neuroanatomical identification and nomenclature abbreviations are those established by Paxinos and Franklin (2001).

Immunofluorescence Microscopy

Brain sections were deparaffinised using Histoclear and rehydrated through decreasing EtOH gradients in dH_2O. Heat-induced epitope retrieval was carried out by submerging slides in sodium citrate buffer (10 mM, pH 6.4) and heating for 10, 7 and 3 min intervals in a 750 W microwave, adding 50 ml room temperature buffer between intervals. Sections were blocked in 5 % bovine serum albumin (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland) in PBS for 30 min, followed by 4 °C overnight incubation in primary antibody diluted in blocking solution. A subset of sections was incubated in blocking solution without primary antibody as a control for non-specific secondary antibody staining. Other sections were preadsorbed with IRβ peptide plus anti-IRβ overnight to control for the primary antibody. Identical control experiments were performed with IRS-1 pS^{616}. Sections were then incubated for 1 h in fluorescent-conjugated secondary antibodies diluted in blocking buffer along with DAPI (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland) as a nuclear counterstain. Prior to mounting in Mowiol 488, sections were treated with Sudan Black (10 min; 0.1 % in 70 % EtOH) to quench lipofuscin autofluorescence. Sections were viewed on a Leica DMI3000 microscope and images captured with a Leica DFC 420C camera (Leica Microsystems GmbH, Wetzlar, Germany). Adobe Photoshop CS3 was used for pseudocolouring and resizing of images (Adobe Systems Incorporated, San Jose, CA, USA).

Quantification

For most experiments, frontal cortices in five mice ($n = 5$) were examined per condition. To calculate plaque number per field, 3 sections were analysed per mouse and these sections were each 10 sections apart. Plaques in five fields per section were counted. For the experiments examining astrocyte activation and heightened nuclear IRS-1 pS^{616} expression, GFAP-labelled astrocytes in 5 fields of one section per mouse or IRS-1 pS^{616} expression in 1 field of two sections per mouse ($n = 3$) were analysed, respectively. Thresholding was applied using the Imaris software (Bitplane AG, Switzerland) or Image J (NIH, USA) whereby an arbitrary background level was imposed and the area of GFAP-immunostaining or IRS-1 pS^{616} expression above this level was calculated. For experiments examining microglial number, Iba1-labelled microglia were counted in 10 fields of one section per mouse. Data were analysed using Graph Pad Prism 5 (Graph Pad Software, CA, USA). Results are represented as mean ± standard error of the mean (SEM). Statistical analysis was carried out using an unpaired Student's t test. Data were deemed significant when $p < 0.05$.

Results

β-Amyloid Plaque Levels, Changes in IR Localisation and Heightened IRS-1 pS^{616} Levels are Significantly Reduced in Liraglutide-Treated APP$_{SWE}$/PS1dE9 Mice

Seven-month-old APP$_{SWE}$/PS1dE9 ($n = 5$) mice and age-matched wild-type animals ($n = 5$) were treated for 8 weeks with either 0.9 % saline or liraglutide (25 nm/kg bw, i.p). Aβ deposition in plaques occurs in the frontal cortex of APP$_{SWE}$/PS1dE9 mice after 6 months of age (Jankowsky et al. 2004) and was visualised here with hAPP-Aβ (6E10) antibody (Fig. 1a). Results show β-amyloid plaque number was significantly reduced (31.5 %, $p < 0.0001$) in the frontal cortex of liraglutide-treated APP$_{SWE}$/PS1dE9 mice (Fig. 1a–c).

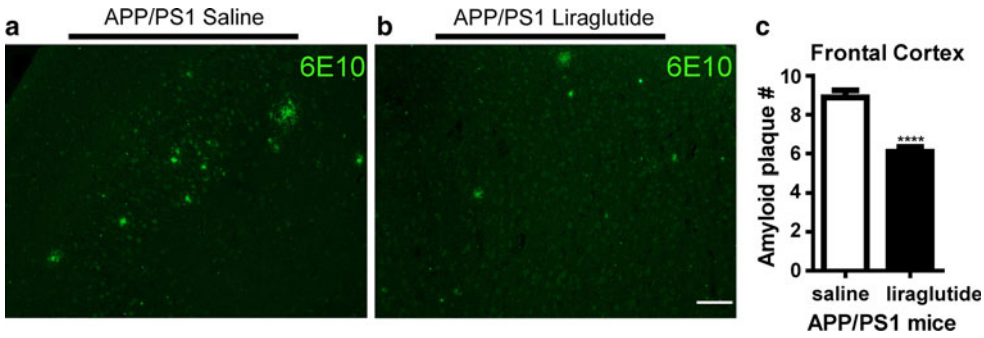

Fig. 1 β-amyloid plaque number is significantly reduced in liraglutide-treated APP_{SWE}/PS1dE9 mice. Representative immunofluorescence analysis showing hAPP-Aβ (6E10) staining in saline- (**a**) and liraglutide- (**b**) treated mice. *Scale bar* 100 µm. **c** Amyloid plaque number per field in frontal cortex was decreased by 31.5 % in liraglutide-treated APP_{SWE}/PS1dE9 mice compared with saline-treated. $^{****}p < 0.0001$ (Student's t test), $n = 5$

Representative images of IRβ immunofluorescence in the frontal cortex of APP_{SWE}/PS1dE9 (Fig. 2b, c, f) and age-matched wild-type mice (Fig. 2a) are shown in Fig. 2. Results show IR is widely expressed throughout frontal cortical layers in non-Tg mice localised within neuronal cell soma and dendrites (Fig. 2a). In the APP_{SWE}/PS1dE9 mice, a significant increase in IR expression is evident in certain areas of the cortex, this is most evident as IR immunoreactivity in dense deposits within the neuropil (Fig. 2b, c, f), assumed to be components of dystrophic neurites. Fragmentation and disorganisation of IR-immunostained neurites are also evident in neurons, and IR can appear increased within some neurons, in the vicinity of IR deposits (Fig. 2b arrow, c, f). This altered distribution of IRβ is never present in wild-type mice (Fig. 2a) and is not reflective of non-specificity of antibody, as controlled by preabsorption of the primary antibody with the immunising synthetic peptide (Fig. 2d) or leaving out the primary antibody (Fig. 2e). No marked difference in the number of neurons expressing IR was detected when comparing APP_{SWE}/PS1dE9 and wild-type mice.

Double immunofluorescence with IRβ with hAPP-Aβ (6E10) antibodies revealed that the pathological IR immunoreactivity in APP_{SWE}/PS1dE9 mice was present in the vicinity of all extracellular Aβ plaques (Fig. 2f, g), where altered IR expression localised selectively within the circumference of all plaques found present. Strikingly, results show liraglutide treatment caused a highly significant reduction in the level of IR detected in the neuropil surrounding Aβ plaques (Fig. 2h, i) compared with saline-treated APP_{SWE}/PS1dE9 mice (Fig. 2f). This occurred in parallel with the above described significant reduction in Aβ plaque burden (Fig. 1c).

Investigation into downstream IR signalling in the APP_{SWE}/PS1dE9 mice demonstrated substantial increases in IRS-1 pS^{616} levels indicative of resistance to insulin signalling. Increased IRS-1 pS^{616} was evident particularly in the cell soma of neurons (Fig. 3b) with a very marked concentric appearance in neurons surrounding plaques (Fig. 3b) and also in neurites in their vicinity (Fig. 3b), compared with levels in WT mice (Fig. 3a), which showed no heightened neuronal IRS-1 pS^{616}, with diffuse expression evident throughout the soma and also neuritic expression especially in deep layers of the frontal cortex. Increased IRS-1 pS^{616} levels were very evident and more widespread than the aberrant changes in IR distribution in APP_{SWE}/PS1dE9 cortical sections. Double-labelling with GFAP and Iba1 confirmed that the increases in IRS-1 pS^{616} were found within neurons and not within astrocytes or microglia, respectively (data not shown). Serial sections of IRS-1 pS^{616}/hAPP-Aβ (6E10) (Fig. 3c) and IRβ/hAPP-Aβ (6E10) (Fig. 3e) immunostaining indicate that IRS-1 pS^{616} is present within a proportion of the same neurons which have obvious alterations in IRβ status (Fig. 3c, e, arrows) and in close vicinity with the increased IR immunoreactivity in deposits, as well as being present in other neurons more distal from the plaques. Interestingly, as well as the extreme levels of heightened IRS-1 pS^{616} in cell soma of APP_{SWE}/PS1dE9 surrounding dense Aβ plaques (Fig. 3c), increased neuronal IRS-1 pS^{616} levels were found in areas which showed only very minor increases in extracellular amyloid-β, suggestive of areas in which nascent plaques may be emerging (not shown). Importantly, heightened IRS-1 pS^{616} was reduced significantly in neurons surrounding remaining plaques in liraglutide-treated APP_{SWE}/PS1dE9 mice (Fig. 3d, f) ($p < 0.05$) concomitantly with plaque load, with the increased cell somal IRS-1 pS^{616} still surrounding any remaining plaques.

Increased Levels of Astrocytic Activation Closely Associate with IRβ and Aβ Pathology in APP_{SWE}/PS1dE9 Mice and is Significantly Reduced by Liraglutide Treatment

An Aβ-associated inflammatory response has been described in APP_{SWE}/PS1dE9 mice linked with increased levels of GFAP-immunopositive activated astrocytes (Gordon et al. 2002; Ruan et al. 2009; Zhang et al. 2012). In the

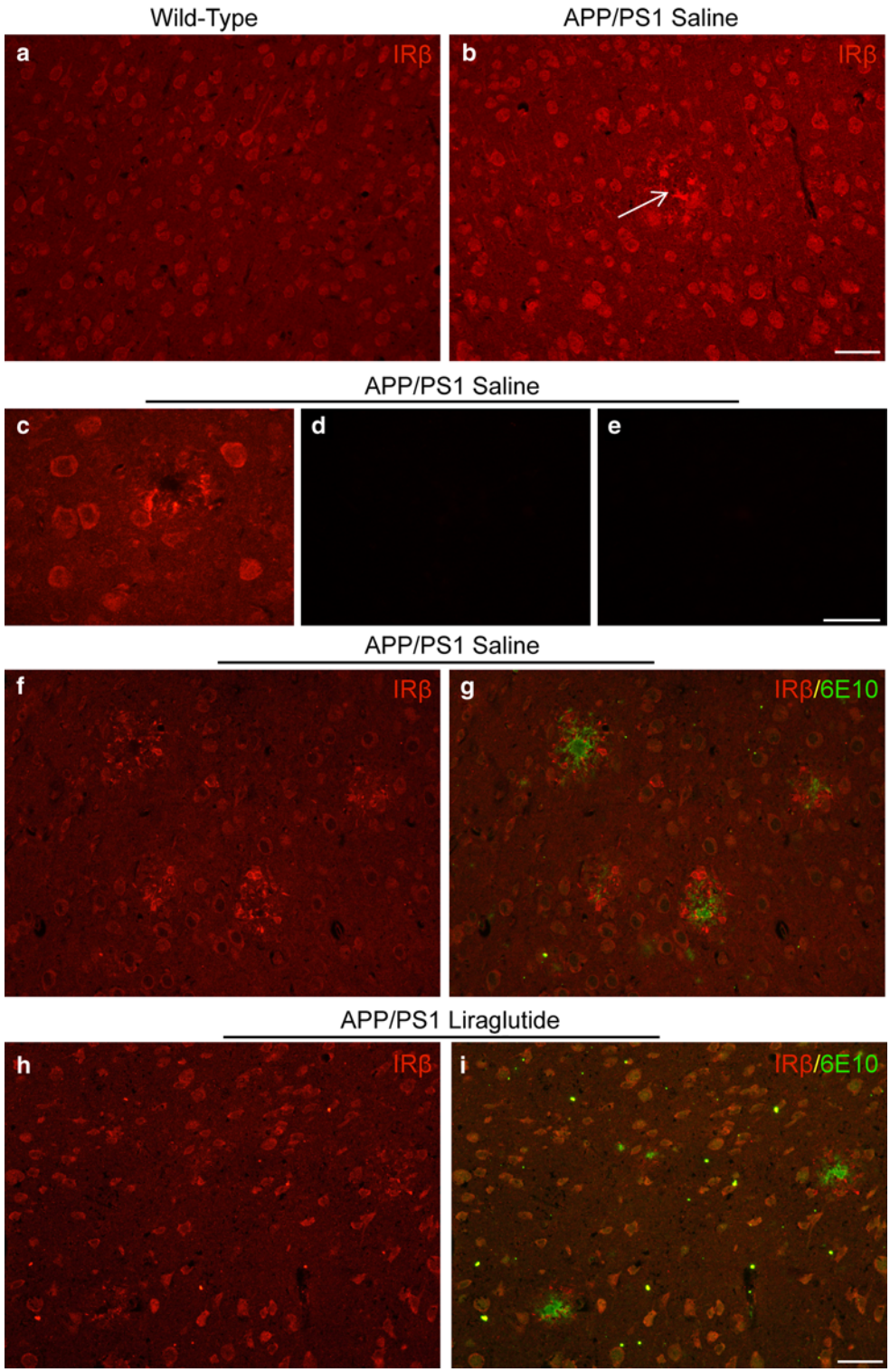

Fig. 2 IR alterations in frontal cortex localise to Aβ plaques and are ameliorated in liraglutide-treated APP_{SWE}/PS1dE9 mice. Representative immunofluorescence analysis shows IRβ in the frontal cortex of wild-type mice (**a**) and APP_{SWE}/PS1dE9 mice (**b**, **c**, **f**) at varying magnifications. Dystrophic IR-immunopositive neurites are shown (**b**, *arrow*, **c**). IRβ peptide block (**d**) and lack of primary antibody applied to APP_{SWE}/PS1dE9 sections (**e**) show the IRβ staining (**c**) is not non-specific. IRβ pathology localises to plaques in APP_{SWE}/PS1dE9 mice (**f**), confirmed by double immunofluorescence with hAPP-Aβ (6E10) (**g**), and this pathology is reduced in liraglutide-treated APP_{SWE}/PS1dE9 mice (**h**, **i**) in parallel with the decrease in Aβ. *Scale bar* 50 μm

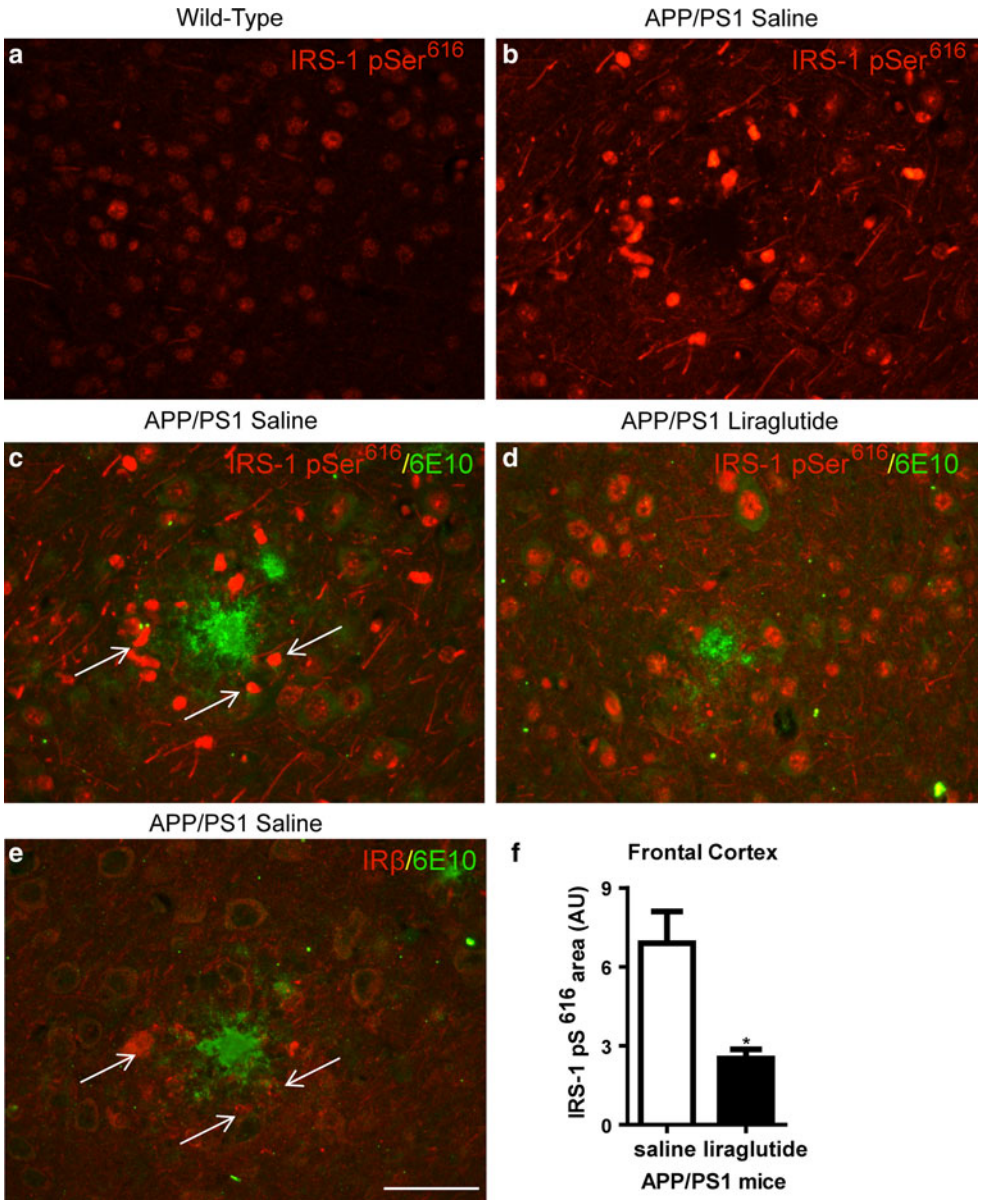

Fig. 3 Substantial increases in IRS-1 pS^{616} occur in frontal cortex of APP_{SWE}/PS1dE9 mice localising with Aβ plaques and is ameliorated in liraglutide-treated APP_{SWE}/PS1dE9 mice. Representative immunofluorescence analysis of IRS-1 pS^{616} in the frontal cortex of wild-type (**a**) and APP_{SWE}/PS1dE9 mice (**b**) showing IRS-1 pS^{616} up-regulation in neurons and neurites in APP_{SWE}/PS1dE9 mice. Double immunofluorescence of IRS-1 pS^{616} with hAPP-Aβ (6E10) shows IRS-1 pS^{616} present at high levels in cell soma of neurons surrounding plaques in APP_{SWE}/PS1dE9 mice and within neurites (**c**) which is significantly decreased in liraglutide-treated APP_{SWE}/PS1dE9 mice (**d**). Serial sections of an Aβ plaque (**c**, **e**) show large increases in IRS-1 pS^{616} in the soma of cells surrounding the plaque (**c**) a subset of which may co-localise with neurons showing aberrations in IRβ status (**c**, **d** *arrows*). Heightened IRS-1 pS^{616} is reduced in liraglutide-treated APP_{SWE}/PS1dE9 mice (**f**). *$p < 0.05$ (Student's t test), $n = 3$. *Scale bar* 50 μm

present study, we detected a very specific and highly significant up-regulation of GFAP-labelled activated astrocytes in the frontal cortex of APP_{SWE}/PS1dE9 mice (Fig. 4b) with extremely minimal to absent GFAP immunoreactivity detected in this region in wild-type litter mate controls (Fig. 4a) or in the liraglutide-treated wild-type group (data not shown). Double immunofluorescence of GFAP with IR in APP_{SWE}/PS1dE9 mice reveals GFAP-positive astrocytes both surround and are closely associated with aberrant IR accumulation in the neuropil (Fig. 4c). IR immunofluorescence in neurites in APP_{SWE}/PS1dE9 frontal cortex can appear fragmented in the vicinity of activated astrocytes (Fig. 4c). In general, IRβ does not co-localise within activated astrocytes (Fig. 4c). The marked astrocytic activation in the frontal cortex of APP_{SWE}/PS1dE9 mice was decreased to a significant

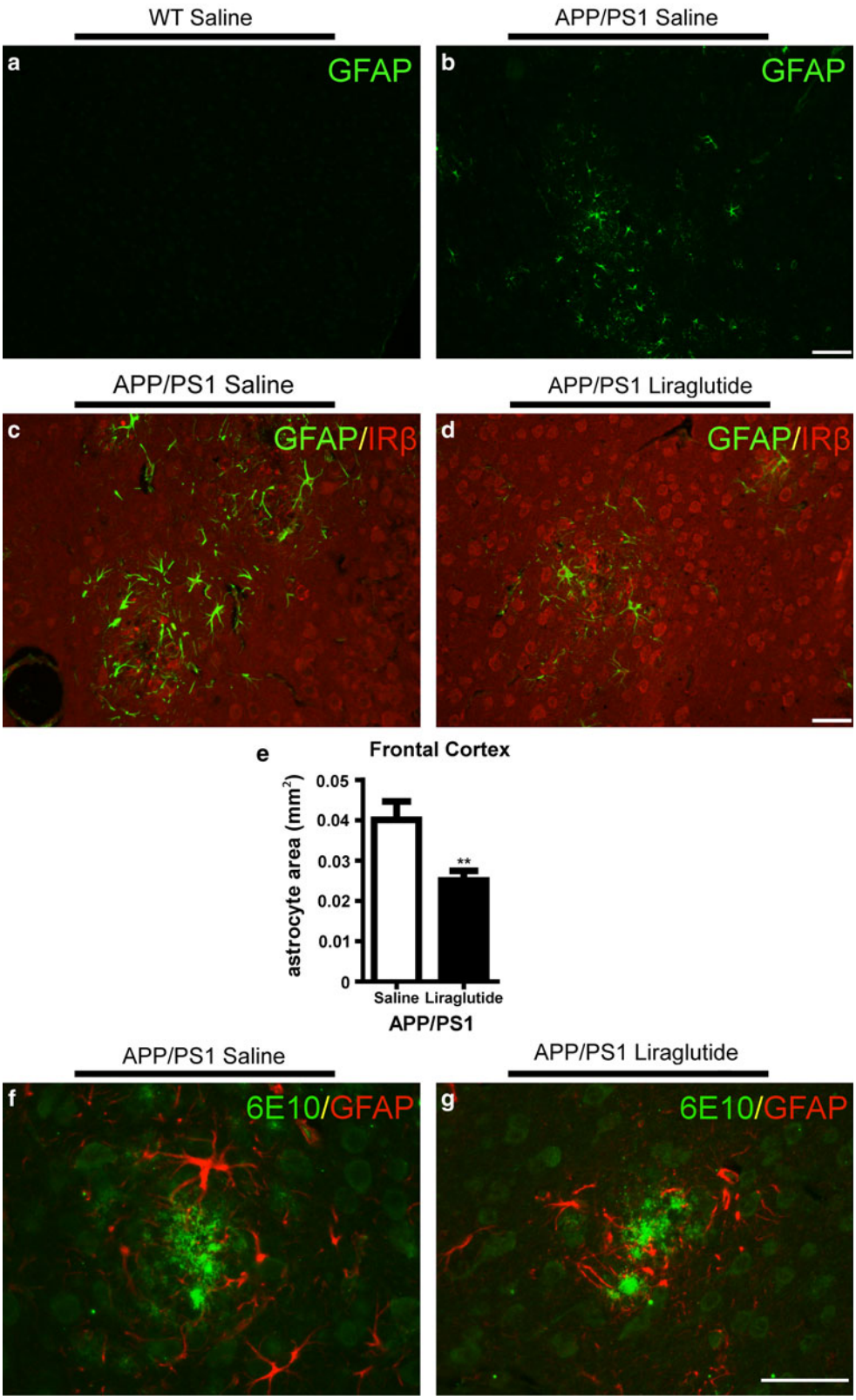

Fig. 4 Astrocytic activation is up-regulated in APP_{SWE}/PS1dE9 mice and associates with Aβ plaque and IR pathology and is abrogated in liraglutide-treated APP_{SWE}/PS1dE9 mice. **a** Representative immunofluorescence analysis shows wild-type mice possess almost no GFAP immunoreactivity in frontal cortical sections. **b** Astrocytic activation is markedly increased in APP_{SWE}/PS1dE9 mice. These astrocytes converge around the aberrantly localised IRβ (**c**, **d**) and the astrocytic load is significantly reduced upon treatment with liraglutide, as seen by representative immunofluorescence (**d**) and by measurement of astrocytic area per field (**e**). The GFAP-positive astrocytes associate proximal to Aβ plaque pathology in saline-treated APP_{SWE}/PS1dE9 mice (**f**), and the few remaining astrocytes also associate very proximal to remaining to Aβ plaque pathology in liraglutide-treated APP_{SWE}/PS1dE9 mice (**g**). $^{**}p < 0.01$ (Student's *t*-test), $n = 5$. *Scale bar* 100 μm (**a**, **b**); 50 μm (**c**–**f**)

degree ($p < 0.01$) in the liraglutide-treated group (Fig. 4e), in parallel with the significant amelioration of the altered localisation and distribution of IR (Figs. 4d, 2h). Reduced levels of astrocytes were most apparent in the distal circumference of abnormal IR immunostaining, and any remaining astrocytes were found in very close proximity to

the minimal residual IR abnormalities in the vicinity of plaques (Fig. 4d). Similarly, double immunofluorescence of GFAP with hAPP-Aβ (6E10) in APP_{SWE}/PS1dE9 brain sections showed astrocytes both distal and proximal to plaques, and upon treatment with liraglutide, remaining astrocytes are found solely proximal to remaining Aβ plaques, shown in high magnification in Fig. 4f, g.

Increased Levels of Activated Microglia Closely Associate with Both Aβ Plaques and Altered IR Localisation in APP_{SWE}/PS1dE9 Mice and Microglial Number is Significantly Reduced by Liraglutide Treatment

The APP_{SWE}/PS1dE9 mice have been determined previously to have increased levels of activated microglia within the cortex (Gordon et al. 2002; Ruan et al. 2009; McClean et al. 2011), which is reduced upon treatment with liraglutide (McClean et al. 2011). The present study confirms this and detected a significant increase in number of Iba1-positive microglia in the frontal cortex of APP_{SWE}/PS1dE9 mice compared with levels in either wild-type mice or liraglutide-treated wild-type mice. Liraglutide treatment caused a significant abrogation in the number of Iba1-positive microglia ($p < 0.05$). Ramified microglia were ubiquitously expressed within the frontal cortex of wild-type mice. In contrast, the up-regulation of microglia in APP_{SWE}/PS1dE9 mice was noted to be focused directly surrounding plaques, and these microglia displayed a more amoeboid macrophage-like appearance (Fig. 5a). The liraglutide-induced reduction of Iba1-positive microglia occurred concomitantly with a reduction in Aβ plaque number (Fig. 5b). Indications of microglia phagocytosing hAPP-Aβ (6E10)-immunoreactive plaques were apparent with liraglutide treatment (Fig. 5b, yellow in merged image).

Double immunofluorescence microscopy of IR and Iba1 was not possible due to antibody constraints. To overcome this, serial sections of APP_{SWE}/PS1dE9 frontal cortex were analysed with hAPP-Aβ (6E10) (Fig. 5c, d), followed by double immunofluorescence with either IRβ (Fig. 5e) or Iba1 (Fig. 5f). This enabled the relationship between IR, Aβ plaques and activated microglia to be investigated. Results indicate both IRβ (Fig. 5e) and Iba1 (Fig. 5f) localise in close proximity to the same plaque (Fig. 5c, d). Some IR immunostaining has morphological similarities to Iba1-immunostaining around the same plaques (arrow Fig. 5e–h), possibly indicating IR within microglia. Mutually exclusive dystrophic IR and microglial activation were also apparent (Fig. 5e–h). This was also evident with liraglutide treatment (not shown).

In conclusion, results from this study demonstrate that liraglutide ameliorates IR abnormalities in the APP_{SWE}/PS1dE9 mice and that this is closely associated with significantly reduced levels of Aβ plaques, as well as a reduction in a marker of insulin resistance IRS-1 pS^{616} and reduced inflammation via activated astrocytes and microglia.

Discussion

The major findings of this study demonstrate that Aβ plaque deposition in APP_{SWE}/PS1dE9 frontal cortex is associated closely with both the altered localisation of neuronal IRs and increased levels of neuronal IRS-1 pS^{616}, the latter a major indicator of resistance to insulin signalling and that a significant amelioration of these distinctive IR and IRS-1 pS^{616} changes is induced by peripheral administration of the GLP-1 analogue liraglutide, a drug currently used in the treatment of T2DM. The beneficial effects of liraglutide on IR status in the APP_{SWE}/PS1dE9 brain were shown to be concomitant with a liraglutide-induced reduction in both Aβ plaque number and the inflammatory response via a significant reduction in both activated astrocytes and microglia. The results draw attention to the potential for liraglutide in alleviating desensitised brain insulin signalling in AD, which is linked to processes of synaptic and cognitive decline in the disease (Ferreira and Klein 2011; Holscher 2011; O'Neill et al. 2012).

This study confirmed a widespread distribution of IRs in neuronal cell bodies, dendrites and synaptic neuropil throughout all cortical layers of the frontal cortex in wild-type mice (for overview see Ferreira and Klein 2011). Results show a very striking and selective association between Aβ plaque deposition and alterations in IR status in the APP_{SWE}/PS1dE9 frontal cortex, with the most marked feature being the accumulation of IR immunoreactivity in distorted deposits that have the appearance of dystrophic neuronal processes/neurites previously described to associate with plaques in the APP_{SWE}/PS1dE9 model (Garcia-Alloza et al. 2006; Wu et al. 2010). Some alterations in the levels of IR could be seen in neurons in the vicinity of plaques. However, the internalisation of IR within neurons and their removal from dendrites that has been described to occur with insulin resistance in the AD brain (Moloney et al. 2010) and concomitant with insulin resistance induced by Aβ oligomer treatment in hippocampal neurons in vitro (Zhao et al. 2008; De Felice et al. 2009) was not evident in the APP_{SWE}/PS1dE9 model at 7–9 months of age.

In order to investigate the status IR signalling in APP_{SWE}/PS1dE9, we selected IRS-1 pS^{616}, a known marker of insulin resistance in T2DM, which increases very significantly in neurons in AD brain (Ma et al. 2009; Moloney et al. 2010; Talbot et al. 2012), in hippocampal neurons in the 3× Tg-AD brain (Ma et al. 2009) and after addition of Aβ oligomers to hippocampal neurons in vitro (Ma et al. 2009; Bomfim et al. 2012). Recent studies show

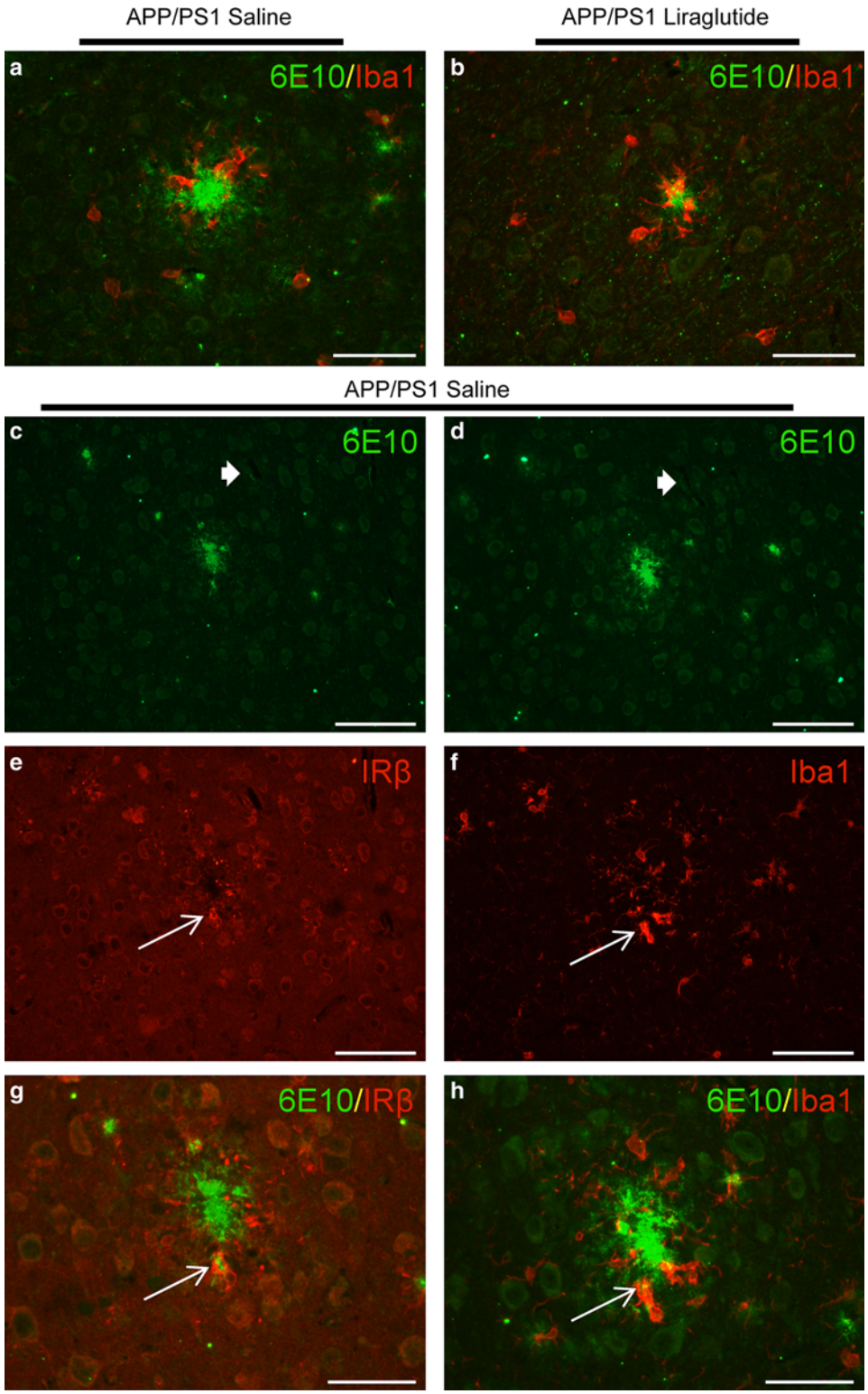

Fig. 5 Microglial activation is associated with Aβ plaque pathology and is decreased in liraglutide-treated APP_{SWE}/PS1dE9 mice, IR which surrounds Aβ plaques in APP_{SWE}/PS1dE9 mice is found both independently of and co-localising with microglia. **a** Iba1-immunoreactive clusters of microglia associate with plaques, confirmed by double immunofluorescence with hAPP-Aβ (6E10), and microglial number per field is significantly reduced ($p < 0.05$) in liraglutide-treated APP_{SWE}/PS1dE9 mice (**b**). Representative immunofluorescence shows a plaque in APP_{SWE}/PS1dE9 serial sections; the *thick arrow* indicates a blood vessel found in both sections (**c**, **d**). IRβ (**e**) and microglial activation (**f**) are localised to the plaque region. IRβ and Iba1 appear in the same location in relation to the plaque, (**g**, **h**, *thin arrows*) indicating a possible co-localisation of IR with microglia. *Scale bar* 50 μm (**a**, **b**, **g**, **h**). 100 μm (**c**–**f**)

increased levels of hippocampal neuronal IRS-1 pS^{616} correlate positively with oligomeric Aβ plaques in AD brain and are a very early and pronounced marker of insulin resistance negatively associated with episodic and working memory in AD, even after adjusting for Aβ plaques, neurofibrillary tangles and APOE-ε4 (Talbot et al. 2012). The present work now reveals highly significant increases in IRS-1 pS^{616} levels within neurons surrounding Aβ plaques and within neurites in the vicinity of plaques in 7-month-old APP_{SWE}/PS1dE9 mice. Although IRS-1 pS^{616} increases co-localised with those observed for IR around Aβ plaques deposits, increased levels of IRS-1 pS^{616} were much more marked and widespread than any expression changes for IR, as has been indicated in AD brain (Moloney et al. 2010; Talbot et al. 2012). Interestingly, increased neuronal and neuritic IRS-1 pS^{616} were detected in regions where nascent plaques were emerging in APP_{SWE}/PS1dE9 and thus may be a very early visible indicator of insulin resistance and emerging AD pathogenesis in this AD model as described in the AD brain (Talbot et al. 2012).

The incretin hormone GLP-1 and long-lasting GLP-1R agonists, exendin-4 and liraglutide are proving very effective in restoring insulin sensitivity in T2DM and are receiving increasing attention as treatments for neurodegenerative disorders. This is because they have been shown to have neuroprotective and neurotrophic roles in models of AD, reducing levels of Aβ, and the neurotoxic effect of Aβ, as well as normalising LTP defects, memory impairments, synapse loss and glial pathologies (Perry et al. 2003; Li et al. 2010; McClean et al. 2011; Klinge et al. 2011; Bomfim et al. 2012), as well as in Parkinson's disease (PD) (Li et al. 2009; Kim et al. 2009) and stroke (Li et al. 2009). Our results show for the first time a striking ability of intraperitoneal liraglutide treatment to target and alleviate impaired insulin signalling in neurons in APP_{SWE}/PS1dE9 brain. This causes the amelioration of assumed pathological alterations in IR distribution and reduction of heightened neuronal and neuritic IRS-1 pS^{616}, in association with diminishing Aβ plaque load and the associated inflammatory glial response. Notably, recent studies show that intraperitoneal injection of exendin-4 can decrease the increased total hippocampal levels of IRS-1 pS^{636} and IRS-1 pS^{312} in the same AD mouse model, which correlated with improvements in behavioural tasks of cognition in these mice (Bomfim et al. 2012). Behaviour was not investigated in the present study, however, the APP_{SWE}/PS1dE9 and wild-type control mice were from the same group as those employed in a previous investigation, wherein liraglutide treatment strategies were identical and liraglutide ameliorated the behavioural memory impairment seen in this mouse model of AD (McClean et al. 2011).

The simplest interpretation of the mechanistic sequence of events for the beneficial effect of liraglutide on IR status is that the liraglutide-induced reduction in Aβ burden deters the potential pathological effect of Aβ species on IR signalling. However, direct effects of liraglutide to restore or resensitise IR signalling and thereby reduce Aβ pathogenesis are also a distinct possibility. This is because insulin-induced stimulation of IR tyrosine kinase activity can block pathological binding of Aβ oligomers to dendritic spines in vitro (De Felice et al. 2009), and functioning IR receptors will increase Aβ clearance, reduce levels of extracellular Aβ oligomers and can impact positively on measures of cognitive function (Townsend et al. 2007; Zhao et al. 2008, 2009).

Our results also emphasise the potent anti-inflammatory mechanism of action of liraglutide in ameliorating both Aβ and also IR pathology. A very early inflammatory response through increased pro-inflammatory mediators and activated microglia occurs prior to the appearance of plaque deposition in APP_{SWE}/PS1dE9 mice (Gordon et al. 2002), and astrocytosis correlates with the levels of soluble Aβ and cognitive defects in this AD model (Zhang et al. 2012). An anti-inflammatory action of liraglutide in reducing microglial activation has been described in the APP_{SWE}/PS1dE9 brain (McClean et al. 2011), whereas the impact of liraglutide on astroglial status has not been examined.

In the present study, focus was placed on investigating the relationship between IR and glial status and related Aβ pathology. The liraglutide-induced reduction in the number of microglia detected was coincident with both a reduced plaque burden and amelioration of IR changes and also revealed an ability of remaining microglia to phagocytose Aβ. Furthermore, the highly significant reduction in the number of GFAP-immunoreactive astrocytes that we show to be induced by liraglutide in the APP_{SWE}/PS1dE9 frontal cortex revealed remaining astroglial activity was exclusively localised to remaining Aβ plaques and highly reduced residual IR pathology. Inflammatory rather than protective behaviour of glia is believed to be an important underlying mechanism of neurodegeneration and cognitive decline in AD (Akiyama et al. 2000; Aisen 2002; McGeer et al. 2006). Our results highlight a central anti-inflammatory mechanism of action for liraglutide that may be important therapeutically in reducing Aβ pathology as well as IR defects and agree with studies showing anti-inflammatory mechanism for GLP-1 agonists in AD (McClean et al. 2011) and PD mouse models (Kim et al. 2009), as well as in in vitro studies (Iwai et al. 2006). This may involve reduced inflammatory TNFα/JNK signalling, as exendin-4 abrogated increases in IRS-1 pS^{636} and IRS-1 pS^{312} levels in APP_{SWE}/PS1dE9 mice via reduced TNFα/JNK signalling (Bomfim et al. 2012).

GLP-1R is a class B G-protein-coupled receptor (GPCR), widely expressed in the brain (Goke et al. 1995; Perry and Greig 2003; Hamilton and Holscher 2009), predominantly in neurons (Goke et al. 1995; Merchenthaler et al. 1999; Hamilton and Holscher 2009), but also in activated microglia and astrocytes (Iwai et al. 2006; Lee et al. 2011). The major

effector for GLP-1R signalling is via activation of the cAMP–PKA–CREB (cAMP-response element binding protein) pathway, an essential mediator of GLP-1 in β-cells (Perry and Greig 2003; Baggio and Drucker 2007; Holst 2007; Holscher and Li 2010) and in primary neurons (Perry et al. 2002). Defects in CREB expression have been reported in AD brain, (Pugazhenthi et al. 2011) and the PKA–CREB pathway has been shown to be essential for memory and can be modulated by Aβ (Vitolo et al. 2002; Caccamo et al. 2010b). Liraglutide-induced activation of PKA–CREB signalling could directly impact on IR signalling to ameliorate IR signalling defects via increasing IRS proteins (Van de Velde et al. 2011). A further convergence of GLP-1R and IR signalling may link to normal transactivation of the PI3-K/Akt pathway, a pathway which is critical for IR signal transduction and which becomes over-activated in AD (An et al. 2003; Griffin et al. 2005; Pei et al. 2008), possibly due to its constitutive activation by Aβ (Bhaskar et al. 2009; Caccamo et al. 2010a, 2011).

The findings in this study are particularly interesting given the complex relationship between T2DM and AD. T2DM, insulin resistance, hyperinsulinaemia and obesity are all apparent risk factors for AD (for reviews see: Luchsinger and Gustafson 2009; Carlsson 2010; Riederer et al. 2011). A lower GLP-1 response to food intake, often described as the 'loss of the incretin effect' is a characteristic pathophysiological finding in T2DM (Drucker and Nauck 2006). Evidence that this may predispose to AD was provided by a large longitudinal study in which a reduced early-phase insulin response in midlife was associated with an increased risk of AD in later life (Ronnemaa et al. 2009). The brain insulin resistance that occurs in AD (for reviews see: de la Monte and Wands 2005; Steen et al. 2005; Craft 2007) is believed to be an early and progressive event that could explain in part the synaptic failure, cognitive decline and impaired Aβ and tau protein homeostasis that characterise the disease (Ferreira and Klein 2011; Holscher 2011; O'Neill et al. 2012). The results of the present study reveal a beneficial impact of liraglutide on IR pathogenesis, indicators of insulin resistance, Aβ burden and inflammatory gliosis in the brain, highlighting the importance of investigating whether the therapeutic use of liraglutide and other related long-lasting GLP-1R agonists may be beneficial for patients with AD.

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