The BACE1 inhibitor LY2886721 improves diabetic phenotypes of *BACE1* knock-in mice

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Abstract

Aim: The β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1) has been identified as the central initiator of amyloid β (Aβ) generation in the brain, the key hallmark of Alzheimer's disease (AD). However, recent studies provided evidence that BACE1 also plays a crucial role in metabolic regulation, and we have shown that neuronal human *BACE1* knock-in mice (PLB4) display type 2 diabetes mellitus (T2DM)-like symptoms alongside AD-like impairments. Hence, we here investigated if targeted BACE1 inhibition using LY2886721, an active site BACE1 inhibitor, would improve glucose homeostasis, insulin sensitivity and motor performance in PLB4 mice.

Materials and methods: LY2886721 was administered as a dietary supplement (0.02% wt/wt) for six consecutive weeks. Physiological, metabolic and motor assessments were performed during the last two weeks of treatment, followed by molecular tissue analyses post-mortem.

Results: LY2886721 treatment improved glucose homeostasis and hepatic gluconeogenesis in diabetic PLB4 mice, as determined by improvements in basal glucose and glucose/pyruvate tolerance tests. Furthermore, LY2886721 improved hepatic insulin sensitivity, as indicated by enhanced basal hyperphosphorylation of insulin receptors. In PLB4 brains, we detected altered basal conditions of APP expression and processing, with beneficial effects on APP processing achieved by LY2886721 treatment. No improvements in motor coordination were found.

Conclusions: Our data provide support for a role of BACE1 as a regulator of systemic glucose homeostasis and suggest BACE1 inhibitors for the treatment of T2DM-associated pathologies, especially in cases where diabetes is comorbid to AD.

Keywords: AD, T2DM, BACE1, LY2886721, Beta Amyloid

1. Introduction

With the increasing prevalence of Type 2 diabetes mellitus (T2DM) and Alzheimer's diseases (AD) worldwide, there is a great need for effective therapeutic interventions to treat these disorders. Strong associations between AD and T2DM have been uncovered in humans [1], and animal model studies report a range of similarities in metabolic, behavioural and pathophysiological profiles of the two conditions [2–4]. Nevertheless, the molecular links and reasons for the comorbidity remain largely unknown.

The aspartyl protease, β-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE1), is the key enzyme initiating the generation of amyloid β (Aβ) in the brain [5]. BACE1 has been identified as a central target for lowering Aβ levels in AD, leading to an intense interest in developing BACE1 inhibitors [6], of which some proceeded to clinical trials [7]. Furthermore, BACE1 may not only be a key target in AD, it also appears to be a regulator of metabolic functions [8–10]. We have recently shown that subtle neuronal expression of human *BACE1* resulted in systemic T2DM-like symptoms (hyperglycaemia, glucose intolerance) alongside AD phenotypes (PLB4 mouse) [9,11]. In addition, several other studies have also reported the importance of BACE1 in metabolic regulation, e.g. lack of murine *Bace1* protected from obesity, insulin resistance and glucose intolerance upon high-fat feeding [8]. BACE1 may also regulate hepatic insulin receptor expression and hypothalamic leptin sensitivity [10,12]. Collectively, this implicates BACE1 as a key player in metabolic control, and as such a likely molecular connection between AD and T2DM.

LY2886721, an inhibitor of the active site of BACE1, was developed by Eli Lilly, and entered phase 2 clinical trials for AD [13,14]. The molecule possesses much higher potency for inhibiting BACE1 compared to previously developed compounds such as LY2811376, has a plasma half-life of ~12h, exhibits decreased efficacy against off-target proteases such as cathepsin D and is permeable across the blood-brain barrier [13,15,16]. Pharmacological properties and safety were also evaluated in a mouse model of AD (PDAPP), whereby oral administration significantly decreased Aβ1-x levels in hippocampus and cortex [13]. Later, its efficacy was tested in canines, and a decrease in different isoforms of Aβ (Aβ1-40, Aβ1-x42, Aβ1-x) in plasma and cerebrospinal fluid (CSF) was confirmed [13]. In subsequent phase 1 clinical trials, LY2886721 lowered Aβ levels in CSF of healthy volunteers [13]. However, during phase 2 trials, LY2886721 testing was terminated due to signs of liver toxicity in 4 out of 70 patients [13,17]. Nevertheless, these data provided strong support for LY2886721 efficacy and suitability to test the effects of BACE1 inhibition in experimental settings.

Neuronal *BACE1* knock in mice (PLB4) exhibit hyperglycaemia and glucose intolerance from ~4 months of age, associated with up-regulated serum and hepatic dipeptidyl peptidase 4 (DPP4) levels, as well as increased liver triglycerides and decreased glycogen storage [9,11]. We recently investigated the effects of anti-diabetic treatments (Liraglutide and Fenretinide) in these mice and reported no substantial improvements in glucose homeostasis with these treatments [11]. Since PLB4 mice exhibit specific elevation in neuronal BACE1, we hypothesised that pharmacological inhibition of BACE1 should be beneficial in alleviating diabetic symptoms in these mice. Therefore, we here assessed effects of LY2886721 in PLB4

and control mice to determine whether this approach would be an effective way for the T2DM-AD comorbid background.

2. Materials and Methods

2.1 Animals

All animals were housed and tested according to the European (2010/63/EU) and UK Home Office regulations. Experiments were approved by the University Ethics Board and carried out in accordance with the Animal (Scientific Procedures) Act 1986 and ARRIVE guidelines. Male transgenic neuronal *BACE1* knock-in (PLB4) and wild-type (WT) control mice were generated as previously described [18]. All animals were singly housed in wire-top cages (12-hour day/night cycle, 20–21°C, 60–65% relative humidity) with *ad libitum* access to water and food. Mice were ~6 months old when treatment started and ~8 months old when tissues were harvested. All mice were 5h fasted and sacrificed by cervical dislocation at the end of the study.

2.2 Treatments

PLB4 and WT control mice received LY2886721 incorporated into rodent chow diet at 0.02% wt/wt (provided by Eli Lilly and Envigo, USA) via *ad libitum* feeding for six consecutive weeks (see Fig. 1i for experimental timeline). All mice were randomised and counterbalanced according to body weight into the control and experimental groups per genotype: **Control** (CON; 2016C Teklad certified global 16% protein rodent diet; TD. 09692, Envigo) (WT n=12; PLB4 n=10) and **LY2886721** (2016C Teklad certified global 16% protein rodent diet + LY2886721, TD.150785 Envigo) (WT n=12; PLB4 n=10). Individual food intake was measured three times a week.

2.3 Body weight and body mass composition

Body weights were monitored three times a week and weekly averages recorded. Fat and lean body mass composition was analysed using an EchoMRI scanner at the end of treatment week 4 (EchoMedical Systems, Houston, TX, U.S.A.).

2.4 Metabolic measurements

Pyruvate tolerance test (PTT) and glucose tolerance test (GTT) were performed at the end of weeks 4 and 5, respectively, as reported previously [19]. All mice were fasted overnight before PTT and 5h-fasted before GTT; tail blood glucose was determined using an AlphaTRAK glucometer (Berkshire, UK) at baseline (time 0) before i.p. injection of sodium pyruvate (PTT: 2mg/g body weight) or glucose (GTT: 2mg/g body weight) and at 15, 30, 60 and 90 min post- injection.

2.5 Serum immunoassays

Trunk-derived blood was collected from 5h-fasted mice into serum separator microtubes and kept at -80C. Serum was used for insulin, leptin, alanine and aspartate aminotransferases (ALT and AST) analyses (insulin, Millipore, Darmstadt, Germany; leptin ELISA, CrystalChem, Zaandam, Netherlands; ALT and AST kits, Abcam, USA).

2.6 RotaRod

RotaRod apparatus (TSE Systems, Bad Homburg, Germany) was used to determine motor coordination and motor learning in mice [9]. Animals were placed on a rotating rod and habituated for 2 min before the first trial at 2rpm. Testing was conducted based on the increasing rotation (5 rpm - 45 rpm over 300s), over 4 trials per day for 2 consecutive days. Latin square design was used to allocate animals on the rotarod

lanes. Motor performance was assessed through the amount of time taken to fall from the rod and motor learning as a comparison of trial 1 versus trial 8.

2.7 Immunoblotting and protein analyses

Immunoblotting and protein analyses were performed as previously described [19]. Brain tissue were dissected from 5h-fasted mice during terminal procedures, snap-frozen in liquid nitrogen and stored at -80°C. Brain tissue was homogenised in NP40 buffer, final concentration: 20mM HEPES, 150mM NaCl, 100uM EDTA, 1% NP40, pH 7.6 supplemented with complete protease inhibitors and PhosStop tablets (1 tablet/10ml, Roche). Homogenates were centrifuged (14,000g, 4°C, 20 min) and the supernatant collected and stored at −80°C. Protein concentrations were measured using a Bicinchonic Acid (BCA) protein assay (Sigma, Dorset UK), samples were prepared at a final concentration of 3µg/µl in NP40 buffer containing lithium dodecyl sulphate (LDS, Thermo Fisher Scientific, Paisley, UK), and 30µg of protein was separated by electrophoresis on NuPage 4-12% sodium Bis-Tris pre-cast gels for 45 mins at 200V in 2-(N-Morpholino)ethanesulfonic acid (MES) buffer. Proteins were dry transferred at 23V for 6 mins onto 0.2µM nitrocellulose membranes using the Nu Page IBlot transfer system (ThermoFisher Scientific). Following protein transfer, membranes were microwaved for 3 mins in phosphate buffered saline (PBS). Membranes were washed in Tris-buffered saline with Tween (TBST) (0.05% Tween, 50mM Trizma base, 150mM NaCl, pH 7.6; 3×5-min washes), blocked in 5% milk powder TBST for 1h at RT and washed 3x 5min in TBST. Membranes were incubated overnight at 4°C in primary antibodies, prepared in 5% BSA, 0.05% sodium azide, TBST (Table 1). After overnight incubation membranes were washed 3 x 5mins in TBST and incubated in appropriate secondary antibodies (Table 1).

Peripheral tissues were dissected from 5h-fasted mice during terminal procedures, snap-frozen in liquid nitrogen and stored at -80°C. Immunoblotting was performed as previously described [19]. Briefly, liver and muscle tissue were homogenized in Radioimmunoprecipitation (RIPA) buffer (10mM Tris-HCl, 150mM NaCl, 0.1% SDS, 1% triton, 1% sodium deoxycholate, 5mM EDTA, 1mM NaF, 1mM NaOV, pH 7.4), homogenates centrifuged (14,000g, 4°C, 20 min), and supernatant collected and stored at −80°C. Protein concentrations were adjusted using a BCA protein assay (Sigma, Dorset UK) and sample proteins were boiled, separated by electrophoresis (10µg/lane,Nu Page 4-12% sodium Bis-Tris gel) and wet transferred onto a nitrocellulose membrane (0.45µM, Invitrogen, UK). Membranes were subsequently washed, blocked in 5% milk TBST and incubated in primary antibody overnight at 4°C, followed by incubation in appropriate secondary antibodies (Table 1).

Proteins were analysed and quantified as described previously [19]. Briefly, immunological detection of proteins was visualised using enhanced chemiluminescent substrate and images captured with a Vilber-Fusion chemiluminescence-imaging camera and Fusion Software (PEQLAB, Germany). Protein was quantified via densitometric analysis of 16-bit Western Blot images using ImageJ software. Protein loading was assessed via Coomassie Blue staining and data for all markers normalised to total protein and expressed relative to WT controls.

2.8 Rodent Aβ (1-x and 1-40) determination

Parenchymal Aβ levels were determined from appropriately diluted guanidine brain homogenates by sandwich ELISA (using monoclonal antibodies 266 for 1-x and 2G3 for 1-40 for capture, and biotinylated N-terminal rodent Aβ antibody b-IBA030-3 as reporting antibody).

Samples were assayed in duplicate, and the concentration of analyte interpolated from a four-parameter fit of the reference curve (XL-Fit for Excel). Aβ ELISA values were normalized to protein levels (determined in duplicate by the Bradford Coomassie Plus Protein method) and expressed as pg/mg.

2.9 Statistical analysis

Results are expressed as means ± SEM. Statistical analyses for time series (body weight, GTT, PTT, Rotarod) were performed using a 3-way ANOVA (with genotype, treatment and time (repeated measures, RM) as factors), and where appropriate, subsequent 2-way ANOVA followed by Bonferroni multiple-comparison post hoc tests. Single endpoint data were also analysed using 2-way ANOVAs followed by Bonferroni post hoc tests or unpaired two-tailed Student's *t*-test. GraphPad Prism (V8, GraphPad Software, Inc., San Diego, California, USA) was used for analyses. P-values < 0.05 were considered reliable.

3. Results

3.1 LY2886721 treatment improved glucose metabolism in PLB4 mice

No gross, overall weight differences were detected between genotypes (3-way ANOVA, $p>0.05$). However, weight changed over time (effect of time: $F(2.98,119) = 3.47$, $p<0.05$; Fig 1A), and this was dependent on genotype (time x genotype interaction: $F(6, 240) = 2,25$, $p<0.05$) and treatment (time x treatment: $F(6, 240) = 3.94$, $p<0.001$). The effect of time and respective interactions can be explained by the reduction in weight in the PLB4 vehicle group over time while both treatment groups showed a subtle weight gain. Echo MRI revealed significantly lower lean body mass in PLB4 mice compared to WT ($p<0.05$; Fig 1. B) and no significant changes in body mass composition of either WT or PLB4 mice on treatment (Fig 1. A and B).

To assess the effect of LY2886721 on hepatic gluconeogenesis and whole-body glucose metabolism, we performed PTTs in overnight-fasted mice at the end of treatment week 4. In agreement with our previous studies[11,9], PLB4 mice had higher basal blood glucose levels compared to WT controls ($p<0.01$; Fig. 1C); However, treatment with LY2886721 did not significantly decrease basal glucose as determined by PTT in PLB4-treated mice (Fig. 1D and E).

Furthermore, PLB4 vehicle-treated mice displayed abnormal hepatic gluconeogenesis, as assessed by PTTs, compared to controls (time: $F(2.63, 100) = 25.9$, $p<0.001$; genotype: $F(1,38) = 33.5$, $p<0.001$; Fig. 1 D and E). The time course also significantly differed between genotypes (time x genotype interaction: $F(4,152) = 4.72$, $p<0.001$). The treatment effect did not depend on genotype ($p>0.05$, but note the trend in the genotype x treatment interaction: $F(1, 38) = 3.07$, $p<0.088$). Importantly, a 3-way interaction was detected, i.e. the treatment effect ultimately

depended on a combination of time and genotype ($F(4, 152) = 3$, $p<0.05$). This was further confirmed by 2-way ANOVAs followed by Bonferroni post hoc tests, yielding a significant difference at 15min post-pyruvate administration in PLB4-treated mice compared to PLB4 controls ($p<0.05$, Fig 1D).

Basal glucose levels and GTTs were assessed at the end of treatment week 5. PLB4 mice displayed severe hyperglycaemia compared to WT controls ($p<0.001$; Fig 1 F), and the BACE1 inhibitor, LY2886721 significantly lowered fasted basal glucose levels in PLB4 treated vs PLB4 control mice ($p<0.05$), but did not affect glucose levels in WT mice (Fig 1. F). Consistent with previous data, PLB4 control mice displayed advanced glucose intolerance in comparison to WT controls (time effect: $F(3, 120) = 77.2$, $p<0.001$; genotype effect: $F(1, 40) = 30.9$, $p<0.001$; Fig. 1 G&H). The time course also significantly differed between genotypes (time x genotype interaction: $F(4, 160) = 6.67$, $p<0.001$), and the treatment effect depended on genotype (genotype x treatment interaction: $F(1, 40) = 4.37$, $p<0.05$) , i.e. treatment was only effective in PLB4 but not WT mice. Further analyses (2-way ANOVA followed by Bonferroni tests) revealed a significant improvement in glucose clearance at 60 min post-glucose injection in PLB4-treated vs. vehicle mice ($p<0.05$; Fig. 1 G), without effects in WT mice (Fig. 1 G).

Figure 1.

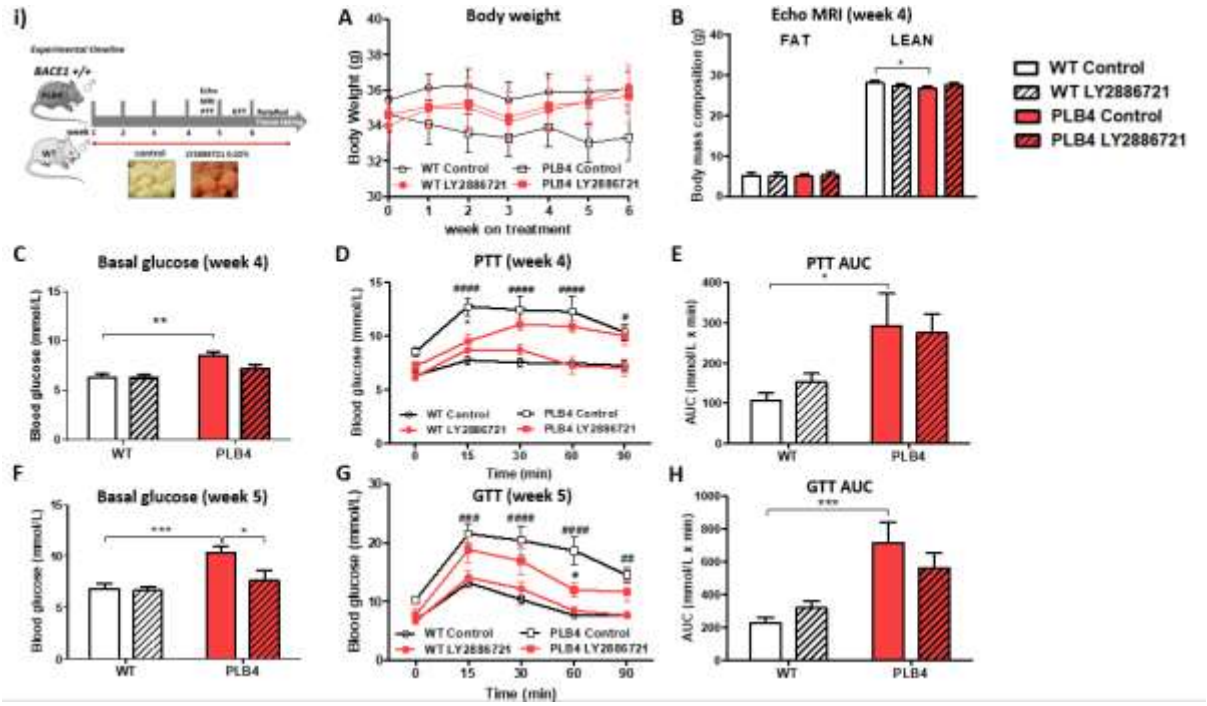

Figure 1. LY2886721 treatment effects on physiological and metabolic assessments in PLB4 and WT mice.

i) Graphic illustration of experimental timeline. (**A**) Body weights of WT and PLB4 mice in LY2886721 and control groups during the six weeks of treatment. (**B**) Fat and lean mass composition of mice at week 4 of treatment as measured using Echo MRI scan. (**C**) Basal glucose levels in overnight-fasted mice at week 4 of treatment. (**D**) PTT graph of PLB4 and WT mice and area under curve (AUC; baseline corrected) analysis of PTT (**E**). Basal glucose in 5h-fasted mice at week 5 of treatment (**F**) and GTT graph and AUC of GTT (**G** and **H**). Number of mice per group: WT control n=12; WT LY2886721 n=12; PLB4 control n=9-10; PLB4 LY2886721 n=9-10. Data are expressed as means ± SEM and analysed using three- or two-way ANOVAs (see text) followed by Bonferroni post hoc test or unpaired two-tailed Student's t-test. Asterisks: *p < 0.05, **p<0.01, ***p<0.001. (D and G) Comparison of WT versus PLB4 control mice is indicated as #p < 0.05, ##p < 0.01, ###p<0.001, ####p<0.0001 and treatment effect within genotype as asterisk *p < 0.05.

3.2 LY2886721 treatment did not improve impaired motor performance of PLB4 mice

To assess motor coordination and learning, RotaRod testing was performed during week 6. A 3-way ANOVA (with genotype, treatment and time (RM) as factors) indicated a gross, highly significant genotype effect (F (1,40) = 24, p<0.001), which did not change over time, i.e. PLB4 mice were overall motorically impaired, which was

further confirmed by a 2-way ANOVA (F (1,140) = 4.65 p<0.05; Fig. 2 A, WT vs PLB4 control groups). We also identified a trend for a genotype-dependent treatment effect (interaction: F (1,40) = 2.95, p=0.093), due to an apparent and additional negative impact of the BACE1 inhibitor, LY2886721 on motor performance in PLB4 mice. The highly significant effect of time (i.e. trial) (F=16.5, p<0.001) also suggested a trend for worse performance of PLB4 mice (time x genotype interaction: F (7, 280) = 1.80, p<0.087), but was overall indicative of intact motor learning in both groups. Further analyses (change from trial 1 to trial 8) indeed suggested an improvement in motor coordination for both genotypes over time (Fig. 2 D and E).

Figure 2.

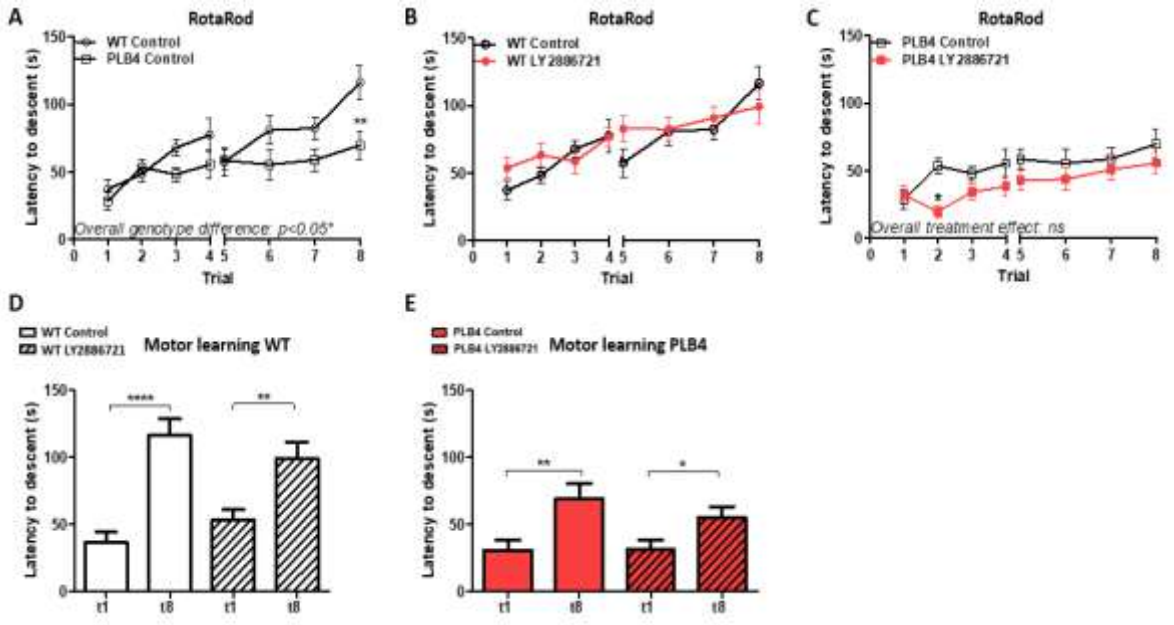

Figure 2. Motor performance on RotaRod

Graphs represent mean scores from individual trials on RotaRod obtained over two days of testing (4 trials /day). (**A**) Comparison of motor performance and motor learning of WT and PLB4 control mice. Effects of LY2886721 treatment on motor performance in WT (**B**) and PLB4 (**C**) mice. Motor learning on RotaRod (trial 1 versus trial 8) in WT (**D**) and PLB4 (**E**) mice. Number of animals per group: WT control n=12; WT LY2886721 n=12; PLB4 control n=10; PLB4 LY2886721 n=10. Data are shown as means ± SEM and were analysed using three- or two-way ANOVA followed by Bonferroni multiple comparison post-hoc or two-tailed Student's t-tests. Asterisks indicate *p < 0.05, **p<0.01,****p<0.0001.

3.3 LY2886721 treatment decreased brain Aβ levels in WT mice and full-length APP in PLB4 mice

As LY2886721 was developed to inhibit BACE1 activity, it was important to investigate how this drug affected Aβ levels in both WT and PLB4 mouse brains. A strong trend for up-regulation of overall Aβ 1-x (Aβ isoforms larger than 16 amino acids) was detected in the brains of PLB4 vs WT controls ($p=0.05$; Fig. 3 A), yet no significant difference was detected in Aβ 1-40 levels between genotypes (Fig. 3 B). Interestingly, LY2886721 treatment markedly decreased both, Aβ 1-x and Aβ 1-40 levels in WT mice ($p<0.01$ Fig 3 A and B), indicative of treatment efficacy. In comparison, no significant changes in Aβ 1-x or Aβ 1-40 levels were detected in PLB4 mice treated with LY2886721 (Fig 3 A and B) even though PLB4 mice had increased flAPP and monomeric Aβ levels ($p<0.01$ and $p<0.05$, respectively; Fig. 3 C and D) and soluble-APPα (sAPPα) trended to be decreased ($p=0.076$; Fig. 3 E) compared to WT controls. LY288671 treatment significantly decreased flAPP ($p<0.05$; Fig. 3 C) and exhibited a trend towards decreasing monomeric Aβ ($p=0.07$; Fig. 3 D), while sAPPα was increased in PLB4 mice ($p<0.05$; Fig. 3 E). Overall, our data suggest altered basal conditions of APP expression and processing in PLB4 mice, alongside beneficial regulation of APP processing achieved with LY2886721, with differing profiles in control vs. transgenic mice.

Figure 3.

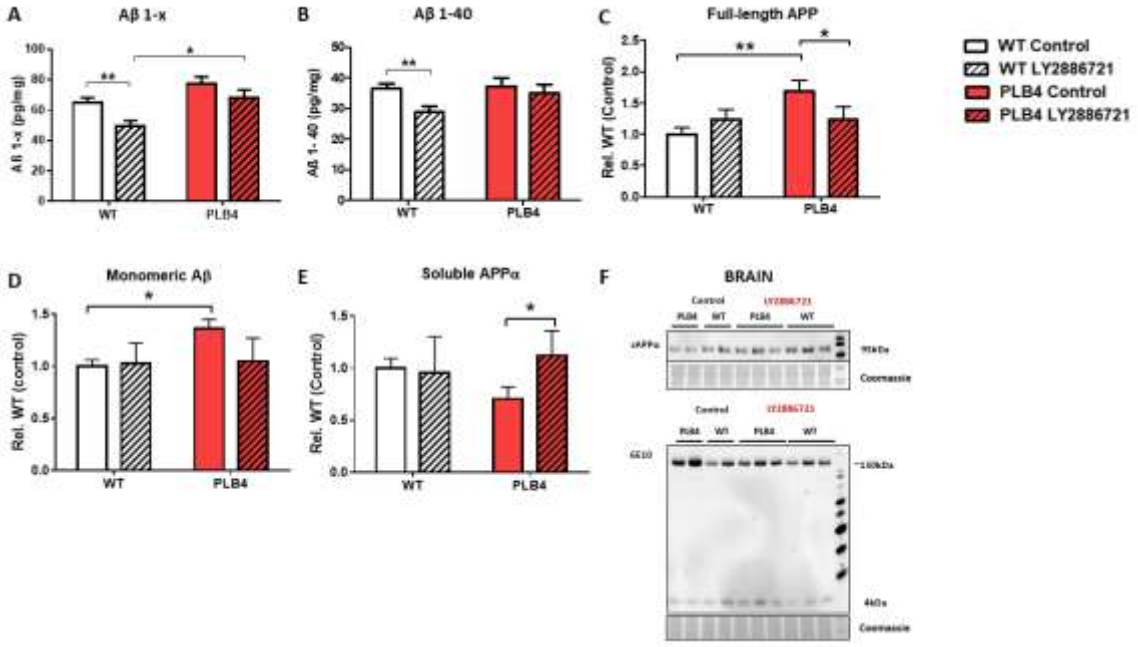

Figure 3. Analyses of Aβ and APP levels in the brain.
Levels of Aβ 1-x (**A**) and Aβ 1-40 (**B**) (measured using ELISA), full-length APP (**C**), monomeric Aβ (**D**) and soluble APPα (**E**) in WT and PLB4 mice brain and effects of LY2886721 treatment. (**F**) Representative immunoblots of soluble APPα, full-length APP (6E10) and monomeric Aβ. Number of mice per group: WT control n=6-8; WT LY2886721 n=6-8; PLB4 control n=6-8; PLB4 LY2886721 =6-8. Data are expressed as means ± SEM and significant differences were determined using a two-way ANOVA followed by Bonferroni *post hoc* or unpaired two-tailed Student's t-tests (see text). Asterisks illustrate *p < 0.05, **p<0.01.

3.4 Differential alterations in AST and ALT activity levels with LY2886721 treatment

The BACE1 inhibitor, LY2886721 did not induce robust effects on serum insulin or leptin levels in either WT or PLB4 mice (Fig. 4 A and B). As phase 2 clinical trials were terminated due to evidence for higher liver enzyme activity in some patients, we also assessed the effects of LY2886721 treatment on serum AST & ALT activity levels, indicators of liver damage. An increase in ALT levels and AST activity was detected in WT mice treated with LY2886721 compared to WT control mice (p<0.05; Fig. 4 C,

p<0.01; Fig. 4 D, respectively), whilst no significant changes were detected in PLB4 mice with treatment (Fig.4 C and D).

Figure 4.

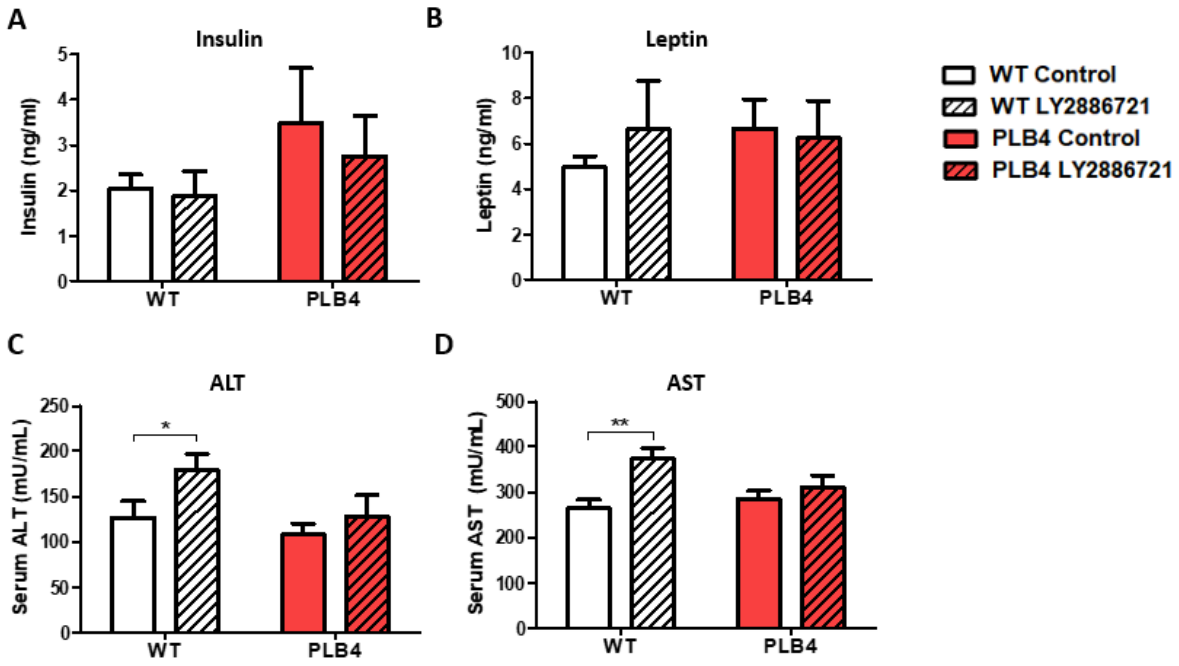

Figure 4. Effects of LY2886721 treatment on serum insulin, leptin, ALT and AST levels

Comparison of 5h-fasted insulin (**A**), leptin (**B**) and serum triglyceride ALT (**C**) and AST (**D**) levels in WT and PLB4 mice. Number of mice per group: (insulin) WT control n=7; WT LY2886721 n=7; PLB4 control n=5; PLB4 LY2886721 = 5; (leptin, ALT, AST) WT control n=12; WT LY2886721 n=12; PLB4 control n=10; PLB4 LY2886721 = 10. Data expressed as means ± SEM and significant differences were determined using two-way ANOVA followed by unpaired two-tailed Student's t-test. Asterisks: *p < 0.05, **p<0.01.

3.5 LY2886721 treatment increased hepatic insulin sensitivity and GSK3β levels in mice

Next, we assessed the effects of the BACE1 inhibitor, LY2886721 on molecular metabolic markers in the liver. Interestingly, LY2886721 treatment increased both phosphorylated and total protein levels of IRβ and total AKT in WT and PLB4 mice (p<0.01 - p<0.001; Fig. 5 A and B). Furthermore, hepatic phosphorylated and total

levels of glycogen synthase kinase 3β (GSK3β) were significantly elevated with treatment in both genotypes of mice ($p<0.001$; Fig. 5 A and B). Hepatic ribosomal protein S6 (rpS6) phosphorylation was increased in PLB4 mice ($p<0.001$ vs. controls), and phospho-rpS6 levels significantly decreased only in LY2886721-treated PLB4 mice ($p<0.001$; Fig. 5 A and B). In addition, DPP4 levels were higher in PLB4 mice compared to WT controls ($p<0.001$, (Fig. 5 A and B).

Figure 5.

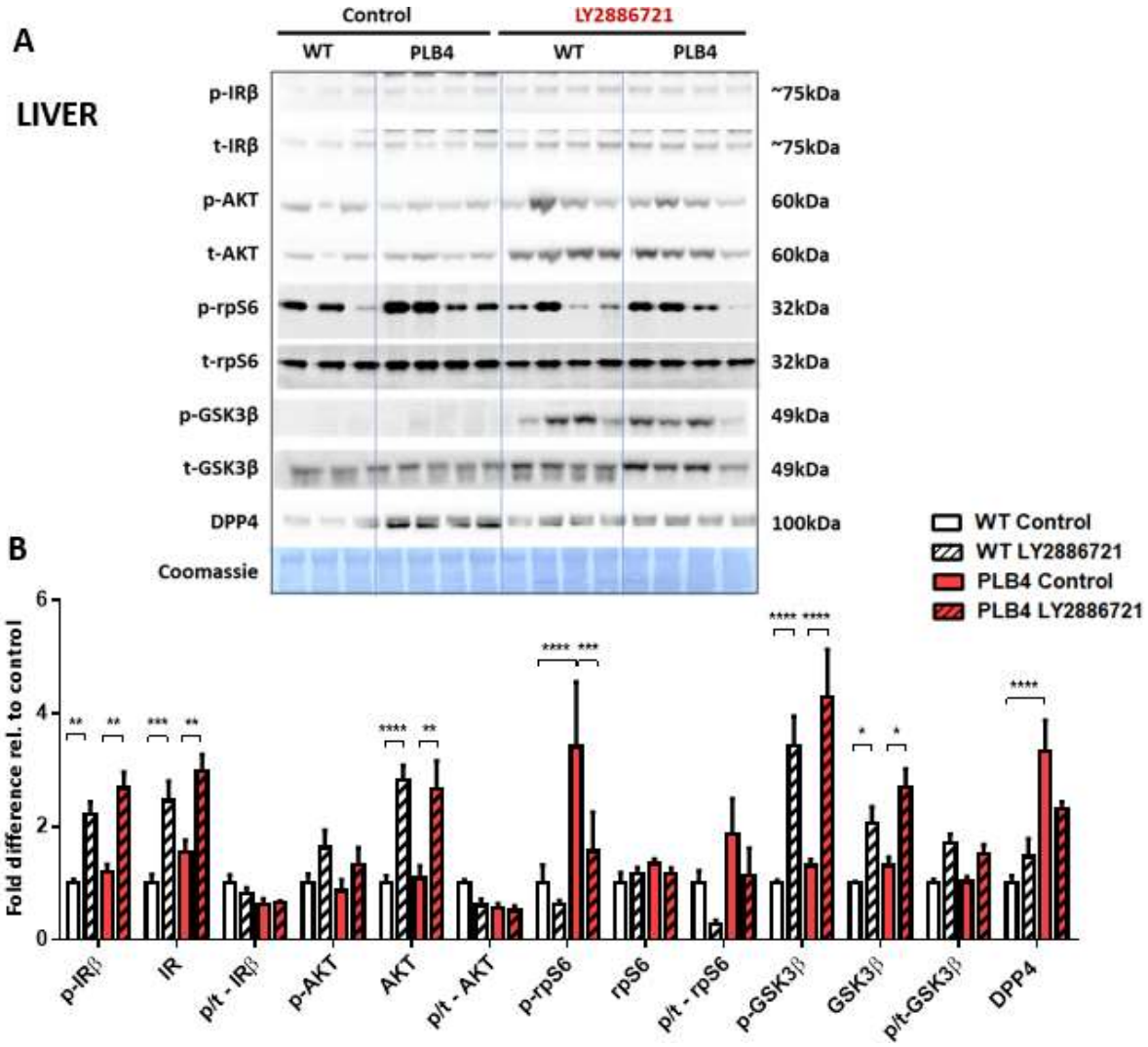

Figure 5. LY2886721 effects on liver protein markers

(**A**) Representative immunoblots of hepatic protein expression of WT and PLB4 mice across control and LY2886721 treatment conditions. (**B**) Quantified and normalised to Coomassie staining hepatic protein levels. Number of mice per group: WT control n=7; WT LY2886721 n=7; PLB4 control n=5;

PLB4 LY2886721 n=4. Data expressed as means ± SEM and significant differences determined using two-way ANOVA followed by Bonferroni multiple comparison post-hoc test. Asterisks: *p < 0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.6 Decreased rpS6 levels with LY2886721 treatment in muscle of PLB4 mice

Muscle tissue, another key compartment for metabolic control and implicated in the impaired motor coordination detected in PLB4 mice (see above), was assessed. LY2886721 treatment led to elevated total and phosphorylated insulin receptor (IR) levels in WT mice ($p<0.05$), but the equivalent effect was not obtained in PLB4 LY2886721-treated mice (Fig. 6 A and B). Comparable to liver, PLB4 mice displayed markedly elevated phospho-rPS6 levels (absolute and relative to total) in muscle ($p<0.0001$; (Fig. 6 A and B), which could indicate increased protein translation to potentially compensate for impaired motor function.

The ratio of phospho/total AKT was also elevated in muscle of PLB4 vs WT mice ($p<0.05$; (Fig. 6 A and B). LY2886721 treatment markedly decreased elevated phospho-rpS6 (and the ratio of phospho/total levels) in muscle of PLB4 mice ($p<0.0001$ & $p<0.001$, respectively; (Fig. 6 A and B). In addition, phosphorylated AKT (and ratio of phospho/total) was significantly lower in muscle of LY288671-treated PLB4 animals ($p<0.05$; (Fig. 6 A and B).

Figure 6.

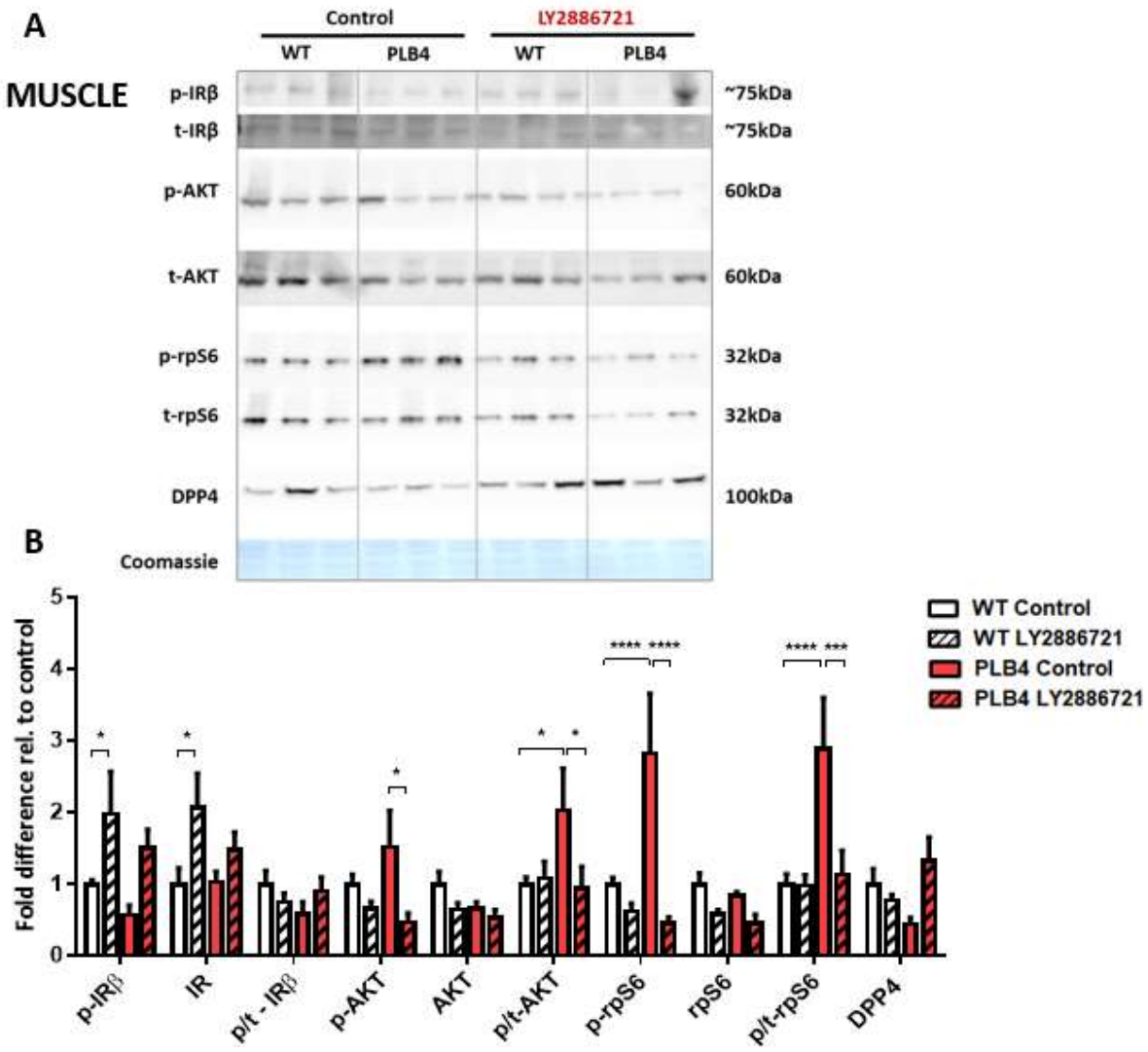

Figure 6. LY2886721 effects on muscle protein markers

(**A**) Representative immunoblots showing protein expression in muscle of WT and PLB4 mice. (**B**) Quantified and normalised to Coomassie staining protein levels in muscle. Number of mice per group: WT control n=7; WT LY2886721 n=7; PLB4 control n=5; PLB4 LY2886721 n=5. Data expressed as means ± SEM and significant differences determined using two-way ANOVA followed by Bonferroni multiple comparison post-hoc test. Asterisks: *p < 0.05, ***p<0.001, ****p<0.0001.

4. Discussion

Although evidence exists regarding BACE1 inhibitor efficacy towards AD-linked pathologies *in vitro* and *in vivo* [5,20], very few studies have investigated the effects of BACE1 inhibition on glucose homeostasis and insulin resistance [10,11]. Therefore, the present study assessed if BACE1 inhibition using LY2886721, a potent small-molecule BACE inhibitor, would alleviate T2DM phenotypes alongside APP-related readouts in transgenic PLB4 mice with a relevant co-morbidity phenotype [9,18].

Treatment with LY2886721 was effective in alleviating T2DM-like symptoms in PLB4 mice as indicated by improved glucose homeostasis and hepatic gluconeogenesis, likely attributable to increased hepatic insulin sensitivity. These data are in line with improved glucose clearance as well as improved insulin sensitivity observed in *Bace1* knock out mice [8]. Additionally, BACE1 inhibition partially restored decreased insulin receptor levels in *db/db* mice [12]. We detected increased phosphorylated and total levels of hepatic IRβ, following treatment, alongside changes in downstream mediators of insulin signalling, including increased total AKT, increased phosphorylated and total levels of GSK3β, and decreased DPP4 and phospho-rpS6, suggesting improved hepatic insulin signalling and glycogenesis, and a decreased translational demand in treated PLB4 mice. Furthermore, LY2886721 treatment did not improve motor performance of PLB4 mice on RotaRod yet decreased (up-regulated) phosphorylation of muscular rpS6, implying that impaired motor coordination may be linked to a compensatory enhancement of rpS6-mediated pathways.

Although LY2886721 has much higher potency towards BACE1 inhibition, it may also decrease BACE2 activity [13]. BACE2 has some homology with BACE1 regarding

their active site, but is primarily expressed in peripheral tissues, with low levels detected in the brain [5,21]. Interestingly, inhibition of BACE2 may also offer beneficial effects against diabetes-like impairments: a BACE2 inhibitor (CpdJ) improved glucose homeostasis in *ob/ob* mice, alongside increased β-cell mass and insulin content [22]. Moreover, deficiency of BACE2 improved glucose tolerance and promoted β-cell survival and insulin secretion in a mouse model of T2DM [23]. While the exact role of BACE2 and its substrates remain largely unknown, we suggest that BACE2 inhibition may have contributed to the improvements of diabetic phenotypes in PLB4 mice. Concurrently, an increasing number of BACE1 substrates (other than APP) exist [5], which would also require consideration in future studies.

Even though we did not detect significant alterations in serum insulin and leptin levels in WT or PLB4 mice with LY2886721 treatment, we observed increases in AST and ALT levels in WT mice. No abnormalities in liver function with administration of LY22886721 were found in mice previously [5], nevertheless, it is noteworthy that this study was conducted in 2-3 month old PDAPP and control mice, whereas here mice were ~8 months old, and the hepatic responses in older mice following LY2886721 treatment may well differ. Intriguingly, we only detected treatment-related effects in WT mice with regards to serum AST and ALT levels, implying that the liver of PLB4s responded differently to BACE1 inhibition. This could be due to disturbed glucose homeostasis and insulin signalling as well as compensatory physiological adjustments in PLB4 mice, in line with previous results suggesting altered responsiveness of PLB4 mice in a number of signalling pathways, affecting for example drug actions and responses to altered diets [11, 24] It is also possible that neuronal BACE1 is directly involved in regulating liver function, potentially through the hypothalamic/liver axis. Our data imply that inhibition of BACE1 activity may impair liver function under

physiological conditions, but responses may differ under pathological conditions such as elevated neuronal BACE1 levels.

With regards to APP and its metabolites, it is noteworthy that flAPP and monomeric Aβ levels were higher in PLB4 vs WT mice, while sAPPα showed the opposite trend, confirming differences in APP processing. Discrepancies with our original report [18], suggesting somewhat lower flAPP levels in PLB4 mice, are likely due to either different antibodies with differing affinity for mouse APP and its metabolites used, as well as potential cohort differences or compensatory drifts.

Importantly, a significant decrease in brain levels of Aβ 1-x and Aβ 1-40 was found in LY2886721 treated WT mice using ELISAs, confirming drug action, yet no significant differences were detected in PLB4 mice with treatment, who displayed elevated levels of Aβ 1-x *per se*. This may suggest that inhibition of BACE1 with LY2886721 was not as effective in lowering Aβ on the background of increased neuronal BACE1 expression and a larger pool of flAPP cf. WT mice. However, we found favourable treatment effects (based on Western Blotting) on amyloid processing, as LY288671 reduced elevated monomeric Aβ levels to WT levels, and increased sAPPα production in PLB4 mice. This is in line with studies suggesting that monomeric Aβ may play a role in sustaining AD pathogenesis [25], and other data indicating that BACE1 inhibition decreased toxic sAPPβ and increased non-amyloidogenic sAPPα levels in both BACE1 transfected neurons and WT mice [26]. Indeed, a debate on the details of BACE1 inhibition required for preventing Aβ production in AD is still ongoing. It is difficult to estimate how BACE1 inhibition translates to Aβ reduction in the brain, since this depends on multiple factors such as age, sex, the stage of the disease and extent of Aβ pathology as well as interaction of BACE1/Aβ with other molecules [27]. Based on evidence from heterozygous Bace1$^{+/-}$ mice, it was hypothesized that ~50% BACE1

inhibition would lead to ~20% decrease in brain Aβ [28,29]. Consequently, a better understanding of the precise pharmacokinetics of effective BACE1 inhibition to abolish Aβ toxicity, the contribution of other BACE1 substrates, as well as signalling pathways affected in other tissues would seem essential to avoid failing clinical trials with BACE1 inhibitors [30]. Nevertheless, BACE1 remains a target of interest in AD, and considering our current evidence, could also be a target for (co-morbid) T2DM.

In summary, our findings provide further support for the role of BACE1 in systemic glucose regulation and suggest that BACE1 inhibitors could also be useful for diabetes -related disorders and co-morbidities.

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Authors' contributions

BP, MD and RD designed the study; BP and MD provided the financial support. RD carried out all the experimental work *in vivo*, with help and assistance from CH, SKS and OH. ZF performed liver Western Blot experiments with aid from PAH and analysed the data; ZF and LC performed muscle immunoblotting and analysed the data. RD performed the serum assays and SKS aided with this. CH performed immunoblotting experiments of Aβ and APP in the brain and analysed the data; ZY performed Aβ (1-x and 1-40) ELISA determination. GR provided advice and equipment for RotaRod experiments. RD and ZF wrote the initial manuscript; BP, MD and GR critically revised and edited the manuscript.

Competing Interests

Zhixiang Yang is an employee of Eli Lilly. The authors declare no other competing interests.

Table 1. Antibodies for immunoblotting analysis

Primary Antibody	Epitope	Dilution	Host species	Supplier
6E10 β-Amyloid	1-16 β-amyloid	1:500	Mouse	Biolegend
β-Amyloid	Soluble APPα	1:500	Mouse	Biolegend
phopho-IR/IGF1R	Tyr1162/Tyr1163	1:500	Rabbit	ThermoFisher
total-IRβ	C-19	1:500	Rabbit	Santa Cruz
phospho-Akt	Ser 473	1:1000	Rabbit	Cell Signalling
total-Akt	Akt1/2/3	1:1000	Rabbit	Cell Signalling
phospho-rpS6	Ser 235-236	1:1000	Rabbit	Cell Signalling
total-rpS6	5G10	1:1000	Rabbit	Cell Signalling
phospho-GSK3β	Ser 9	1:1000	Rabbit	Cell signalling
total-GSK3β	Total GSK-3β protein	1:1000	Rabbit	Cell Signalling
DPP4	n/a	1:1000	Rabbit	Abcam
Secondary Antibody				
mouse anti-rabbit	n/a	1:2500		Merck Millipore
goat anti-mouse	n/a	1:2500		Merck Millipore

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