Prophylactic liraglutide treatment prevents amyloid plaque deposition, chronic inflammation and memory impairment in APP/PS1 mice

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Abstract

Type 2 diabetes is a risk factor for Alzheimer’s disease (AD). Previously, we have shown that the diabetes drug liraglutide is protective in middle aged and in old APP/PS1 mice. Here, we show that liraglutide has prophylactic properties. When injecting liraglutide once-daily ip. in two months old mice for 8 months, the main hallmarks of AD were much reduced. Memory formation in object recognition and Morris water maze were normalised and synapse loss and the loss of synaptic plasticity was prevented. In addition, amyloid plaque load, including dense core congophilic plaques, was much reduced. Chronic inflammation (activated microglia) was also reduced in the cortex, and neurogenesis was enhanced in the dentate gyrus. The results demonstrate that liraglutide may protect from progressive neurodegeneration that develops in AD. The drug is currently in clinical trials in patients with AD.

Keywords: memory loss; synaptic loss; growth factor; insulin; incretin; LTP
1. Introduction

Type 2 diabetes has been identified as a risk factor for Alzheimer’s disease (Luchsinger, Tang, Shea, & Mayeux, 2004; Ohara et al., 2011), presumably linked to an impairment in insulin signalling in the brain. In the brains of people with Alzheimer’s disease, insulin signalling was found to be much impaired, even independently of a medical history of diabetes (Moloney et al., 2010; Talbot et al., 2012). The incretin hormone Glucagon like peptide-1 (GLP-1) facilitates insulin signalling, and long-lasting GLP-1 analogues such as Liraglutide (Victoza®) are on the market as diabetes therapies (Lovshin & Drucker, 2009). Liraglutide crosses the blood-brain barrier and increases synaptic plasticity (Hunter & Holscher, 2012; P. L. McClean, Gault, Harriott, & Holscher, 2010). Liraglutide and other GLP-1 mimetics have been found to be neuroprotective in a range of neurodegenerative disorders (Holscher, 2013; Perry & Greig, 2005).

We reported previously that liraglutide treatment improved cognitive function, reduced amyloid plaque deposition, inflammation, overall amyloid precursor protein (APP) and amyloid oligomer levels and enhanced long-term potentiation of synaptic transmission (LTP) in the hippocampus in 9 months old APPswe/PS1 ΔE9 (APP/PS1) mice that had been treated for 8 weeks (P. McClean, Parthsarathy, Faivre, & Hölscher, 2011). At this stage, animals had already developed amyloid plaques and the first synaptic and memory impairments, comparable to patients in the early stage of Alzheimer’s disease. Liraglutide also reversed disease progression in 16 months old APP/PS1 mice that had been treated for two months. Liraglutide significantly improved recognition and spatial memory, reduced beta amyloid plaque load, total APP and aggregated beta amyloid levels and chronic inflammation in the brain (P. L. McClean & Holscher, 2014). At 14 months, the amyloid plaque load of APP/PS1 mice had increased to maximum levels, and damage in the brain had already been established.

Insulin signalling is impaired in the brain of this APP/PS1 mouse model, similar to the insulin signalling impairment observed in the human brain (Bomfim et al., 2012). Importantly, liraglutide has been shown to prevent this insulin de-sensitisation in the brain, as well as an ability to reverse some of the insulin signalling impairments in human AD brain tissue in an ex vivo assay (Talbot et al., 2011).

The current study was designed to elucidate if liraglutide has a prophylactic effect when administered before plaque deposition and associated memory impairments are
present in the APP/PS1 model. Animals were treated from 8 weeks of age onwards for 8 months once-daily at the previously established effective dose (P. McClean et al., 2011).

2. Material and Methods

2.1 Animals

APP\textsubscript{swe}/PS1\textsubscript{\Delta E9} mice with a C57Bl/6 background were bred at the animal unit of the University of Ulster. Heterozygous males were bred with wild-type C57/Bl6 females bought locally (Harlan, UK). Offspring were ear punched and genotyped using PCR with primers specific for the APP-sequence (Forward “GAATTCCGACATGACTCAGG”, Reverse: “GTTCTGCTGCATCTTGGACA”). For details see (Gengler, Hamilton, & Holscher, 2010). Mice not expressing the transgene were used as wild-type controls. Male animals were used in all studies. Animals were caged individually and maintained on a 12/12 light-dark cycle (lights on at 08h00, off at 20h00), in temperature-controlled room (T: 21.5°C±1). Food and water were available ad libitum. Animals were handled daily for two weeks prior to commencement of the study.

APP/PS1 and wild-type animals were 8 weeks of age when treatment began. Mice were randomized and had blood glucose measured before initiation of their designated treatment, liraglutide (25 nm/kg bw) or saline (0.9% w/v). Liraglutide and saline were made up to a final volume of 10ml/kg, with 0.9% saline used as vehicle for liraglutide and were injected intraperitoneally (i.p.) once daily (at 15:00h). Treatment groups comprised n =12. All experiments were licensed by the UK home office in accordance with the Animal (scientific procedures) Act of 1986.

2.2 Peptides

Liraglutide was purchased from GL Biochem Ltd. (Shanghai). The purity of the peptide was analysed by reversed-phase HPLC and characterised using matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) mass spectrometry, with a purity > 99%.

Peptides were reconstituted in ultrapure® water to a concentration of 1 mg/ml in polypropylene tubes and frozen in aliquots to permit fresh preparation of doses required for injection.

2.3 Blood glucose measurement
Blood glucose was measured from tail vein blood blood by an automated glucose oxidase procedure using the Ascencia® Contour® Blood Glucose Meter and corresponding analysis strips (Bayer Healthcare, Berkshire, UK).

2.4 Object recognition task

The object recognition task was conducted in a grey-coloured aluminium open-field arena (58cm in diameter; 31cm high wall) as previously described (Abbas, Faivre, & Hölscher, 2009). Objects for exploration were red cubes (1.8cm diameter) and white balls (2.6cm diameter). The arena and objects were cleaned with 70% ethanol between trials to prevent the build-up of olfactory cues.

Mice received a session of 5min in the empty open-field, 24h prior to exposure to objects, in order to habituate them to the apparatus and test room. Motor activity was recorded by total path, number of lines crossed, and speed. The number of rearing events (forepaws elevated from the floor) was considered to be an index of exploratory behaviour. The number of grooming sessions and the number of fecal pellets were recorded as measures of anxiety in mice.

Twenty-four hours after habituation, each mouse was subject to a 10min acquisition trial, during which they were placed in the open-field in presence of two identical objects (cube or ball) situated at 15cm from the arena wall. After a 3h retention interval, the mice were placed back into the arena and exposed to the familiar object and to a novel object for a further 10min. Object location and novel and familiar objects were randomized throughout the trial.

Total time spent exploring each of the two objects (when the animal’s snout was directly toward the object at a distance ≤2cm), was recorded. Recognition index was defined as the amount of time exploring the novel object over the total time spent exploring both objects multiplied by 100, and was used to measure recognition memory (TB/(TA + TB))*100 where A represents familiar object and B, novel object. Object recognition data from all groups was analysed by student t-test for each group, to compare time spent exploring the familiar object to time spent exploring the novel object. Animals were injected for 8 months prior to, and received their injection as normal at 3pm on the day prior to open field and object recognition tasks, which were commenced at 9am to avoid acute drug effects.

2.5 Morris Water maze task
The maze was made of white opaque plastic with a diameter of 120cm and 40 cm high walls, and was filled with water at 25°C to avoid hypothermia. A small escape platform (10 x 6.5 x 21.5 cm) was placed at a fixed position in the centre of one quadrant, 25cm from the perimeter, and was hidden 1cm beneath the water surface. The room contained a number of fixed visual cues on the walls.

**Acquisition phase**: The acquisition trial phase consisted of 4 training days (Day1-4) and four trials per day with a 15-min inter-trial interval. Four points equally spaced along the circumference of the pool (North, South, East, West) served as the starting position, which was randomised across the four trials each day. If an animal did not reach the platform within 90s, it was guided to the platform where it had to remain for 30s, before being returned to its home cage. Mice were kept dry, between trials, in a plastic holding cage filled with paper towels. The path length and escape latencies were recorded (n=12 per group).

**Probe Trial** - One day after finishing the acquisition task (Day 5), a probe trial was performed in order to assess the spatial memory (after a 24h delay). The platform was removed from the maze and animals were allowed to swim freely for 60s. Time spent in the target quadrant was assessed, as was spatial acuity, which measured the amount of time spent in the exact area where the escape platform had been located.

Reversal Morris Water maze task
One day after completion of the Morris water maze task, a reversal training programme commenced. This involved changing the location of the escape platform and a 3-day acquisition phase, followed by a probe trial on day 4 (conducted as outlined above; n=12 per group).

2.6 Surgery and LTP recording in the hippocampus area CA1
Mice were anesthetized with urethane (ethyl carbamate, 1.8g/kg ip.) for the duration of all experiments. The skull was exposed and 3 holes with 0.8mm diameter were drilled. Electrodes (tungsten with Teflon coating, Bilaney, Kent, United Kingdom) were implanted, 1.5mm posterior and 1.0mm lateral to the midline for the recording electrode, and 2.0mm posterior to bregma and 1.5mm lateral to the midline for the stimulating electrode. The electrodes were slowly lowered through the cortex and the upper layers of the hippocampus and into the CA1 region until the appearance of a negative deflecting excitatory postsynaptic potential (EPSP) that had a latency of ca
10msec. Recordings of field excitatory postsynaptic potentials (fEPSPs) were made from the stratum radiatum in the CA1 region of the right hippocampal hemisphere in response to stimulation of the Schaffer collateral/commissural pathway. fEPSPs were recorded on a computerized stimulating and recording unit (PowerLab, ADI Instruments) in which the trigger threshold was adjustable. The triggered unit activated a constant current stimulus isolation unit (Neurolog, United Kingdom). The data acquisition system was triggered simultaneously to record all events. Sampling speed was at 20kHz for recordings of fEPSPs. In order to assess the excitability and determine an appropriate voltage for high frequency stimulation and recording, the response of each of the mouse to increasing voltage was measured in an input/output curve at intervals of 0.25 V from 2.0 V to a maximum of 5 V (or until a maximal response was elicited). Stimulation voltage was then adjusted to generate approximately 60% of the maximum fEPSP, and the recording of the baseline was measured at this value. The high frequency stimulation protocol for inducing LTP consisted of 4 trains of 100 stimuli with an interstimulus interval of 5msec (200Hz) and an inter-train interval of 2 sec. This LTP induction protocol was chosen to prevent saturation of LTP and thus allow the possibility to detect facilitation of LTP in the APP/PS1 mice. LTP was measured as a percentage of baseline fEPSP slope recorded over a 15-minute period before application of high frequency stimulation. This value was taken as 100% of the excitatory postsynaptic potential slope and all recorded values were normalized to this baseline value. The post high frequency stimulation LTP data were then analysed by a two-way repeated measures ANOVA with treatment group as the between subject factor and time as the within subject factor.

Paired-pulse facilitation (PPF) was measured to analyse pre-synaptic functions and interneuron activity. Two stimuli were given at 60% of max fEPSP response. The interval between two stimuli was changed from 25ms to 50, 80, 120, 160 and 200ms to analyse PPF in relation to time. PPF induced at short interstimulus intervals is considered to be triggered by pre-synaptic transmitter release facilitating processes (Chen, Chad, & Wheal, 1996) while later PPF is considered to be linked to GABAa (80-120msec) and GABAb (160-200msec) inter-neuronal synaptic transmission (Schulz, Cook, & Johnston, 1995; Tsai, Shen, & Leung, 2008). The size of the fEPSP response was measured by analysing the change from baseline to the lowest point of the fEPSP. Data were normalised by taking the first fEPSP value as 100% and comparing the second fEPSP with it. The normalised PPF values were then analysed by two-way repeated measures
ANOVA with treatment group as the between subject factor and time as the within subject factor.

2.7 Histology

After the LTP studies, animals were perfused trans-cardially with PBS buffer followed by ice-cold 4% paraformaldehyde in PBS. Brains were removed and fixed in 4% paraformaldehyde for at least 24h before being transferred to 30% sucrose solution overnight. Brains were then snap frozen using Envirofreeze™ and coronal sections of 40-micron thickness were cut at a depth of -2 to -3 Bregma using a Leica cryostat. Sections were chosen according to stereological rules (Bondolfi et al., 2002) with the first section taken at random and every 6th section afterwards.

Staining was carried out for Iba-1, a marker for activated microglia to measure the inflammation response (Paresce, Chung, & Maxfield, 1997), beta-amyloid plaques, and congophilic dense-core amyloid plaques. Staining was also carried out for synaptophysin, in order to determine synapse numbers, and Doublecortin (DCX), a marker for immature neurons, as a measure of neurogenesis. All sections were incubated in 3% H₂O₂ to quench endogenous peroxidase activity. For Iba1 staining, sections were incubated in 0.05M trisodium citrate (pH 9) at 90°C for 30 minutes to enhance antigen recognition. After blocking the sections in 5% normal serum to avoid non-specific antibody binding, they were incubated with rabbit polyclonal anti ionized calcium binding adaptor molecule 1 (Iba1) (1:2000, Wako, Germany, 016-20001) or rabbit polyclonal anti amyloid beta peptide (1:250, Invitrogen, UK, 71-5800) as shown in (Radde et al., 2006) or goat polyclonal anti doublecortin (1:200, Santacruz, USA, sc-710), or rabbit anti-synaptophysin (1:200, Abcam, Cambridge, MA, 7837-500). After overnight incubation at 4°C, the sections were incubated in respective secondary antibodies. For visualisation Vectastain Elite and SG substrate (Vector laboratories, Burlingame, CA, USA) were used. Congo red staining for congophilic plaques was carried out as described by (Wilcock, Gordon, & Morgan, 2006). All staining was visualized by Axio Scope 1 (Zeiss, Germany) and analysis of percentage stained area was conducted for plaque load, dense core plaques and inflammation. The percentage stained area of each image (2 images per section, approximately 8-10 sections per mouse, 16-20 images total) was quantified using a multi threshold plug in with Image J (NIH, USA). N=12 per group.
2.8 Statistics
Data were analysed using the program Prism (Graphpad software Inc., USA), with the level of probability set at 95%. Results are expressed as means ± standard error of the mean (SEM). Data were analyzed by 1-way or 2-way ANOVA, followed by post hoc tests, or student t-tests.

3. Results

3.1 Chronic 8 month injection has no impact on bodyweight, plasma glucose and general behaviour in wild-type and APP/PS1 mice in the open field
Bodyweight of mice was comparable among all groups throughout the duration of the study, with no significant differences observed between groups. APP/PS1 saline 21.4±0.8g (start) 30.6±0.8g (end), APP/PS1 liraglutide 22.0±0.6g (start) 28.6±0.7g (end), Wild-type saline 22.2±0.3g (start), 30.4±1.0g (end). In line with this, a 2-way repeated measures ANOVA demonstrated that fasted plasma glucose concentrations were not significantly altered by long-term administration of liraglutide, with no significant treatment effect observed. APP/PS1 saline 6.3±0.2mmol/L (start) 5.2±0.1 mmol/L (end), APP liraglutide 6.0±0.2 mmol/L (start) 5.1±0.3 mmol/L (end), Wild-type saline 5.7±0.2 mmol/L (start), 5.0±0.3 mmol/L (end).

APP/PS1 mice were injected once-daily with saline or 25nmol/kg liraglutide i.p., while wild-type mice were injected with saline for 8 months prior to exposure to the open field arena. In a one-way ANOVA, despite such long-term injection no significant difference was observed in path length (A; F=0.99; p>0.05), speed (B; F=0.75; p>0.05), number of line crosses (C; F=0.87; p>0.05), exploratory behaviour (D; F=0.38; p>0.05), number of grooming episodes (E; F=2.58; p>0.05) or amount of time spent in the centre vs. periphery of the arena (F; F=2.62; p>0.05) between groups. This indicates that anxiety levels and locomotor activity are not affected by long-term administration of liraglutide in APP/PS1 mice (Figure. 1).

3.2 Liraglutide treatment maintains recognition memory in APP/PS1 mice
In a two way ANOVA of object recognition data there was a significant preference for novel object location in APP/PS1 liraglutide (t=4.28, p<0.001) and wild-type saline-treated mice (t=2.78; p<0.05), but not APP/PS1 saline-treated mice (t=1.3; p>0.05). Illustrated in Figure 2 (upper) are the recognition indices for novel and familiar objects.
and results of a student t-tests per treatment. The object recognition task was conducted at 10 months of age after 8 months saline or liraglutide. Wild-type saline-treated mice demonstrated a significant preference for novel object (A; \( t=3.3; \ p=0.004 \)). Saline-treated APP/PS1 mice failed to discriminate novel and familiar object (B; \( t=0.89; \ p>0.05 \)), while APP/PS1 mice treated with 25nmol/kg liraglutide demonstrated a strong preference for novel object (C; \( t=3.6; \ p=0.0016 \)), indicating recognition memory is preserved by the drug. Looking at the difference scores between groups, a one-way ANOVA, demonstrates there is a significant difference overall (Figure 2D; \( F=5.0; \ p=0.014 \)). Bonferroni post hoc analysis shows that there was a significant difference between the APP/PS1 liraglutide and APP/PS1 saline-treated groups (Figure 2D;\( t=3.1; \ p<0.05 \)).

3.3 Liraglutide treatment improves Morris water maze performance in APP/PS1 mice

In a Morris water maze task APP/PS1-saline treated mice performed significantly worse than wild-type saline and APP/PS1 liraglutide-treated mice overall (Figure 2 E,F,G). Escape latency significantly decreased over time (2E; \( F=46.91; \ P<0.0001 \)). Acquisition, as analysed by 2-way repeated measures ANOVA, was significantly different between treatment groups (\( F=3.28; \ p<0.05 \)), however in bonferroni post hoc tests, escape latency of APP/PS1 saline treated mice was significantly greater than that of wild-type saline-treated mice on day 1 (Figure 2E; \( t=3.5; \ p<0.01 \)). In a probe trial (Figure 2F, the wild-type saline (\( t=2.7; \ p<0.05 \)), and APP/PS1 liraglutide-treated mice (\( t=4.78; \ p<0.001 \) spent significantly more time than expected by chance (25%) in the target quadrant, while APP/PS1 saline-treated mice did not. Moreover, APP/PS1 saline-treated mice spent significantly less time in the target quadrant than the APP/PS1 liraglutide-treated group (Figure 2F; \( t=3.7; \ p<0.05 \)). Spatial acuity was analysed by assessing the amount of time spent exactly where the escape platform was located during training. The performance of liraglutide-treated APP/PS1 mice was comparable with their wild-type counterparts, with both groups performing significantly better that the APP/PS1 saline-treated group (Figure 2G; \( f=2.8 \) and 2.6 respectively; \( p<0.05 \)).

In the acquisition phase of the reversal water maze task (Figure 2H), liraglutide-treated APP/PS1 mice performed similarly to wild-type mice, and significantly better than the APP/PS1 saline group. Escape latency decreased over time (\( F=53.5; \ p<0.0001 \)), and a significant group effect was observed (\( F=3.8; \ p=0.025 \)). In the reversal probe trial the APP/PS1 saline-treated group spent significantly less time in the target quadrant than the
APP/PS1 liraglutide-treated group (Figure 2I; t=3.2; p<0.05). There were no significant differences in spatial acuity between groups in the reversal probe trial (Figure 2F).

3.4 Liraglutide enhances LTP in APP/PS1 mice
When testing the inducibility of LTP in the hippocampus in vivo, using a weak stimulation protocol, saline-treated wild-type mice induced weak LTP, saline-treated APP/PS1 animals were unable to induce LTP, while the APP/PS1 liraglutide-treated group showed enhanced LTP (Figure 3A; overall 2-way repeated measures ANOVA of post HFS baseline demonstrated a significant group effect p=0.012, F=5.72). There was no significant difference between wild-type saline and APP/PS1 liraglutide-treated groups (p=0.09, F=3.38), or wild-type saline and APP/PS1 saline treated groups (p=0.11, F=3.07). However comparing post-HFS baseline of APP/PS1 saline and APP/PS1 liraglutide groups, there was a significant drug effect (p=0.013, F=8.395).

Analysing the final 15 minutes of recordings by 2-way repeated measures ANOVA also demonstrated a significant group effect p=0.026, F=4.52). There was no significant difference between wild-type saline and APP/PS1 liraglutide-treated groups (p=0.10, F=3.14), or wild-type saline and APP/PS1 saline treated groups (p=0.26, F=1.43). However comparing the final 15 minutes of post-HFS recordings of APP/PS1 saline and APP/PS1 liraglutide groups, there was a significant drug effect (p=0.02, F=7.1).

Paired-pulse facilitation was comparable between wild-type and both groups of APP/PS1 mice at 25, 50, 80, 160 and 200ms (Figure 3B).

3.5 Histological hallmarks of Alzheimer’s disease are dramatically reduced in the cortex and in area CA1 of liraglutide-treated APP/PS1 mice
In 10 months old APP/PS1 mice, after 8 months treatment with 0.9% saline or liraglutide (25 nm/kg bw), there was a 40% reduction in plaque load of in the cortex of liraglutide-treated APP/PS1 mice (Figure 4 A-C; p<0.001). Furthermore, the percentage area stained positively for dense-core Congo-red plaques was reduced by 59% with liraglutide treatment (Figure 4 D-F; p<0.001). The inflammatory response in the cortex, as measured by number of activated microglia (IBA-1 stain), was also reduced by 50% in the liraglutide-treated group, in line with the reductions observed in plaque load (The P value is < 0.0001, F = 82.41, DFn=2, DFd=187, For the interaction: P value is 0.9797, F = 0.4314, DFn=18, DFd=187, Figure 4 G-I). In comparison, wt brains showed very low
levels of inflammation in comparison with APP/PS1 mice that were injected with saline or liraglutide (Figure 4 I; p<0.0001). Additionally the number of young neurons (doublecortin-positive cells) in the dentate gyrus was increased by 61% by liraglutide, normalising cell proliferation compared to wt brain tissue (The P value is < 0.0001, F = 23.55, DFn=2, DFd=98, For the interaction: P value is 0.1592, F = 1.478, DFn=10, DFd=98, Figure 4 J-L).

In the area CA1, the plaque load was reduced by 45% in liraglutide-treated APP/PS1 mice (p<0.001; Figure 5A-C). The Congo red-positive dense core plaque load was reduced by 64% (p<0.001; Figure 5D-F). The inflammatory response, as shown by activated microglia staining (Iba-1), was decreased by 47% with liraglutide treatment (The P value is < 0.0001, F = 227.94, DFn=2, DFd=186; For the interaction: P value is 0.0008, F = 3.208, DFn=10, DFd=186; Figure 5G-I). In comparison, wt brains showed very low levels of inflammation in comparison with APP/PS1 mice that were injected with saline or liraglutide (Figure 5 I; p<0.0001). Data analysed by regular two-way ANOVA (not repeated measures) with Bonferroni post-hoc test, n=6.

3.6 Synaptophysin levels are increased in Liraglutide-treated APP/PS1 mice

Representative micrographs of synaptophysin staining in APP/PS1 saline (A, D), APP/PS1 liraglutide (B, E), and wild-type saline (C, F) mice are illustrated in Figure 6. Quantification of levels of expression of synaptophysin demonstrates that wild-type saline–treated mice had significantly higher expression levels than APP/PS1 saline-treated mice in all brain regions studied (Figure 6 G-N; p<0.05-p<0.0001). APP/PS1 liraglutide-treated mice, meanwhile, had comparable density of synaptophysin staining as wild-type mice in the polymorph layer (Figure G), granule cell layer (Figure H), molecular layer (Figure I) and stratum pyramidale (Figure K), but significantly reduced levels in stratum radiatum (P<0.01, Figure J), stratum oriens (P<0.0001, Figure L), interior (P<0.0001, Figure M), and exterior cortex (P<0.0001, Figure N), when compared to the wild-type mice. Statistics for the treatment effect (column factor in the ANOVA analysis) for each layer: polymorph layer (The P value is < 0.0001, F = 45.60, DFn=2, DFd=88, Figure 5 G), granule cell layer (The P value is 0.0011, F = 7.388, DFn=2, DFd=89, Figure 5 H), molecular layer (The P value is < 0.0001, F = 16.60, DFn=2, DFd=89, Figure 5 I), stratum radiatum (The P value is < 0.0001, F = 43.96, DFn=2, DFd=90, Figure 5 J), stratum pyramidale (The P value is 0.0006, F = 8.046, DFn=2, DFd=89, Figure 5 K).
Statistics for the interaction: polymorph layer (The P value is 0.6155,  F = 0.8144, DFn=10, DFd=88, Figure 5 G), granule cell layer (The P value is 0.0179,  F = 2.317, DFn=10, DFd=89, Figure 5 H), molecular layer (The P value is 0.6527,  F = 0.7746, DFn=10, DFd=89, Figure 5 I), stratum radiatum (The P value is 0.4965,  F = 0.9452, DFn=10, DFd=90, Figure 5 J), stratum pyramidale (The P value is 0.9458,  F = 0.3948, DFn=10, DFd=90, Figure 5 K), stratum oriens (The P value is 0.3376,  F = 1.146, DFn=10, DFd=90, Figure 5 L) interior cortex (The P value is 0.1614,  F = 1.105, DFn=10, DFd=90, Figure 5 M).  exterior cortex (The P value is 0.1614,  F = 1.476, DFn=10, DFd=90, Figure 5 N). Overall, APP/PS1 liraglutide-treated animals had significantly higher expression levels than saline-treated APP/PS1 mice in all layers of the hippocampus (Figure 6G-L), as well as the interior (M) and exterior (N) cortex, indicating that liraglutide has a protective effect on synapse numbers. Data analysed by regular two-way ANOVA (not repeated measures) with Bonferroni post-hoc test, n=6.

4. Discussion

The results show that liraglutide treatment, initiated before amyloid plaque deposition and memory impairments are apparent significantly reduces histological and behavioural biomarkers of AD in the APP/PS1 mouse model. Notably similar improvements were observed when treatment was initiated after memory impairments and significant amyloid plaque load are apparent (P. McClean et al., 2011), indicating prophylactic administration does not appear to offer additional benefits in this transgenic model of AD. When testing the general behaviour in the open field, no overt changes in motor activity, exploration or anxiety could be detected. Liraglutide has been on the market as a treatment for diabetes in Europe since 2009 (Davies, Kela, & Khunti, 2011). It is a safe and well-tolerated drug with few side effects. An initial report that GLP-1 mimetics may enhance the likelihood of developing pancreatitis or even pancreatic cancer has not been confirmed in a subsequent large scale analysis (EMA, 2013; Roy et al., 2014; Sadry & Drucker, 2013).
Therefore, this drug is considered safe and can be taken safely over a long period of time. Recently, liraglutide has been licenced as a weight-loss drug under the brand name Saxenda® and is safe to be taken by people who are not diabetic (Wadden et al., 2013), which is an important finding with respect to its potential use in normoglycaemic Alzheimer’s disease patients.

In our study, chronic drug treatment that started before the onset of amyloid plaque formation and the associated neurodegenerative processes showed good effects. In the object recognition and Morris water maze memory tests, drug treated APP/PS1 mice performed as well as littermate wild type mice, while saline treated APP/PS1 mice demonstrated impairments in both recognition and spatial memory. This clear result was also reflected in the enhancement of long-term potentiation of synaptic activity in the hippocampus (LTP). The saline treated APP/PS1 mice failed to develop stable LTP, while drug treated APP/PS1 mice were able to express strong LTP that was superior to that of littermate controls. When quantifying synapse numbers in the cortex and hippocampus, we demonstrated that there was a substantial loss of synapses in the saline treated APP/PS1 mice. This loss was prevented to some degree by liraglutide treatment. Synapse loss is commonly observed in human AD brains (Robinson et al., 2014). This loss of connections between neurons is most likely the reason for the loss of cognitive function in AD patients. The preservation of memory formation and the demonstration of healthy LTP in the hippocampus in the APP/PS1 mice show promise that liraglutide may protect the brain from this synaptic deterioration. The results reported here are comparable to our study of the effects of liraglutide in middle-aged APP/PS1 mice that already showed first signs of AD pathology and amyloid build-up before initiation of treatment (P. McClean et al., 2011) and aged mice that have established AD pathology and a high amyloid plaque load (P. L. McClean & Holscher, 2014).

In the current study, long-term liraglutide treatment greatly reduced the formation of amyloid plaques. The Congo red positive crystalised amyloid core plaque load was more significantly reduced than overall plaque load. As these dense-core amyloid plaques are considered to induce the inflammation response (Saura, 2010), this result is of great interest. The formation of amyloid plaques was not completely prevented with chronic liraglutide treatment, however, one has to keep in mind that this transgenic mouse model of AD expresses human mutated APP in an unregulated way (Jankowsky et al., 2001). The expression of APP in such a model cannot be regulated as is in wild-type mice. APP expression is modulated by several factors, so that it may be possible that in humans, APP
gene expression in the brain may be reduced by this drug (Kovacs et al., 1995). The current observation that there are still moderately high levels of amyloid plaques found in the brain should be interpreted with caution. GLP-1 signalling modulates BACE1 and alpha-secretase expression and activity. Previous research has shown that amyloid formation is reduced by a shift from beta- to alpha-secretase processing of APP (Ohtake, Saito, Eto, & Seki, 2014; Perry et al., 2003). This process may explain the reduced amyloid levels found here.

In line with reduced plaque load, the chronic inflammation response, as measured in levels of activated microglia, was concurrently and significantly reduced. Chronic inflammation has detrimental effects on the brain and is an important part of chronic neurodegenerative disorders (Holmes et al., 2009; Najem, Bamji-Mirza, Chang, Liu, & Zhang, 2014). Neurogenesis was also found to be normalised in the dentate gyrus of APP/PS1 mice. The impairment of neurogenesis is an indication that growth factor signalling is reduced in the brains of these animals, and pro-inflammatory cytokines have been shown to inhibit this process (Lee, Han, Nam, Oh, & Hong, 2010). Chronic treatment with liraglutide reversed this impairment, demonstrating that growth signalling has been normalised. We have previously shown that liraglutide reduces the chronic inflammation response and the associated release of pro-inflammatory cytokines (Parthsarathy & Holscher, 2013). GLP-1 is a growth factor, and activation of GLP-1 receptors activates second messenger cell signalling pathways that enhance gene expression, reduce apoptosis, normalise energy utilisation and cell proliferation (Li, Tweedie, Mattson, Holloway, & Greig, 2010; Sharma, Jalewa, & Holscher, 2013). Growth factor signalling has been shown to be impaired in the brains of AD patients (Moloney et al., 2010; Talbot et al., 2012) and in APP/PS1 mice (Long-Smith et al., 2013), and the treatment with GLP-1 receptor agonists normalised this (Bomfim et al., 2012; Isacson et al., 2011; Long-Smith et al., 2013). The normalisation of growth factor signalling will normalise the repair and growth processes in neurons, including the normalisation of energy metabolism and synaptogenesis (Holscher, 2014).

In conclusion, the study presented here supports the concept that liraglutide or similar GLP-1 mimetics have prophylactic effects and reduce Alzheimer’s disease progression in the APP/PS1 mouse model. Our previous study in middle-aged mice demonstrated that liraglutide has protective effects at a stage where the disease has already advanced to a stage where the first behavioural symptoms appear (P. McClean et al., 2011). The drug also showed some improvement in aged APP/PS1 mice which represent the late stage of
AD (P. L. McClean & Holscher, 2014), raising hopes that liraglutide may show some positive effects even in the later stages of Alzheimer’s disease. We also showed in a separate study that the extensive vascular pathology induced by the high expression of amyloid in the APP/PS1 mouse model could be ameliorated by liraglutide (Kelly et al., 2015). As vascular pathology plays an important role in AD, this drug effect may be an important factor in the overall protective drug effect. Other studies also report good neuroprotective effects of liraglutide in a different mouse model of AD. In the senescence-accelerated mouse prone 8 (SAMP8) mouse model, liraglutide significantly enhanced memory formation and also protected from neuronal loss in area CA1 (Hansen et al., 2015).

Our data add to the growing evidence base demonstrating GLP-1 mimetics are neuroprotective (Holscher, 2013). Several GLP-1 mimetics such as Exendin-4, liraglutide and lixisenatide are licenced as treatments for type 2 diabetes (Campbell & Drucker, 2013), and this facilitates the testing of these drugs in clinical trials of neurodegenerative diseases. A clinical trial of exendin-4 in Parkinson patients had been conducted as a proof of concept in a randomised open label trial. Drug treated patients showed improvement at 12 months in motor tasks, compared with decline in control patients. Most interestingly, exendin-4 showed a clear improvement in the Mattis DRS-2 cognitive score, suggesting that exendin-4 has beneficial effects on cognition (Aviles-Olmos et al., 2013). This effect was still measurable 12 months after the clinical trial had finished (Aviles-Olmos et al., 2014). A larger study using a double blind randomised design is currently ongoing.

In addition, based on the encouraging preclinical effects that we found with liraglutide, a pilot study testing liraglutide in AD patients had been conducted. In a double-blind placebo controlled trial lasting 6 months, the placebo group showed a decline in brain activity and energy metabolism as shown in 18FGD-PET imaging. The liraglutide treated group showed no decline at all, and even displayed a tendency of improvement over baseline. The 18FGD-PET signal correlates well with cognitive impairment, synaptic dysfunction and general disease progression (Gejl et al., 2015). This result is a proof of concept that the data obtained in animal studies translate to human patients. A phase II clinical trial testing liraglutide in MCI/AD patients has started at Imperial College London (clinical trial identifier NCT01843075). This trial will deliver more detailed information on how liraglutide affects or improves the disease progression of AD.
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References


EMA. (2013). Assessment report for GLP-1 based therapies.


Figure 1:
Open field observation of 10 month old liraglutide treated APP/PS1 mice, compared with saline-injected APP/PS1 and wild-type mice treated for 8 months, beginning at 8 weeks of age. APP/PS1 mice were injected once-daily with saline or 25nmol/kg liraglutide, while wild-type mice were injected with saline for 8 months prior to exposure to the open field arena. Long-term treatment had no impact on motor activity or anxiety measures, as no significant difference was observed in path length (A), speed (B), number of line crosses (C), exploratory behaviour (D), number of grooming episodes (E) or amount of time spent in the centre vs. periphery of the arena (F) between all groups. (n=12 per group).

Figure 2:
Top: Recognition memory is preserved in liraglutide-treated APP/PS1 mice.
The object recognition task was conducted, in 10 month old mice, after 8 months daily treatment of APP/PS1 mice with either 0.9% saline or liraglutide (25nm/kg bw). Their performance was compared with wild-type mice injected with saline for 8 months, which served as a control. In the task two identical objects were shown to mice for 10min. After a 3h interval mice were exposed to one novel and one familiar object. Shown is the recognition index (RI), which is the % time spent exploring the novel object vs. the overall exploration time. Wild-type mice demonstrated a significant preference for novel object (A). Saline-treated APP/PS1 mice failed to discriminate novel and familiar object (B), while APP/PS1 mice treated with 25nmol/kg liraglutide demonstrated a strong preference for novel object (C). Overall difference scores were significantly reduced in the APP/PS1 saline-treated group compared with the APP/PS1 liraglutide treated group (D). **p<0.01 vs. familiar object, Δp<0.05 vs. APP/PS1 liraglutide group, n=12 per group.
Bottom: Liraglutide treatment improves Morris water maze performance in APP/PS1 mice
The Morris water maze task was conducted in 10 month old APP/PS1 mice, after 8 months treatment with 0.9% saline or Liraglutide (25nm/kg bw). Their performance was compared with control wild-type mice injected with saline. Mice were exposed to a
Morris water maze learning paradigm involving 4 days acquisition, followed by a probe trial. Mice then received 3 days reversal training (with platform location moved) followed by a reversal probe trial. Escape latency of APP/PS1 saline treated mice was significantly greater than that of wild-type saline-treated mice on day 1 (E). In the probe trial (F), wild-type saline and APP/PS1 liraglutide-treated mice performed better than the APP/PS1 saline group. In fact the liraglutide APP/PS1 group spent significantly more time in the target quadrant than the saline-treated APP/PS1 group (F). Spatial acuity of wild-type saline and APP/PS1 liraglutide groups was significantly better than the APP/PS1 saline-treated group (C).

Escape latencies of liraglutide-treated APP/PS1 mice and wild-type mice were significantly faster than the APP/PS1 saline group the reversal water maze task (H). In the reversal probe trial the APP/PS1 saline-treated group spent significantly less time in the target quadrant than the APP/PS1 liraglutide-treated group (G). There were no significant differences in spatial acuity between groups in the reversal probe trial (H). *p<0.05, ***p<0.001 vs chance (25%). Δp<0.05, vs. APP/PS1 liraglutide, Οp<0.05 vs wild-type saline. N=12 per group

**Figure 3: Liraglutide enhances LTP in APP/PS1 mice**

Liraglutide, injected chronically for 8 months protected and potentiated the induction and maintenance of LTP in the CA1 of the hippocampus in APP/PS1 mice when compared with APP/PS1 saline-treated mice, who failed to induce LTP (A). Paired-pulse facilitation was comparable between wild-type and both groups of APP/PS1 at 50, 80, 160 and 200ms (B). N=9-12 per group.

**Figure 4: Histological hallmarks of Alzheimer’s disease are improved by liraglutide treatment.** After chronic 8 month treatment with 0.9% saline or liraglutide (25nm/kg bw) the number of plaques in the cortex of liraglutide-treated APP/PS1 mice was reduced by 40% (A, B, C). The number of Congo-red positive dense core plaques was reduced by 59% (D, E, F). The inflammatory response, as shown by activated microglia (IBA-1stain), was also halved (G, H, I). Mice treated with liraglutide also had a significant increase in neurogenesis (Doublecortin positive cells) compared with saline-treated animals (J, K, L). Wild type brain sections are shown for comparison (split panels H, K, graph I, L). Sample micrographs are shown. ***P<0.001 vs APP/PS1 saline-treated group, n=6 per group.
**Figure 5: Plaque load and inflammation in the CA1 are reduced with chronic liraglutide treatment.**

After chronic 8 month treatment with 0.9% saline or liraglutide (25nm/kg bw) the number of plaques in the CA1 of liraglutide-treated APP/PS1 mice was reduced by 45% (A, B, C). The number of Congo-red positive dense core plaques was reduced by 63% (D, E, F), and the inflammatory response, as shown by activated microglial load (IBA-1stain), was reduced by 47% (G, H, I). Wild type IBA-1 data are shown for comparison (split panel H, graph I). Sample micrographs are shown. ***p<0.0001 vs APP/PS1 saline-treated group n=6.

**Figure 6: Synaptophysin levels are partially restored by Liraglutide treatment in APP/PS1 mice**

Representative hippocampal images of synaptophysin-stained brains of APP/PS1 saline (A, B), APP/PS1 liraglutide (C, D) and wild-type saline (E, F) mice are shown. In each image the layers of the hippocampus are numbered as 1: Polymorph layer, 2: Granule cell layer, 3: Molecular layer, 4: Stratum radiatum, 5: Stratum pyramidale, 6: Stratum oriens

Quantification of levels of expression of synaptophysin in each of the layers, Polymorph layer (G), Granule cell layer (H), Molecular layer (I), Stratum radiatum (J), Stratum pyramidale (K), Stratum oriens (L), as well as interior cortex (M), and exterior cortex (N) demonstrates that wild-type animals had significantly higher expression levels in all regions analysed than both saline and Liraglutide-treated APP/PS1 mice. Liraglutide increased hippocampal and cortical synaptophysin levels in APP/PS1 mice. *p<0.05, **p<0.01, ***p<0.001, n=6 per group.
Fig 1
Object recognition memory task

Wild-type saline

APP/PS1 saline

APP/PS1 liraglutide

Fig. 2
Fig. 3
Fig. 4

A. APP/PS1 saline
B. APP/PS1 liraglutide
C. Amyloid plaque load

D. APP/PS1 saline
E. APP/PS1 liraglutide
F. Dense Core plaques

G. APP/PS1 saline
H. APP/PS1 WT saline liraglutide
I. Inflammation Response in Cortex

J. APP/PS1 saline
K. APP/PS1 WT saline liraglutide
L. Neurogenesis

Fig. 4
Fig 5