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Peripheral elevation of IGF-1 fails to alter Aβ clearance in multiple in vivo models

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ABSTRACT

Increasing beta-amyloid (Aβ) clearance may alter the course of Alzheimer's disease progression and attenuate amyloid plaque pathology. Insulin-like growth factor I (IGF-1) augmentation has been suggested to increase Aβ clearance by facilitating transport of Aβ out of the brain. The availability of safe agents that increase IGF-1 levels therefore makes IGF-1 elevation an attractive target for disease modifying therapy in AD. The present series of studies sought to replicate published paradigms in which peripheral IGF-1 administration lowered brain Aβ acutely, with reduction in plaque pathology after chronic treatment. Thus Aβ levels were measured in several animal models following treatments that elevated IGF-1. Administration of IGF-1 to young or old rats for up to 3 days had no effect on Aβ levels in brain, CSF, or plasma. In adult beagles, 4 days of dosing with the growth hormone secretagogue, CP-424391, doubled baseline plasma IGF-1 levels, yet failed to alter CSF or plasma Aβ. 5-day treatment of young Tg2576 mice with IGF-1 produced robust elevations of IGF-1 levels in plasma, but no effects on Aβ were detected in brain, CSF, or plasma. Finally, 11-month-old Tg2576 mice were implanted with subcutaneous minipumps delivering IGF-1 for 1 month. No significant changes in Aβ (by ELISA or Western blot), plaque pathology, or phospho-tau epitopes were detected. These results do not demonstrate acute or chronic actions of peripherally administered IGF-1 on Aβ levels or the phosphorylation state of tau and therefore do not suggest any disease-modifying benefits of IGF-1 restorative therapy for AD through these mechanisms.

1. Introduction

One of the hallmark pathological characteristics of Alzheimer's disease (AD) brains is the accumulation of plaques composed primarily of amyloid-beta (Aβ) peptide. In both sporadic and familial AD, the deposition of Aβ into plaques requires decades. Even in transgenic mouse models that overexpress mutant APP at very high levels, such as the Tg2576 mouse, brain Aβ levels remain stable for 8-9 months before any plaque deposition begins [1]. Once seeded, however, plaques grow rapidly and the brain accumulates Aβ peptide in this insoluble form. The initial trigger for this cascade of deposition is unclear, but some have suggested that a deficit in the clearance of Aβ from the brain could play a role. Even after plaques have seeded, a disruption in the efflux of soluble Aβ from the brain could promote further deposition. Thus, improving Aβ clearance has become an attractive strategy for disease-modification in AD.

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A number of groups have shown that passive immunization is capable of preventing and even reversing Aβ deposition in transgenic mice [2–5], possibly by enhancing Aβ clearance from the brain. In addition, reducing expression of proteins implicated in transporting Aβ out of the brain, such as low-density lipoprotein receptor-related protein (LRP) or P-glycoprotein, has been shown to increase levels of brain Aβ and enhance plaque deposition [6,7]. It has been suggested that a decline in circulating insulin-like growth-factor I (IGF-1), as has been observed in AD patients [8], might reduce Aβ clearance from the brain and result in accumulation of Aβ that then fosters plaque formation [9,10]. Administration of human IGF-1 to aged rats or transgenic mice was reported to reduce brain Aβ, as assessed by Western blot and immunohistochemistry, while elevating levels of Aβ and associated carrier proteins in cerebrospinal fluid (CSF) [11]. IGF-1 treatment of APPxPS2 mice was additionally shown to improve cognition [10], presumably as a result of enhancing Aβ clearance. However, chronic elevation of plasma IGF-1 levels was not shown to have cognitive benefit in Alzheimer's disease patients in a 1-year clinical trial of a growth hormone secretagogue [12].

The present series of experiments sought to further examine the potential for IGF-1 to improve Aβ clearance in multiple in vivo models. Using the acute, subchronic, and chronic dosing paradigms of Carro et al. [11], the impact of IGF-1 upon Aβ levels in brain, CSF, and plasma was evaluated in rats and Tg2576 mice. In addition, the growth hormone secretagogue, CP-424391, was used to raise endogenous plasma IGF-1 in beagles, and CSF and plasma Aβ levels were monitored. IGF-1 levels were measured in all studies to ensure adequate exposure. Despite robust elevations in circulating IGF-1 levels, however, no changes in Aβ were detected by quantitative ELISA assays in any of these models. Tau phosphorylation was also assessed in Tg2576 mice after chronic IGF-1 administration, but no significant effects were observed.

2. Materials and methods

2.1. *In vitro IGF-1 activity*

Recombinant human IGF-1 (hIGF-1) and Long®R^{3}IGF-1 (LongIGF) from Gropep Ltd. (Adelaide, Australia) as well as hIGF-1 from Peprotech Inc. (Rocky Hill, NJ) were evaluated for functional activity using cultured myoblast cells in vitro. L6 rat myoblast cells (American Type Culture Collection, Manassas, VA) were cultured for four passages before being plated at a density of 10,000 cells per well in a 96 well plate (Costar) in DMEM supplemented with, 1% L-glutamine, 0.1% gentamicin, and 10% FBS (Gemini Bio-Products, West Sacramento, CA). The plate was incubated at 37 °C, 5% CO_2 for 24 h. The cells were then treated with hIGF-1 (Gropep or Peprotech) or LongIGF in triplicate at concentrations ranging from 10 to 100,000 ng/mL in DMEM plus 2% horse serum and 0.1% gentamicin. After a 72 h incubation at 37 °C, 5% CO_2, the cells were lysed and stored at −80 °C. All cell culture products were purchased from Gibco-Invitrogen (Carlsbad, CA) unless otherwise stated.

IGF-1 will increase myosin levels in myoblasts in culture. Quantification of embryonic myosin heavy chain (eMHC) was conducted using the MesoScale Discovery (MSD) platform. The cell lysate was added to a high-binding plate and incubated at room temperature for 1 h. 150 μL of blocker A was added to each well. After 1 h, the plate was washed with phosphate-buffered saline (PBS) and incubated with 50 μL of a monoclonal antibody to eMHC, F1.652 (American Type Culture Collection) for 90 min. After another PBS wash, 50 μL of a 1:1000 dilution of ruthenium-labeled anti-mouse IgG was added and incubated for 90 min. Following a third wash with PBS, 150 μL of T Buffer was added and the plate read using a Sector Imager 6000. Equipment and reagents were all purchased from MSD (Gaithersburg, MD) unless otherwise stated. Data are presented as percentage of vehicle level.

2.2. *In vivo experiments*

All animal treatment protocols were approved by Pfizer's Institutional Animal Care and Use Committee and were compliant with Animal Welfare Act Regulations.

2-month-old Wistar and Sprague–Dawley (SD) rats were purchased from Charles River Laboratories and acclimated for at least 1 week prior to dosing. Aged SD rats originally purchased from Charles River Laboratories were kept in an aging colony at Pfizer until 22 months of age. Peprotech hIGF-1 was used in all in vivo studies. Wistar rats received dual cannulation of the jugular vein and carotid artery. After a 1-week acclimation period, vehicle (saline) or hIGF-1 was delivered in a volume of 2 mL/kg via intracarotid (i.c.) infusion over 20 s. Blood was drawn from the venous catheter 2 h post-infusion. 48 h later, rats were euthanized with CO_2, blood was collected by cardiac puncture, CSF was obtained from the cisterna magna, and brains were harvested and frozen on liquid N_2. Plasma was obtained from EDTA tubes, and all tissues were stored at −80 °C. 2-month-old SD rats were implanted with s.c. minipumps containing saline or hIGF-1 at a flow rate of 1 μL/h. 22-month-old SD rats received s.c. BID injections of hIGF-1. Both minipump and BID injection treatments were administered for 3 days, after which time rats were euthanized and tissue collected as described above.

Beagles aged 3.5–6.5 years (n = 4 males, n = 1 female) with cannulated cisterna magna and jugular veins were used for a non-terminal experiment. Dogs were fasted overnight, but had unlimited access to water. On the first day, baseline CSF (0.6 mL) and blood samples (2 mL) were collected at 8:00, 10:30, 13:00, and 15:30 h. Starting on the second day, dogs were dosed orally with the growth hormone secretagogue CP-424391 formulated in 0.5% methylcellulose, BID for 5 days. Efficacy in the beagle model has previously been demonstrated using this dosing paradigm [13]. Each dosing was followed by a 20 mL washout with water. CSF and blood samples were collected at the same intervals on the second and fifth day. AM dosing occurred after the first sample collection, and PM dosing occurred following the last sample collection of the day. Plasma was isolated from EDTA-coated tubes; both CSF and plasma were stored at −80 °C until assay.

Tg2576 mice were bred and aged at Charles River. 3-month-old mice were dosed s.c., BID with either saline, hIGF-1, or

murine IGF-1 (rIGF-1, Peprotech) for 5 days. 2 h following the final dose, mice were anaesthetized with a ketamine/xylazine mixture, and CSF was harvested as described previously [14]. Blood was collected via cardiac puncture into EDTA tubes for plasma separation. Brains were bisected and each hemisphere was frozen separately. 11-month-old mice were anaesthetized with a ketamine/xylazine mixture, then implanted with s.c. minipumps delivering saline or hIGF-1 for 14 days at a flow rate of 0.25 μL/h. After 14 days the surgery was repeated to implant a fresh pump. After 28 days of dosing, mice were euthanized and blood was collected by cardiac puncture for EDTA plasma isolation. Brains were bisected, and one hemisphere was fixed in 4% paraformaldehyde for immunohistochemistry. From the other hemisphere, hippocampus and cortex were dissected out and frozen separately for biochemical analysis.

2.3. Tissue processing

CSF and plasma samples were directly diluted into multiple assays. Brain tissue required homogenization and further processing before assay. Rat brains were homogenized in 5 M guanidine hydrochloride (GuHCl) at 100 mg/mL, and the Aβ-containing fraction was concentrated by solid-phase extraction according to methods detailed previously [15]. Young Tg2576 brains were divided, with one half homogenized in 5 M GuHCl, and one half homogenized in 0.2% diethylamine (DEA) at 100 mg/mL. DEA homogenates incubated 3 h at 4 °C, then were centrifuged at 100,000 × *g* for 1 h. Supernatant was frozen at −80 °C until assay. No concentration step was performed for DEA or GuHCl Tg2576 homogenates.

For 12-month-old Tg2576 mice, hippocampus was homogenized as described in GuHCl, while cortex was homogenized in two steps. The first step was designed to preserve tau phosphorylation and provide a measure of soluble Aβ. Tissue was homogenized at 150 mg/mL in a buffer containing: 50 mM Tris–HCl, 5 mM EGTA, 0.5% NP-40, 1 mM phenyl phosphate, 1 mM sodium orthovanadate, 50 mM β-glycerophosphate, 1 mM dithiothreitol, 1.2 μM okadaic acid, and 1× complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN; all other chemicals purchased from Sigma–Aldrich, St. Louis, MO). Samples were centrifuged at 100,000 × *g* for 1 h, and supernatant was decanted and frozen. For tau analysis, this supernatant was analyzed without further treatment. Due to low signal of soluble Aβ, for this analysis samples were concentrated by solid-phase extraction as described for rat brain samples [15]. The pellet was then homogenized in 5 M GuHCl at 150 mg/mL.

2.4. Biochemical tissue analyses

Plasma IGF-1 levels were analyzed using ELISA kits specific for human or rodent IGF-1 from R&D systems (Minneapolis, MN). Samples values were interpolated from a standard curve using 4-parameter logistical fit (Softmax Pro 4.6).

Albumin content was measured using a sensitive ELISA kit from Bethyl Laboratories (Montgomery, TX). Tissue and biofluid albumin concentrations were interpolated from a standard curve using 4-parameter logistical fit (Softmax Pro 4.6).

Aβ fluorescence ELISAs were performed on brain, CSF, and plasma as detailed previously [15]. 6E10 was used to capture dog and Tg2576 Aβ, and rodent-specific N-terminal pAb #9153 was used to capture rat Aβ (Signet Labs, Dedham, MA). C-terminal polyclonal antibodies (purchased from P. Mehta) were used to detect Aβ1–40 and 1–42, and 4G8 was used to detect total Aβ, also referred to as (Aβ1-X). Sample values were extrapolated from a standard curve using 4-parameter regression (GraphPad Prism 4.0).

Multiple tau phosphorylation sites were assessed in soluble brain fractions from aged Tg2576 mice using an electrochemiluminescence assay. A biotinylated polyclonal antibody was used to capture tau. Monoclonal antibodies were used to detect tau phosphorylated at S202/T205 (AT8), T181 (AT270), T231 (AT180), S396 (PHF-13, Abcam, Cambridge, MA); de-phosphorylated resides at 195, 198, 199, 202 were detected with Tau-1 (Biosource, Carlsbad, CA). AT8, AT270, and AT180 were purchased from Pierce (Rockford, IL). Streptavidin M-28 Dynabeads were used to pull down the antibody complex, with ruthenylated anti-mouse antibody providing an electrochemilumiscent signal detectable by an Origen M-8 analyzer (reagents and equipment from BioVeris, Gaithersburg, MD). Specific counts were normalized to total protein (Bio-Rad, Hercules, CA). Samples are reported as percentage of vehicle for individual epitopes.

2.5. Immunohistochemistry

10 μM sagittal sections of hemibrain were cut from paraffin blocks. Antigen retrieval was performed by immersion in 70% formic acid for 10 min prior to blocking the sections. 4G8 was used to detect Aβ. Biotin-conjugated Fab fragment goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) was used as a secondary antibody. Immunostaining was visualized using avidin-biotin HRP complex followed by 3,3′-diaminobenzidine substrate (both reagents from Vector Labs, Burlingame, CA).

2.6. Western blot

Brain samples or synethic Aβ1–40 peptide were run in 16% Tris–tricine gels alongside SeeBlue Plus 2 molecular weight markers (Invitrogen, Carlsbad, CA) at 120 V for 90 min. The gel was transferred to a nitrocellulose membrane in 1× Novex transfer buffer (Invitrogen, Carlsbad, CA) with 10% MeOH at 25 V for 90 min. Following transfer, the blot was boiled (microwaved 3 min) in PBS to enhance Aβ detection. Blots were blocked for a minimum of 2 h in Odyssey blocking buffer (LiCor, Lincoln, NE). Blots then incubated with primary antibody in Odyssey blocking buffer overnight at 4 °C. 4G8 was obtained from Signet Labs (Dedham, MA) and run at 250 ng/mL. 9TL, a proprietary Aβ1–40 C-terminal-specific antibody [16] was used at 125 ng/mL. After five washes in TBS with 0.2% Tween 20, blots were incubated with species-specific antibodies coupled to AlexaFluor 680 at a 1:10,000 dilution for 1 h at RT (Invitrogen, Carlsbad, CA). Blots were washed further and imaged on a LiCor Odyssey (Lincoln, NE). Bands were quantified using Odyssey software version 2.1.

2.7. Statistical analysis

Between-group one-way analysis of variance (ANOVA) was performed on each set of extrapolated values from rats and mice. For p-tau samples, as there was no standard curve, background-subtracted counts were used to determine statistically significant treatment effects. Following $p < 0.05$ in the ANOVA, individual changes versus vehicle were assessed post-hoc using Dunnett's multiple comparison test. For the dog samples, within-group ANOVA was performed for each timepoint, and significant treatment effects were described as any interval with a $p < 0.05$ versus time-matched baseline levels.

3. Results

3.1. Activity of hIGF-1 in L6 cells

To confirm biological activity of recombinant human IGF-1 (hIGF-1) to be used in vivo, three sources of IGF-1 were examined in an established in vitro model of IGF-1 activity. The IGF-1 signaling pathway has been shown to increase muscle cell proliferation and differentiation [17–19]. Thus, L6 myoblast cells were treated with hIGF-1 from Peprotech or Gropep, or with longIGF, an analog of hIGF-1 extended by 13 amino acids to improve in vitro stability (Gropep). All three forms of IGF-1 induced myotube formation. As myotube formation in these cells is accompanied by an increase in the production of embryonic myosin heavy chain (eMHC) [20], this endpoint was used as a more quantifiable measure of the changes in cellular morphology. A concentration-dependent rise in eMHC expression can be seen in Fig. 1. While all three forms of IGF-1 elevated eMHC, Gropep hIGF-1 exhibited lower potency than longIGF or the Peprotech lot of hIGF-1. Peprotech hIGF-1 was used in all in vivo experiments described below.

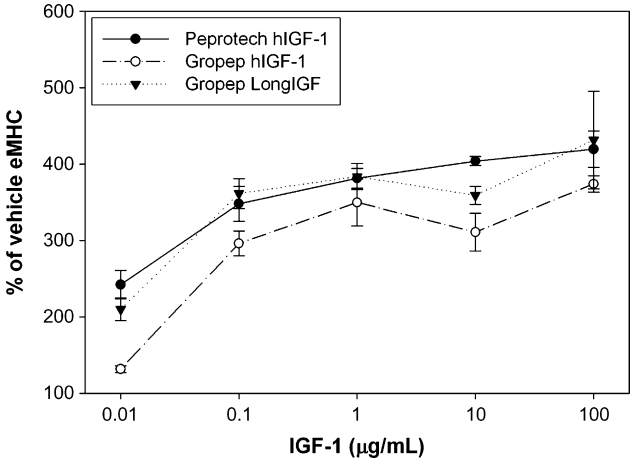

Fig. 1 – Concentration-dependent increase in embryonic myosin heavy chain (eMHC) expression by L6 cells treated with hIGF-1. Cells were treated in triplicate wells for 3 days with vehicle or Peprotech hIGF-1 (filled circles), Gropep hIGF-1 (open circles), or with Gropep LongIGF (filled triangles). eMHC in cell lysates is presented as mean ± SEM % of vehicle.

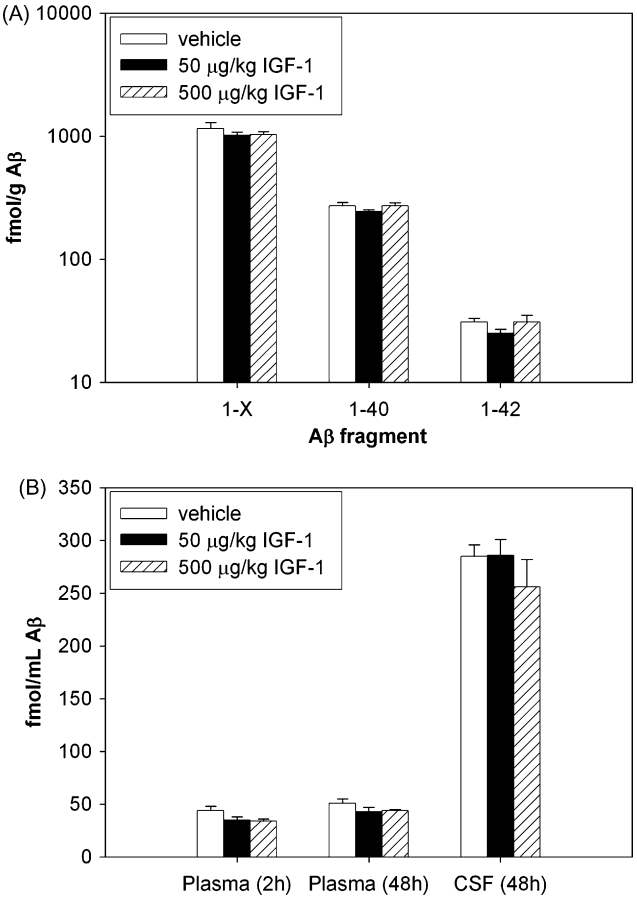

Fig. 2 – Aβ levels in 2-month-old Wistar rats ($n = 5$) following an i.c. injection of saline (open bars) or hIGF-1 at 50 μg/kg (black bars) or 500 μg/kg (striped bars). Bars and error bars show mean ± SEM. (A) Brain Aβ1-X, 1–40, and 1–42 was measured in GuHCl homogenates 48 h post-dose. (B) Aβ1-X levels were analyzed in plasma at 2 h post-dose, and in both CSF and plasma 48 h post-dose. No treatment effects were significantly different from vehicle.

3.2. Acute and subchronic dosing of rats with hIGF-1

To evaluate the plasma exposure following intracarotid administration of IGF-1, human IGF-1 (hIGF-1) was administered to young male Wistar rats via i.c. cannula at 50 or 500 μg/kg. 2 h following injection, plasma obtained from a jugular cannula showed hIGF-1 levels of 36 ± 1 ng/mL at 50 μg/kg, and 182 ± 11 ng/mL at 500 μg/kg. Endogenous rIGF-1 levels in rat plasma were 1061 ± 65 ng/mL after vehicle treatment, 987 ± 70 ng/mL after 50 μg/kg hIGF-1, and 1148 ± 112 ng/mL after 500 μg/kg hIGF-1 treatment (no significant treatment effect). CSF levels of rodent IGF-1 were below the limit of detection (30 pg/mL) for the assays used here and hIGF-1 was also below the detection limit (94 pg/mL) in CSF following i.c. administration (data not shown). 48 h following dosing, rats were euthanized. Aβ1-X, 1–40, and 1–42 levels in GuHCl homogenates of whole brain are shown in Fig. 2A. Neither dose of hIGF-1 resulted in a statistically significant change in any Aβ fragment. Aβ levels were assessed in plasma at both 2 h and 48 h, and in CSF at the 48 h termination (Fig. 2B). As in brain, no

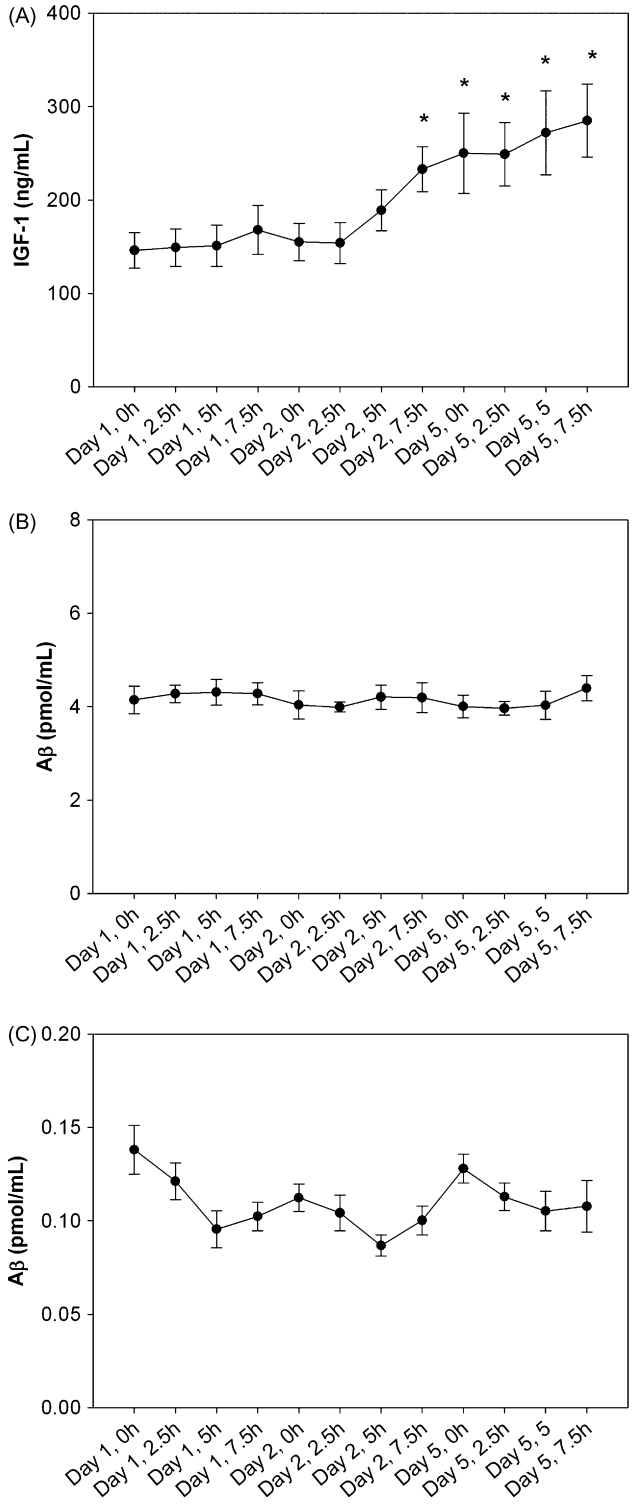

Fig. 3 – CSF and plasma samples were collected from cannulated beagles ($n = 5$) every 2.5 h between 8:00 and 16:00 h on day 1. Starting on day 2, all dogs were orally dosed BID with 5 mg/kg CP-424391 following the collection of the 0 and 7.5 h samples. CSF and plasma samples were collected every 2.5 h and day 5 at the same time as the day 1 baseline collection. (A) Mean ± SEM. IGF-1 concentration in plasma. Asterisks denote significant difference ($p < 0.05$ within-group) from the time-matched baseline sample. (B) Mean ± SEM. Aβ1-X levels in CSF. (C) Mean ± SEM. Aβ1-X in plasma. Treatment did not produce any significant difference from time-matched baseline in either CSF or plasma.

treatment effect was observed. Aβ1–40 was also analyzed in CSF and plasma (Aβ1–42 was below the limit of detection in these biofluids), but no treatment effect was observed (data not shown). To assess a potential impact of hIGF-1 treatment on albumin trafficking, albumin concentration was determined by ELISA in all compartments (data not shown). As with Aβ, no statistically significant treatment effects were observed.

To evaluate whether a continuous administration paradigm could enhance the exposure of IGF-1, young SD rats ($n = 3$) were implanted with s.c. minipumps containing saline or hIGF-1 (50 μg/kg/day). After 3 days of infusion, brain, CSF and plasma were harvested for Aβ1-X analysis. hIGF-1 levels in plasma were below the limit of detection (1 ng/mL), and no statistically significant differences in IGF-1 levels between hIGF-1-treated and vehicle-treated animals were detected (data not shown).

Since effects of IGF-1 administration on brain and CSF Aβ levels were first reported in aged rats, 22-month-old rats ($n = 5$) were dosed for 3 days with saline or hIGF-1 (100 μg/kg/day) administered by s.c. BID injection. Brain, CSF, and plasma were harvested 2 h following the final dose. Using this injection paradigm, hIGF-1 levels in plasma were 234 ± 23 ng/mL in rats treated with hIGF-1. Rodent IGF-1 levels were 1250 ± 64 ng/mL in vehicle-treated rats and 1430 ± 180 ng/mL in the hIGF-1 treatment group. Aβ1-X levels were assessed from brain and biofluids, though no treatment effects were statistically significant: 1565 ± 66 fmol/g in vehicle brain versus 1538 ± 49 fmol/g in treated brain, 227 ± 36 fmol/mL in vehicle CSF versus 231 ± 17 fmol/mL in treated CSF, and 64 ± 4 fmol/mL in vehicle plasma versus 67 ± 5 fmol/mL in treated plasma Aβ1-X.

3.3. *Subchronic treatment of beagles with the growth-hormone secretagogue, CP-424391*

Beagles, aged 3.5–6.5 years, were dosed orally with a compound known to stimulate growth hormone release, and thereby augment endogenous IGF-1 levels, CP-424391. This larger animal model was used to facilitate repeated sampling of CSF and plasma. After 1 day of baseline collection, all dogs were treated with 5 mg/kg CP-424391, BID. Plasma IGF-1 levels rose during the first day of treatment, and had approximately doubled by day 5 (Fig. 3A). CSF Aβ1-X (Fig. 3B), 1–40 and 1–42 (not shown) levels were unchanged over the course of the study. Plasma Aβ1-X (Fig. 3C), 1–40 and 1–42 (not shown) levels exhibited diurnal variation, but at no time were levels significantly different from the time-matched baseline.

3.4. *Subchronic dosing of young Tg2576 mice with IGF-1*

After failing to detect changes in Aβ clearance due to IGF-1 augmentation in models with endogenous APP expression, an APP over-expressing model, the Tg2576 mouse, was evaluated next. Mice were dosed at 3 months (well before the onset of

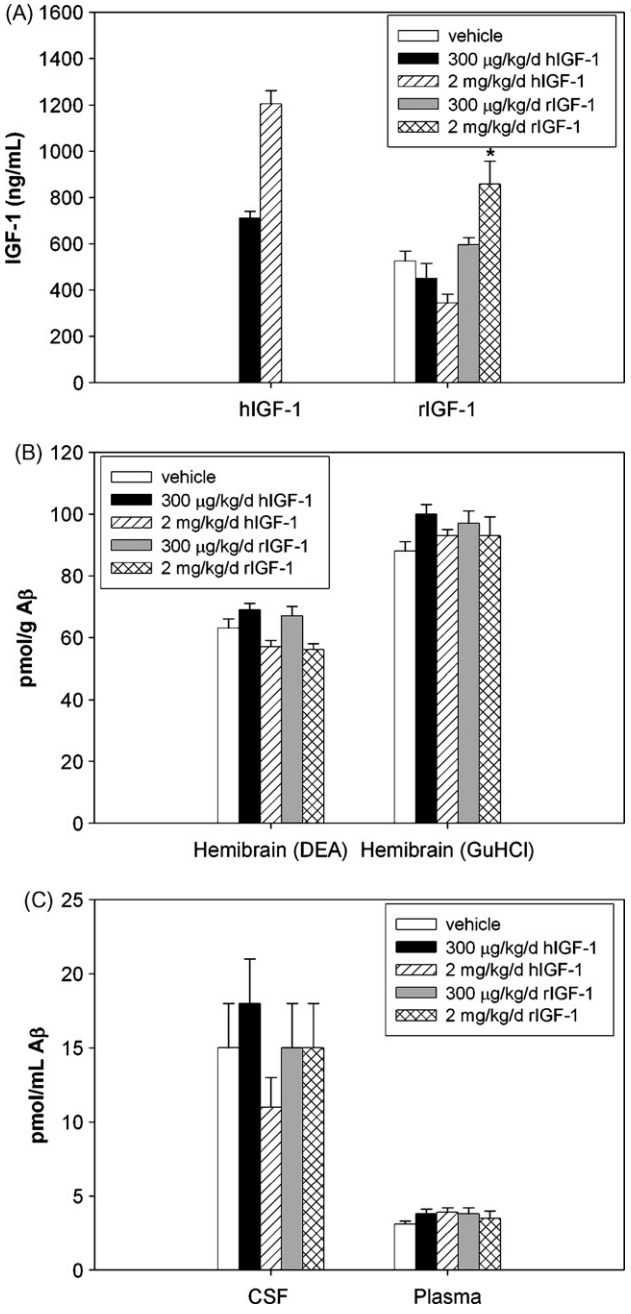

Fig. 4 – 3-month-old Tg2576 mice were treated s.c., BID for 5 days with vehicle (saline, open bars), 300 μg/kg/day human IGF-1 (black bars), 2 mg/kg/day human IGF-1 (striped bars), 300 mg/kg/day rodent IGF-1 (grey bars), or 2 mg/kg/day rodent IGF-1 (cross-hatched bars). (A) Mean ± SEM human and rodent IGF-1 levels were measured in rat plasma 2 h following the final dose. Human IGF-1 was not present in mice treated with vehicle or rIGF-1. An asterisk at 2 mg/kg/day rIGF-1 denotes $p < 0.05$ versus vehicle levels. Brain Aβ levels were measured in DEA and GuHCl extracts (B) and in CSF and plasma (C). No statistically significant changes in Aβ were observed.

plaque deposition) for 5 days with human or rodent IGF-1 via s.c., BID injections. Doses of 0.3 and 2 mg/kg were used to ensure measurable IGF-1 exposure in plasma. 2 h after the final dose, concentrations of hIGF-1 were 711 ± 30 ng/mL at the 0.3 mg/kg dose and 1204 ± 58 ng/mL at the 2 mg/kg dose (Fig. 4A). Rodent IGF-1 levels trended toward a decline in mice treated with hIGF-1, but the reductions were not statistically significant. In mice treated with rIGF-1, plasma concentrations of rIGF-1 rose from 525 ± 43 (vehicle) to 596 ± 30 (0.3 mg/kg) and 857 ± 100 (2 mg/kg, $p < 0.05$).

To assess changes in Aβ levels, two different extraction techniques were compared. Brains were split, with one half extracted in 0.2% DEA and the other in 5 M GuHCl. Based upon a series of studies comparing younger, non-depositing mice with older plaque-depositing mice we have determined that GuHCl is capable of extracting both soluble and insoluble Aβ, while DEA is only capable of extracting soluble Aβ (unpublished data). In response to hIGF-1 or rIGF-1 treatment, neither homogenate showed any statistically significant change in Aβ1-X (Fig. 4B), 1–40 or 1–42 (data not shown). CSF and plasma Aβ levels were also unchanged by this regimen of IGF-1 treatment (Fig. 4C). Furthermore, albumin levels as measured by ELISA in brain and CSF were unchanged relative to vehicle (data not shown). Thus despite maintaining robust IGF-1 exposure for several days, subchronic treatment was unable to influence Aβ or albumin clearance in young Tg2576 mice.

3.5. *Chronic dosing study in plaque-bearing Tg2576 mice*

As a final test of an IGF-1 connection to Aβ clearance, 11-month-old Tg2576 mice (an age at which these mice have Aβ deposits) were implanted with s.c. minipumps delivering saline or 50 μg/kg/day hIGF-1 for 1 month according to the experimental design of Carro et al. [11]. An additional dose of 500 μg/kg/day IGF-1 was included to ensure that circulating hIGF-1 levels could be measured ($n = 9$ in all groups). After 1 month of hIGF-1 delivery, plasma hIGF-1 levels at the 50 μg/kg/day dose were below the limit of detection (1 ng/mL) in five of nine mice. The four mice with detectable levels at this dose showed 5 ± 2 ng/mL hIGF-1. At the 500 μg/kg/day dose, all mice had detectable levels of plasma hIGF-1, with a mean ± SEM of 78 ± 10 ng/mL (Fig. 5A). Rodent IGF-1 levels showed no significant differences between the three groups.

Brains were bisected, with one half fixed for immunohistochemistry. From the other hemisphere, hippocampus was homogenized directly in 5 M GuHCl. Cortex was homogenized first in a mild detergent containing NP-40, phosphatase inhibitors and protease inhibitors (for analysis of tau phosphorylation and a minor soluble Aβ fraction) and the pellet from this homogenization was subsequently extracted in 5M GuHCl (to recover remaining soluble and insoluble Aβ). Aβ1-X levels were measured in all three extracts, but no significant differences were detected in response to chronic IGF-1 treatment (Fig. 5B). Aβ1–40 and 1–42 were measured from GuHCl extracts of cortex and hippocampus, and Aβ1–40 was measured in the soluble cortical fraction (Aβ1–42 was below the limit of quantitation in this pool), but as with Aβ1-X, there were no significant treatment effects (data not shown). As expected, based on the biochemical data, immunohistochemical analysis of 4G8 staining revealed no clear drug effect

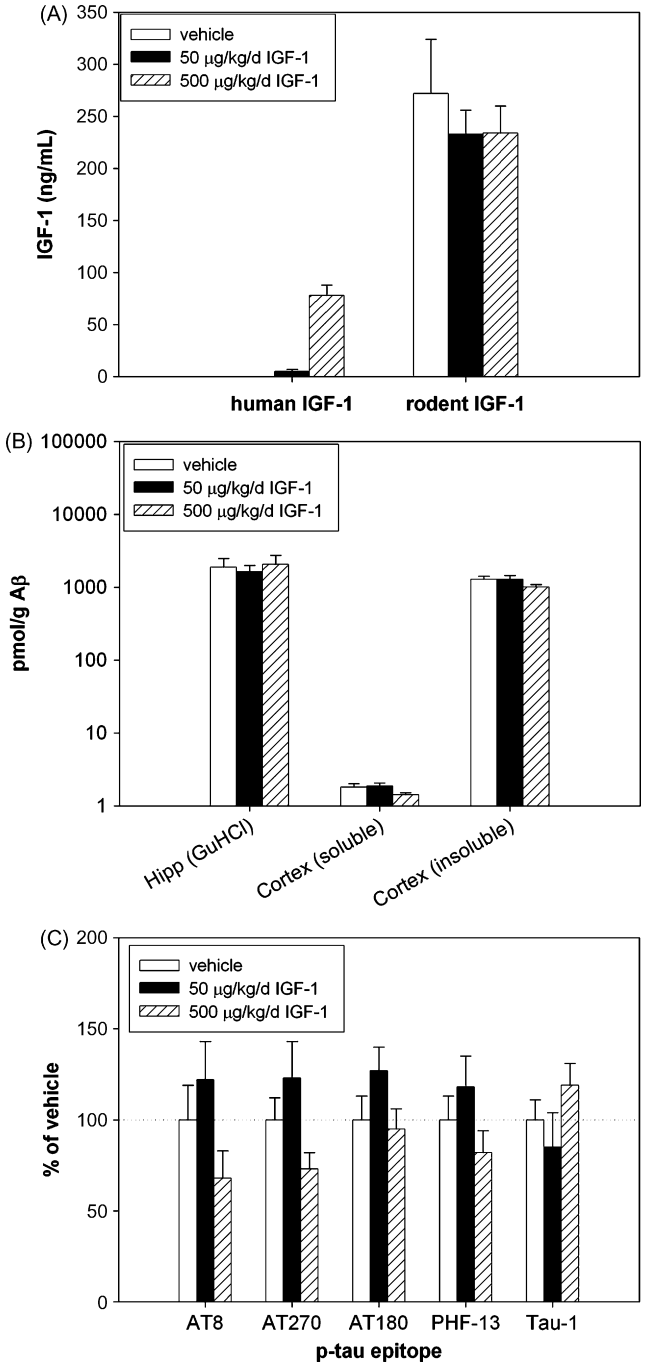

Fig. 5 – 11-month-old Tg2576 mice were implanted with s.c. minipumps delivering vehicle (open bars) or hIGF-1 (50 μg/kg/day, black bars; 500 μg/kg/day, striped bars). After 4 weeks of treatment, blood and brains were harvested. (A) Mean ± SEM hIGF-1 showed dose-responsive exposure in plasma. Treatment had no effect on rIGF-1 levels in plasma. (B) Mean ± SEM brain Aβ levels were measured by ELISA. Hippocampus was homogenized in GuHCl. Cortex was extracted in buffer containing mild detergent to measure soluble Aβ, and the resulting pellet was homogenized in GuHCl to assess insoluble Aβ. No extract showed statistically significant changes in Aβ. (C) Tau phosphorylation at a number of different epitopes was evaluated in the soluble cortical homogenates. Data are presented as mean ± SEM % of vehicle. Asterisks denote significant treatment effect ($p < 0.05$ versus vehicle).

(data not shown). Due to variability in plaque load at this age, however, the present study was powered such that anything short of a 60% reduction in plaque load would not be considered statistically significant.

Because tau phosphorylation state is preserved in the soluble cortical extracts, ELISA assays were used to assess multiple tau phosphorylation sites. Tau was captured and various phosphorylated epitopes were assessed by electrochemiluminescence (Fig. 5C). While some epitopes showed trends toward reduction, no differences were statistically significant. The antibody Tau-1, which selectively binds dephosphorylated epitopes, showed inverse trends to the phosphorylated epitopes as expected, but no significant treatment effect was detected.

3.6. *Western blot analysis of Aβ in rat and Tg2576 brain*

As much of the Aβ data reported in Carro et al. [11] was derived from Western blots, selected samples from the present studies were evaluated by Western blot to determine if there are changes in Aβ that can be observed by this method that could not be detected by ELISA or immunohistochemical analysis. Numerous pilot studies suggested that 4G8 Western blot is not a sensitive method for detecting Aβ, thus multiple sample types were analyzed by 4G8 (Fig. 6A) and also by the sensitive C-terminal Aβ antibody 9TL (Fig. 6B). As shown previously [15], native Tg2576 (lane 2) and rat (lane 4) brain extracts display high molecular weight bands that are greatly reduced after purification by SPE (lanes 3, 5, and 6). In samples denatured in GuHCl prior to denaturing gel conditions, all Aβ (soluble and insoluble) should be reduced to monomeric form (<5 kDa). This band is clearly observed in 12-month-old Tg2576 mice with either 4G8 or 9TL detection and SPE purification demonstrates high recovery of the Aβ band (lanes 2 and 3). Because rat brains have much lower Aβ levels versus plaque-bearing Tg2576 mice, this band is not visible in rat brain extracts without SPE. Thus, rat brain samples were enriched and concentrated via SPE. In vehicle-treated rat brain extracts, multiple bands can still be observed. To confirm the identity of the Aβ-specific band(s), a vehicle-treated rat was compared with a rat receiving a high dose of the potent γ-secretase inhibitor, LY-411575. ELISA analysis of the same samples previously showed approximately 75% inhibition of brain Aβ following treatment with the γ-secretase inhibitor. While a 4.5 kDa band is clearly reduced in the 9TL blot (Fig. 6B, lane 6), this band is not visible using 4G8 detection (Fig. 6A, lane 6). Based on background-subtracted integrated intensity, sample treated with LY-411575 showed 48% inhibition compared with control on the 9TL blot; the Aβ band could not be detected in the 4G8 blot. The higher sensitivity of 9TL versus 4G8 was demonstrated using synthetic Aβ1–40 peptide standards (lanes 7–9). The lowest concentration clearly detectable was 100 pg in the 9TL blot, or 1000 pg in the 4G8 blot.

Thus 9TL was used to evaluate subsequent samples by Western blot, and analysis was restricted to Tg2576 mice, because the Aβ level in rat brain was deemed to low to be

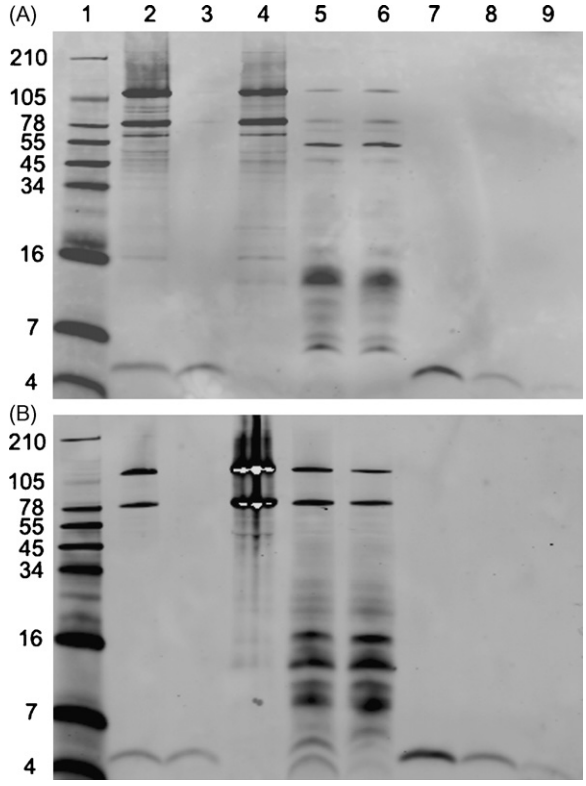

Fig. 6 – Aβ Western blots using 4G8 (A) or 9TL (B) detection antibody. Molecular weight markers (kDa) were loaded into lane 1. 12-month-old Tg2576 GuHCl brain homogenates (equal to 5 μg wet tissue weight) were loaded at 1:20 dilution directly into lane 2, or after SPE into lane 3. Neat rat GuHCl brain homogenate equal to 100 μg wet tissue weight was loaded into lane 4. Lanes 5 and 6 were loaded with rat brain GuHCl homogenates concentrated by SPE from 40 mg wet tissue weight. Lane 5 was a vehicle-treated rat, while lane 6 was a rat treated with 3 mg/kg LY-411575, a potent γ-secretase inhibitor. Synthetic Aβ1–40 peptide was loaded into lanes 7–9. For the 4G8 blot (A), lanes 7, 8 and 9 contain 4000, 2000, and 1000 pg, respectively. For the more sensitive 9TL blot (B), lanes 7, 8, and 9 contain 500, 250, and 100 pg of synthetic peptide, respectively.

accurately assessed by this method. GuHCl brain extracts from the Tg2576 mice treated with IGF-1 or vehicle minipumps for 1 month were run on a Western blot using 9TL detection (Fig. 7). As suggested by the ELISA and immunohistochemical data, no effect of IGF-1 treatment could be observed by Aβ Western blot. The mean ± SEM background-subtracted integrated intensity was 9.95 ± 1.12 (arbitrary units) in the vehicle group, and 9.51 ± 2.15 in the IGF-1 treated group.

4. Discussion

In recent years, a large literature has developed around the role of IGF-1 in neurodegeneration and neuroprotection. IGF-1 signaling is reported to be impaired in Alzheimer's brain [21–23]. In contrast, IGF-1 administration is reported to show neuroprotective activities [24–26] to increase cortical microvascular density [27,28] and to ameliorate age-related declines in hippocampal neurogenesis [29]. An IGF-1 knockout mouse displays neuronal loss, reduced brain size [30] and hyperphosphorylation of the microtubule-associated protein tau [31], suggesting that loss of IGF-1 signaling may underlie certain changes associated with neurodegeneration and neuropathology in Alzheimer's disease.

A series of recent publications have reported that peripheral administration of IGF-1 (50 μg/kg/day) facilitates the clearance of Aβ from the brains of aged rats, young rats, and plaque-depositing transgenic mice [9–11,32]. Chronic administration of this amount of IGF-1 daily, via peripheral minipumps, was reported to induce plaque-lowering and behavioral improvements in different strains of plaque-depositing transgenic mice. The putative mechanism proposed was enhanced clearance, whereby IGF-1 activates a megalin transporter and facilitates the movement of Aβ-binding proteins, such as albumin and transthyretin, across the choroid plexus to shuttle Aβ out of the brain [32]. In the initial report [11] IGF-1 was reported to increase CSF Aβ, as it was cleared from the brain on route to elimination through the choroid plexus. Brain albumin and transthyretin levels were also reported to be increased in IGF-1 treated animals.

The present series of studies failed to replicate Aβ-lowering effects of IGF-1 in a number of models reported by Carro et al. [11,32]. We measured plasma IGF-1 levels following administrations of hIGF-1 at the levels used by Carro et al. (50 μg/kg/day) and observed no detectable levels of hIGF-1, using an ELISA assay that detects as little as 90 pg/mL of hIGF-1. We thus administered hIGF-1 at this dose and at higher doses, where we could measure plasma exposures of hIGF-1. In our hands, acute intra-carotid injection of IGF-1 in young rats elicited no changes in brain, CSF, or plasma Aβ or in brain albumin, and 3-day exposure of either young or old rats to s.c. delivery of IGF-1 also failed to show effects as reported. In Tg2576 mice, neither subchronic nor chronic treatment

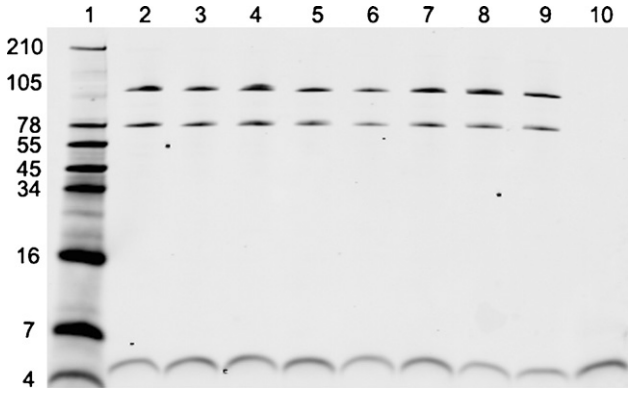

Fig. 7 – Aβ Western blot using 9TL as detection antibody. Lane 1 contains molecular weight markers, and lane 10 contains 175 pg of synthetic Aβ1–40. GuHCl brain homogenates of 12-month-old Tg2576 mice, treated with vehicle or IGF-1 for 1 month by minipumps, were loaded into the remaining lanes. Odd lanes are vehicle-treated, and even lanes received 500 μg/kg/day IGF-1.

of IGF-1 in a wide range of concentrations produced any measurable effects on amyloid plaque load or on Aβ levels as measured by ELISA and Western blot. Furthermore, when endogenous IGF-1 was continuously elevated for several days in beagles using a growth hormone secretagogue (GHS), CSF and plasma Aβ levels remained unchanged.

While small differences in methodologies could account for the divergent outcomes of the present studies as compared to the previous reports, we have made every attempt to replicate the treatment conditions as reported. Our analysis of the reported data, coupled with our experience in measurements of amyloid peptide lead us to conclude that the primary differences are accounted for in the assay methodologies employed.

We have recently described a nonspecific binding artifact that is present in brain extracts and compromises plate-based immunoassays of low abundance analytes [15]. This artifact is especially apparent in Aβ assays due to the relatively low abundance of this peptide in the brains of non-transgenic animals. Assaying brain Aβ by ELISA using various homogenization buffers has been shown to elicit nonspecific signal in immunoassays that obscures the specific signal [15,33]. The nonspecific signal occurs in the presence or the absence of a capture antibody and is observed with a wide variety of detection antibodies and blocking conditions. Thus a clearing step, such as the solid-phase extraction procedure used in the present studies, is essential to accurately quantify Aβ levels in non-transgenic rodent brains. Aβ recovery following this extraction protocol for brain samples has been shown to be greater than 90% [15] using either Tg2576 mouse brain homogenates or wild type mouse or rat brain homogenates that have been spiked with various levels of Aβ peptides. Nonspecific binding elements are likely to show much less interference with Aβ assays in Tg2576 brain, due to the relatively high peptide concentrations requiring large sample dilutions, prior to assay. In contrast, nonspecific binding components are more concentrated in measurements of Aβ in wild type rodent brains and this can give rise to considerable inaccuracies in quantitation.

The absolute Aβ values reported in rat brain by Carro et al. [11] suggest that they were indeed tracking a nonspecific artifact in rat brain. They reported measuring over 1 nmol/g Aβ1–40 in rat brain (which does not deposit amyloid plaque), whereas they reported 1.7 nmol/g in plaque-bearing Tg2576 mouse brain. The Tg2576 mouse, engineered to produce APP at much higher levels than wild type rodents, produces Aβ levels far in excess of the wild type rat. Thus the fact that Carro et al. report similar levels in wild type rat as in the Tg2576 mouse appears inconsistent. While their quantification of Aβ in Tg2576 mouse is similar to the one derived in the present analysis of 12-month-old Tg2576 mice, our analysis of wild type rat brain after removal of nonspecific binding, indicates levels of Aβ that are 1000-fold lower than their report, or about 1 pmol/g of Aβ1–40. We also measure low levels of Aβ in rat CSF (on the order of <1 pmol/mL), where sample extraction is not required. Thus we speculate that measures of Aβ changes in rats reported in Carro et al. [11] are not actually Aβ at all. Indeed, much of the data in the Carro et al. [11] report consisted of Western blots of Aβ from rat brain homogenates. As shown in Fig. 7, Western blot has both a lower degree of sensitivity and a high propensity for the appearance of nonspecific bands, especially in wild-type rodents. Even with significant concentration using SPE, rat brain Aβ cannot be robustly measured on a Western blot. ELISA following SPE offers increased sensitivity, elimination of nonspecific binding artifact, and more accurate quantitation versus Western blot analysis.

In the Tg2576 mouse studies, the discrepancy between the current results and the previous report could be attributed to biological variability, which can be quite large in 12-month-old Tg2576 mice. The early stages of Aβ deposition (8–13 months) are characterized by higher variability as compared with younger or older mice (unpublished observation). In the present studies, using $n = 9$ per group, the standard error of the mean for vehicle-treated mice ranged from 11% in cortex to 31% in hippocampus. In hippocampus in particular, Aβ1-X levels for individual mice ranged from 68 to 5545 pmol/g. The sample size reported in Carro et al. [11] was only $n = 4$–6 per group, thus increasing the risk for observing a result that is not representative of the population in terms of both biochemistry and immunohistochemistry. This biological variability could account for the appearance of plaque-lowering in the IGF-1 treatment group.

In their studies, Carro et al. did not show that the dose of IGF-1 used, 50 μg/kg, produced measurable levels of hIGF-1, or changes in the total level of IGF-1 in circulation. In the present studies, this dose produced undetectable exposure in rats, and exposure in mice that was just at the edge of detection by ELISA. A 10-fold higher dose elicited measurable hIGF-1 levels, yet still failed to impact Aβ levels in brain or biofluids.

At high doses IGF-1 injections produced a transient rise in plasma IGF-1 levels. To achieve sustained increases in plasma IGF-1 levels, a growth hormone secretagogue (GHS) was utilized. In dogs and humans GHS compounds elevate circulating growth hormone, which in turn augments production and release of IGF-1 [13,34,35]. After several days of dosing beagle dogs with the GHS CP-424391, plasma IGF-1 levels reached double the basal levels. Despite analysis of multiple timepoints, however, no alterations in CSF or plasma Aβ were detected. While the study was not terminal and brain tissue was therefore not examined, any reduction in Aβ via a clearance mechanism would be expected to alter Aβ dynamics in biofluid compartments.

In summary, the present series of experiments failed to show any effect of IGF-1 elevation on Aβ clearance in a number of acute and chronic in vivo models, both transgenic and non-transgenic. Additionally, while an association between IGF-1 and tau phosphorylation has been described in a cell culture model [36], no significant effects on tau phosphorylation were achieved in a 1-month infusion of hIGF-1. Using a cellular myoblast differentiation model, we have shown that the samples of IGF-1 used here were biologically active at the expected potencies. Thus we conclude that peripheral administration of IGF-1 to rodents has no effects on the processes that lead to the two prominent histopathologies of human Alzheimer's disease brain, Aβ accumulation or tau hyperphosphorylation. The notion that elevation of growth hormone and IGF-1 levels could be beneficial in AD has already been evaluated in a 563-patient clinical trial. The GHS MK-0677 was administered for 1 year to AD patients, but had no

beneficial effect on cognition despite raising circulating IGF-1 levels by 60%. The failure of a large clinical trial to show cognitive benefit from GHS treatment of AD patients, taken together with the negative biochemical results presented here suggest that IGF-1 elevation has little merit as a target for the treatment of Alzheimer's disease.

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