JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.104.081174

Title: Quantitative measurement of changes in Aβ40 in the rat brain and CSF following treatment with the γ-secretase inhibitor, *N*2-[(2*S*)-2-(3,5-Difluorophenyl)-2-hydroxyethanoyl]-*N*1-[(7*S*)-5-methyl-6-oxo-6,7-dihydro-5*H*dibenzo[*b*,*d*]azepin-7-yl]-L-alaninamide (LY-411575).

Jonathan D. Best[1]*†, Mark T. Jay[1]*, Franklin Otu[1], Jerome Ma[1], Alan Nadin[2], Semantha Ellis[3], Huw D. Lewis[3], Christine Pattison[3], Michael Reilly[3], Timothy Harrison[2], Mark S. Shearman[4], Toni L. Williamson[1], John R. Atack[1]

Departments of In Vivo Neuroscience[1], Medicinal Chemistry[2], Drug Metabolism and Pharmacokinetics[3] and Molecular and Cellular Neuroscience[4], The Neuroscience Research Centre, Merck Sharp and Dohme Research Laboratories, Terlings Park, Harlow, Essex, CM20 2QR

* These authors contributed equally to this publication.

†- corresponding author.

Running title: Gamma secretase inhibition in the rat.

Corresponding author:

Jonathan Best

Address: Department of In Vivo Neuroscience,

The Neuroscience Research Centre,

Merck, Sharp & Dohme Research Laboratories,

Terlings Park,

Harlow,

Essex, CM20 2QR, UK

Tel: 44-1279 440 383

Fax: 44-1279 440 733

Email: jonathan_best@merck.com

Number of text pages: 23

Number of tables: 2

Number of figures: 4

Number of references: 40

Number of words in Abstract: 241

Number of words in Introduction: 748

Number of words in Discussion: 1500

Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer's disease; APP, Amyloid Precursor Protein; APP-KO, Amyloid Precursor Protein- Knockout; FAD, Familial Alzheimer's Disease; CNS, Central Nervous system; CSF, cerebrospinal fluid; GnHCl, Guanidine hydrochloride.

Section: Neuropharmacology

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Abstract

The efficacy of γ-secretase inhibitors *in vivo* has to date been generally assessed in transgenic mouse models expressing increased levels of Aβ peptide thereby allowing the detection of changes in Aβ production. However, it is not clear whether the *in vivo* potency of γ-secretase inhibitors is independent of the level of APP expression: in other words does a γ-secretase inhibitor have the same effect in non-transgenic physiological animals versus transgenic over-expressing animals? In the present study, an immunoassay has been developed which can detect Aβ(40) in the rat brain, where concentrations are much lower than those seen in transgenic mice such as the Tg2576 (c.0.7 nM and c. 25 nM respectively) and in cerebrospinal fluid (CSF-c.0.3nM). Using this immunoassay, the effects of the γ-secretase inhibitor LY-411575 were assessed and robust dose-dependent reductions in rat brain and CSF Aβ(40) levels were observed with ID_{50} values of 1.3 mg/kg for both brain and CSF. These values were comparable to those calculated for LY-411575 in transgenic mice. Time course experiments using LY-411575 demonstrated comparable temporal reductions in rat brain and CSF Aβ(40) further suggesting these two pools of Aβ are related. Accordingly, when all the data for the dose response curve and time course were correlated, a strong association was observed between the brain and CSF Aβ(40) levels. These data demonstrate the utility of the rat as a novel approach for assessing the effects of γ-secretase inhibitors on CNS Aβ(40) levels *in vivo*.

Introduction

Alzheimer's disease (AD) is one the major neurological diseases of the elderly, characterised histopathologically by protein deposits in the brain parenchyma (plaques) or blood vessels, and intracellular neurofibrillary tangles of abnormally phosphorylated tau protein. The plaques mainly consist of the Aβ peptides originating from cleavage of the amyloid precursor protein (APP), with the majority being the more hydrophobic Aβ(42) which is particularly prone to aggregation (Selkoe 2001). Furthermore, the identification of APP gene mutations in familial Alzheimer's Disease (FAD) cases gives evidence of the involvement of APP processing in AD (Goate et al., 1991; Hardy 1997).

The enzymes responsible for processing APP into Aβ are the aspartyl proteases, β-amyloid cleaving enzyme (BACE or β-secretase) and γ-secretase (Selkoe and Schenk 2003). Although an attractive target for drug discovery, inhibition of β-secretase has proved challenging in terms of identification of small molecules for therapeutic use (Middendorp et al., 2004; Selkoe and Schenk 2003). As a result, considerable effort has centred on the inhibition of γ-secretase, an enzyme complex composed of at least four different protein subunits; presenilin (the putative aspartyl protease), nicastrin, APH-1 and PEN-2. γ-secretase is responsible for the intra-membrane proteolytic cleavage of the C-terminal fragment of APP resulting in mainly Aβ(40) or Aβ(42) production (reviewed in Haas 2004; Selkoe and Schenk 2003).

There have been a number of reports describing the inhibition of γ-secretase *in vitro*, but relatively few describing inhibition *in vivo* (for review: Harrison et al., 2004). Recently, bioavailable, brain penetrant γ-secretase inhibitors have permitted the characterization of the reduction of Aβ *in vivo* (Dovey et al., 2001; Lanz et al., 2003, 2004; Wong et al., 2004). These studies used strains of transgenic mice over-expressing the 695 amino acid isoform of the human APP sequence containing FAD mutations (PDAPP- Games et al., 1995; Tg2576- Hsiao et al.,

1996; TgCRND8- Chishti et al., 2001), to demonstrate Aβ lowering effects with two different γ-secretase inhibitors, DAPT and LY-411575. DAPT, a dipeptide, gave dose dependent decreases of Aβ in the brain, CSF and plasma of PDAPP and Tg2576 mice (Dovey et al., 2001; Lanz et al., 2003). More recently, evaluation of the potent lactam LY-411575 demonstrated a dose dependent effect in TgCRND8 and Tg2576 mice (Wong et al., 2004; Lanz et al., 2004).

These FAD mutations result in increases in Aβ(42) production and lead to the development of a number of neuropathological hallmarks of AD at various time intervals after birth. Clinically, FAD is only a small percentage of cases, the majority being sporadic AD. Although important as models of amyloidosis, the effect of artificially high Aβ levels on the animal's physiology, is unclear at present.

Recently, a study using the inhibitor DAPT, revealed less of a reduction in Aβ in guinea pig compared to two strains of transgenic mouse suggesting non-transgenic animals may respond differently to γ-secretase inhibitors than transgenic mice (Harrison et al. 2004). Therefore, as well as assessing efficacy of γ-secretase inhibitors in these transgenic mice, it is important to determine whether these inhibitors are as effective in other non-transgenic rodents such as rats. In addition, rats are used preclinically as a toxicology safety species. With concerns raised about pharmacological toxicity resulting from inhibition of γ-secretase (Searfoss et al., 2003), being able to determine toxicity and CNS efficacy in the same animal is critical to enable determination of therapeutic windows *in vivo*.

Numerous studies have demonstrated that transport Aβ in and out of the brain is regulated by different mechanisms such as low-density lipoprotein receptor-related protein (LRP) and Receptor for Advanced Glycation End-products (RAGE) (Reviewed in Tanzi et al., 2004). Determining *in vivo* changes in CNS Aβ production resulting from γ-secretase inhibition will aid understanding of these dynamics in a physiological system.

Although a previous study used immunoprecipitation and western blotting to ascertain changes in rat brain and CSF Aβ after administration of IGF-1 (Carro et al., 2002), this method is limited as it is only semi-quantitative. Using an immunoassay-based method, quantitative changes in Aβ(40) levels can be determined with better resolution. Here, we describe a method for the reliable quantitative detection of Aβ(40) in rat brain and CSF, and demonstrate robust reductions of Aβ(40) in rat brain and CSF using the γ-secretase inhibitor LY-411575, demonstrating its validity as a physiological model to test Aβ lowering compounds. Moreover, the correlation of rat brain and CSF Aβ(40) concentrations reinforces the concept that these two pools of Aβ peptide are in dynamic equilibrium, with changes in rat CSF Aβ(40) reflecting changes in brain Aβ(40) concentrations.

Materials and Methods

Chemistry

LY-411575 (*N*2-[(2*S*)-2-(3,5-Difluorophenyl)-2-hydroxyethanoyl]-*N*1-[(7*S*)-5-methyl-6-oxo-6,7-dihydro-5*H*dibenzo[*b*,*d*]azepin-7-yl]-L-alaninamide) was prepared in-house by methods as described elsewhere (Wu et al., 1998; Audia et al., 2000), with the exception that the 5-methyl-5,7-dihydro-6H-dibenzo[b,d]azepin-6-one moiety was prepared by Pd(0)-catalyzed cross-coupling of 2-aminophenylboronic acid with 2-bromophenylacetonitrile, followed by base-mediated hydrolysis, cyclization and methylation (35% yield, 3 steps) (Baudoin et al., 2002).

Animals and Dosing

All procedures were conducted, in accordance with the Animals (Scientific Procedures) Act of 1986 and its associated guidelines. Four to six month old Tg2576 transgenic mice (male and female) over-expressing human APP harbouring the Swedish mutation (K670N, M671L, Hsiao et al., 1996) were bred in-house whereas male Sprague Dawley rats (250-300g) were obtained from Charles River. All animals were maintained on a 12:12 light:dark cycle with unrestricted access to food and water until use. Rats were dosed orally with a suspension in 0.5% methylcellulose at 1ml/kg.

Pharmacokinetic measurement of LY-411575

Six male rats, weighing approximately 350 g, were deprived of food overnight. Carotid arteries were cannulated under anaesthesia (isofluroane), and each rat given 100 unit dose of heparin (0.1 ml, 1000 units/ml) via the cannula. Each cannulated rat was connected to an AccuSampler, an automatic blood sampler (Dilab, Lund, Sweden) and the rats were allowed to recover for a least 36 hours. LY-411575 was administered at 1 mg/kg intravenously to three animals via a bolus injection (1 ml/kg) in PEG300:water 3:1 solution. LY-411575 was also administered at 1 mg/kg

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orally to the remaining 3 animals (5 ml/kg) in 0.5% methylcellulose in water as an amorphous suspension. Serial blood samples were collected using the AccuSampler at specified time points upto 24 hours after dosing. Plasma was separated by centrifugation and the samples frozen at -80°C prior to analysis by tandem mass spectrometry.

LY-411575 analysis

Plasma samples were extracted by protein precipitation using an automated method on a Beckman Biomek 2000 liquid handling robot. The resultant samples were then analysed using an Agilent 1100 HPLC and CTC autosampler interfaced to a Sciex API4000 triple quadruple mass spectrometer (Applied Biosystems, Cheshire). Chromatography was performed on a Kromasil KR100 5C18 column, 150 x 3.2 mm i.d. (Hichrom, Theale) with a mobile phase consisting of 60% acetonitrile in 25 mM ammonium formate buffer at pH3, and a flow rate of 500 µl/min. Injections of 50 µl were made with detection of LY-411575 by MRM monitoring of transition 480-239.

The terminal phase rate constant (k_{el}) was determined by linear regression of the natural log plasma concentration-time profile. The terminal elimination half-life ($t_{1/2}$) was calculated from $0.693/k_{el}$. Area under the plasma concentration-time curve (AUC_{0-T}) was calculated using the linear trapezoid rule and extrapolated to infinity ($AUC_{T-\infty}$) using k_{el}. The mean residence time (MRT) was calculated as the ratio AUMC/AUC following an intravenous dose, where AUMC is the area under the first moment curve from $0 - \infty$. Systemic clearance was calculated from intravenous data using dose/$AUC_{0-\infty}$. Volume of distribution was calculated from MRT multiplied by systemic clearance. Oral bioavailability was calculated from the ratio of $AUC_{0-\infty}$ values after oral and intravenous doses.

Tissue sample preparation

Tg2576 mice were euthanased by stunning followed by decapitation. Rats were anaesthetised using isofluorane and CSF removed by puncturing the cisterna magna with a 21G butterfly cannula and then euthanased by decapitation. Brains were removed and along with the CSF, immediately frozen on dry ice and stored at –80°C until use. CSF samples with visible blood contaminants were discarded.

The frozen brains were homogenised in 10 volumes (w:v) of 0.2% Diethylamine (DEA) containing 50 mM NaCl (pH 10) and protease inhibitors (Roche, Mannheim, Germany) (Savage et al., 1998), then centrifuged at 355,000 *g*, 4°C, for 30 min (Beckman Coulter Ultra Centrifuge, Optima Max). The resulting supernatant was retained as the soluble fraction and neutralised by addition of 10% 0.5M TrisHCl, pH 6.8. Samples were frozen at -80^{o}C awaiting analysis.

Prior to analysis the CSF was thawed, centrifuged at 2300 *g* and the supernatant was diluted 1:4 with PBS, 2% Bovine Serum Albumin (BSA), 0.5% Tween-20 (Sigma, Poole, UK) plus protease inhibitors.

Measurement of Aβ(40)

In this study, monoclonal antibodies G2-10 (Ida et al., 1996) are used with biotinylated antibody 4G8 (Signet Laboratories PLC, Dedham, MA., Kim et al., 1988) to detect Aβ peptides in solution ending at residue 40, with negligible cross-reactivity. These species, referred to as Aβ(40), reflect subpopulations of peptides with heterogeneous N-termini encompassing at least the 4G8 epitope at residues 17-24.

Analysis of the samples was performed using the Meso Scale Discovery (MSD) Sector Imager 6000 (Gaithersburg, MD). The MSD methodology is essentially an immunoassay which utilises electrochemiluminesce (ECL) to measure protein levels. The Aβ(40) (California Peptide Research, Inc., Napa, CA) standards were generated from a 100μM stock in DMSO diluted into

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BSA buffer (2% BSA, 0.5% Tween in PBS-Sigma, plus protease inhibitors-Roche). Twenty-five microlitres of 4G8–biotinylated antibody at 4µg/ml (capture antibody-Signet) were added to avidin coated multi-array 96-well plate and incubated on a plate shaker (Heidolph titramax 100) at 600rpm overnight at 4ºC. The plates were then washed three times with 200µl PBS and 100µl of blocking Buffer Solution (100ml H_20, 6g Blocker A, 20ml Blocking Buffer A) added to all wells. The plates were then sealed, wrapped in tin foil and incubated at room temperature on a plate shaker (600rpm) for 1h. At the end of the incubation the wells were washed three times with 200µl PBS. Twenty-five microlitres of the standards or samples were then added to the wells followed by G210 antibody at 1µg/ml (licensed from the University of Heidelberg, Germany) labelled with a Ruthenium (II) tris-bipyridine NHS ester which when in close proximity to the bottom of the well emits light following electrical stimulation of the plate. The plates were then aspirated and washed 3 times with PBS. Finally MSD-S read buffer was added to the plates and they were read on the Sector Imager 6000. To correct for non-specific effects of the brain assay, brains taken from transgenic mice deficient in APP (Zheng et al., 1996- APP-KO) were also processed similarly to the experimental groups and extracts were included on each plate (Supplied by Taconic Farms USA). The counts from these samples (non-specific) were subtracted from all samples and standards to give specific counts.

Statistical Analysis.

Standard curves generated from known concentrations of synthetic Aβ(40) were logged to remove bias from high concentration counts and values for brain and CSF Aβ(40) interpolated using y=mx+c. Groups were analysed using two-way and one-way ANOVA and where appropriate *post-hoc* Dunnett's t-test with vehicle/control group, or Bonferroni's t-test between experimental groups applied (Prism 3.00, GraphPad Software Inc., CA).

Results

Assay Characterisation

Concentrations of Aβ(40) in CSF and DEA extracted brain tissue for vehicle treated animals were typically in the upper mid range of the standards in both the logged and linear graphs (Figure 1a and insert) with the limit of detection usually being about 1-3 pM. The signal to noise ratio of the vehicle samples with respect to APP-KO samples was usually about 6 (figure 1b). The intra-assay variability of the immunoassay was typically about 5% whereas inter-assay variability was approximately 15%, both were assessed using control tissue from naïve rats.

Comparison of Aβ (40) levels between the rat and transgenic mice

The concentrations of Aβ(40) measured in CSF and DEA-extracted rat brain was approximately 0.3 nM and 0.7 nM respectively (Table 1). The value for brain Aβ(40) in the rat is about 35 times lower than in-house data generated for brain Aβ(40) levels in 3-5 month old Tg2576 mice using the same extraction and immunoassay protocol (Table 1). The ratio of brain Aβ(40) to CSF Aβ(40) in the rat was about 2.3. Overall, despite much lower levels of Aβ in the rat compared to transgenic mice, using the immunoassay based technology we were able to consistently measure both brain and CSF Aβ(40).

Pharmacokinetic parameters of LY-411575 in the rat

To determine whether LY-411575 had comparable pharmacokinetic in rats to the published Tg2576 mouse data (Lanz et al., 2004), 1mg/kg was dosed to male rats. Plasma C_{max} was 11nM $\pm$ 6nM, T_{max} was about 30 minutes and the $t_{1/2}$ was calculated to be about 2 hours. The plasma concentration from a 10mg/kg dose at 4h was 633nM$\pm$ 81nM, whereas the brain concentration was 117nM$\pm$ 22nM (mean $\pm$ S.E.M).

Dose related reduction of rat brain and CSF Aβ(40) by LY-411575

To determine whether the levels of Aβ(40) in the rat brain and CSF could be reduced by inhibition of γ-secretase *in vivo*, a dose response experiment was performed in male Sprague Dawley rats using the potent γ-secretase inhibitor LY-411575 (Figure 2).

Robust dose-dependent reductions in Aβ(40) levels were demonstrated in both the brain and CSF with increasing dose. Complete reduction was obtained in the brain at 10 and 30 mg/kg (i.e. the assay signal was reduced to background levels as defined by the signal obtained in APP-KO mouse brain tissue). The levels of Aβ(40) in the CSF were not completely reduced to zero (APP-KO CSF was not available to account for non-specific signal), although the reductions did not significantly increase between 10mg/kg and 30mg/kg.

To determine whether the reductions in CSF Aβ(40) tracked the reductions in brain Aβ(40) levels, ID_{50} values were calculated using the dose response data (figure 2a & b). The ID_{50} for the rat was 1.3 mg/kg for both brain and CSF (table 2).

Time course data

To determine if the reductions in brain and CSF Aβ(40) were maintained with time, two separate time course experiments using 3 and 10 mg/kg doses of LY-411575 were conducted. At both 3 and 10 mg/kg the reductions in the CSF Aβ(40) levels at 4 hours were comparable with those seen in the dose response curve ($p>0.05$, figure 3 vs. 2). Both doses of LY-411575 had significant effects on Aβ(40) levels in the brain (figure 3a & b: 3 mg/kg- $F= 47.96$, $p<0.0001$; 10mg/kg- $F=42.06$, $p<0.0001$) and CSF (figure 3a & b: 3 mg/kg- $F= 18.75$, $p<0.0001$; 10mg/kg- $F=41.28$, $p<0.0001$).

In the 3 mg/kg time course, the maximum reduction of Aβ(40) by LY-411575 was seen by 8 hours in both CSF and brain ($p<0.001$ compared to vehicle levels). The levels of both brain and CSF Aβ(40) had begun to rise at 16h and had returned to vehicle levels by 24h post dose. When normalised to account for the difference in levels, analysis by 2 way ANOVA (figure 3) revealed an effect of time on the Aβ(40) levels ($p<0.0001$) and tissue (CSF vs brain, $p<0.0001$) with a significant interaction between them ($p<0.001$). This was borne out in the post-hoc analysis as there was a significant difference between brain and CSF levels at 1 and 2 hours ($t=5.11$, $p<0.001$ and $t=3.56$, $p<0.01$ respectively) suggesting a time lag between the brain reductions and CSF. However from 4h onwards there was no difference in the reductions between brain and CSF Aβ(40).

With the 10 mg/kg time course, maximum reductions of Aβ(40) levels by LY-411575 were seen by 4 hours in both CSF and brain. These reductions did not significantly change in either the CSF or brain up to 24 hours post dose (figure 3 b).

Correlation between brain and CSF Aβ(40) levels.

To determine if the reduction of Aβ(40) in the CSF paralleled the brain Aβ(40) reduction, the values for brain and CSF from the dose response and time course experiments were correlated using linear regression analysis (Figure 4). When all the values were included the data demonstrated a good correlation ($F=226.5$, $p<0.0001$) and good fit of the data to the line ($r^2= 0.69$). However, if the 1 and 2 hour points from the 3mg/kg time course were excluded (open circles-due to there being a time lag) there was a better fit of the data ($r^2= 0.79$).

Discussion

Evidence from clinical studies (Naslund et al., 2000; Wang et al., 1999), *in vitro* experiments (Lambert et al., 1998; Hartley et al., 1999; Chromy et al., 2003), and *in vivo* experiments (Shin et al., 1997; Hsia et al., 1999) suggest that the initial pathogenesis of AD is due to the build up of neurotoxic aggregates of the soluble Aβ peptide species Aβ(1-40) and Aβ(1-42). As a result, a number of therapeutic approaches for lowering amyloid are in progress, one of which is the use of γ-secretase inhibitors (Hardy and Selkoe 2002; Harrison et al., 2004). With the advent of orally available γ-secretase inhibitors, studies in transgenic mice using these inhibitors have demonstrated reductions of Aβ levels in the brain, CSF and plasma (Dovey et al., 2001; Lanz et al., 2003, 2004; Wong et al., 2004). However, these models have high levels of Aβ not representative of normal physiological rodent levels.

A previous study used immunoprecipitation and western blotting to determine the effect of IGF on changes of Aβ in the brain and CSF of rats, though the semi-quantitative nature of this assay limits this technique (Carro et al., 2002). In the present study, we have established an immunoassay used to determine whether reproducible reductions in Aβ levels could be demonstrated in the rat in response to administration of the γ-secretase inhibitor LY-411575 in a comparable way to that seen in transgenic mice (Lanz et al., 2004; Wong et al., 2004).

A number of studies have investigated the concentrations of Aβ peptide obtained from brain following different extraction methods in transgenic mice and human post-mortem tissue (Lewis et al., 2004; Kawarabayashi et al., 2001; Wang et al., 1999). The study here examined Aβ extracted from rat brain using DEA. In normal aging human brains, sequential extraction using Tris buffer saline, RIPA buffer and formic acid extracted a total of about 1nM of Aβ(40) (Wang et al., 1999), levels similar to those extracted from rat brain using DEA (see table 1). The levels of Aβ extracted change as both Tg2576 mice and humans begin to develop plaques, with the amount

of total Aβ extracted by formic acid increasing with plaque burden (Kawarabayashi et al., 2001; Wang et al., 1999). The difficulty of comparing absolute levels across studies due to different extraction and antibody methods has been discussed previously (Lewis et al. 2004). However, taking these caveats into account we can conclude that the levels seen in the rat are significantly lower than those reported for transgenic mice ranging from 35 to 600 times in the brain and about 15 to 40 times in CSF (see Table 1). Interestingly, despite these differences, the ratio of brain to CSF Aβ(40) in the rat was about 2.3, similar to the 1.9 seen in the Tg2576 mouse. Thus, it is possible using this immunoassay system to quantifiably compare effects of γ-secretase inhibitors in the rat with different species both non-transgenic and transgenic.

LY-411575 caused a dose-dependent reduction of Aβ(40) in the brains of TgCRND8 and Tg2576 mice, with a maximal effect between 3-6 hours (Wong et al., 2004; Lanz et al., 2004). A dose response study conducted with LY-411575 at 4 hours in the rat demonstrated a dose-dependent reduction of brain Aβ(40) with 30 mg/kg causing complete reduction.

The pharmacokinetic profile of LY-411575 in the rat was very similar to the transgenic mouse with a 1mg/kg oral dose, despite different dosing vehicles. The 10mg/kg dose gave slightly higher plasma and brain drug concentrations at 4 hour in the rat compared to 10mg/kg at 3 hour in the Tg2576 mouse (Lanz et al., 2004) suggesting better bioavailability of LY-411575 in the rat compared to the mouse. Therefore, changes in the pharmacodynamics of Aβ between the mouse and rat are unlikely to be related to the pharmacokinetic profile of the compound.

Previous studies have demonstrated that changes in CSF Aβ correlated with changes in brain Aβ in the transgenic mouse (Lanz et al., 2004) and disease state in humans (Maruyama et al., 2001; Mehta et al., 2000). The rat has the added advantage of having easily accessible CSF for collection and measurement of Aβ involving relatively simple sampling techniques compared to the mouse (DeMattos et al., 2002). The dose response data for LY-411575 demonstrated that a

good correlation between CSF and brain Aβ(40) also holds true in the rat. To further characterise the dynamics of this relationship, time course experiments using a high dose (10mg/kg) and low dose (3mg/kg) of LY-411575 were performed. At 10mg/kg, both the CSF and brain were maximally reduced by 4h, in keeping with data seen in the transgenic mice (Wong et al., 2004). Interestingly, at the lower dose there appeared to be a slight lag between the reduction of Aβ(40) in the brain and CSF at the early time points, though after this time point the levels in CSF and brain followed each other very closely. Since no lag is seen in the higher dose, whether this is truly significant needs to be investigated further.

These results appear to contrast with the studies in the Tg2576 mouse, where 1 mg/kg was dosed to Tg2576 and brain and CSF Aβ(40) taken at 3, 9 and 24 hours (Lanz et al., 2004). The authors observed that both the CSF and brain Aβ(40) were reduced similarly at 3 hours and then the CSF appeared to recover more quickly than brain Aβ(40) by 9 hours with CSF Aβ(40) recovering fully and brain Aβ(40) partially recovered by 24 hours. The lack of time points earlier than 3 hours make comparisons between the studies difficult and if the rat 3 mg/kg data were replotted without the 1 and 2 hours points then the reductions in the brain and CSF would parallel each other without an apparent lag. The recovery noted in the Tg2576 mice at 9 hours was not seen in the rat at 8 hours though this is unlikely to be due to the difference in time points. One explanation for the difference could be that the Tg2576 mouse study used GnHCl extraction whereas the present study used DEA.

It has been shown that the DEA extraction contains very little membrane associated C99 or APP compared to GnHCl extraction (Savage et al., 1998), and so may reflect different pools of Aβ (soluble versus deposited/aggregated, as well as possibly extracellular versus intracellular). Thus, the DEA method maybe extracting Aβ from a pool more akin to CSF Aβ, though it would be interesting to investigate whether the pharmacodynamics of Aβ change using different

extraction protocols in the rat. However, using this extraction protocol the strong association seen between the two compartments when these data were correlated reinforces the idea that CSF Aβ(40) can be used as a *bone fide* biomarker for brain Aβ(40) levels for this type of dosing regime.

As well as the relationship between brain and CSF, the mechanism of Aβ transport into and out of the brain via the blood brain barrier (BBB) is of significant interest. Of these RAGE and LRP have been proposed as major candidates in this process (reviewed in Tanzi et al., 2004). It has been demonstrated that removal of Aβ(1-40) from the brain to the plasma is very rapid and appears to be regulated by LRP (Shibata et al., 2000). Furthermore, at a low dose of 0.1mg/kg, the Tg2576 mouse showed an increase in plasma Aβ without effects on brain or CSF Aβ (Lanz et al., 2004). The importance of these findings make the generation of an assay able to measure pM levels of Aβ expected in the rat plasma an important future objective.

It has also been shown that Aβ(1-40) and Aβ(1-42) appear to have differential transport with LRP favouring clearance of Aβ(1-40) (Deane et al., 2004). Again being able to determine the relationship between different species of Aβ especially Aβ(1-42) in the rat as a result of γ-secretase inhibition would be an important in understanding the relationship of different isoforms of Aβ in a physiological model.

In addition to its role in processing APP, γ-secretase is involved in other regulatory functions. One of the best characterised is control of the Notch pathway, which has been implicated in a number of functions, including peripheral organ toxicity (Searfoss et al., 2003; Wong et al., 2004). Since the rat is widely used as a safety species, the ability to measure brain and CSF Aβ(40) in the rat allows quantitative efficacy - toxicity relationships to be derived in a non-transgenic species.

In summary, the current study demonstrates the utility of the rat as an alternative model for investigating the effects of therapeutic Aβ lowering agents. These data also further reinforce observations made in previous studies that CSF Aβ levels are markers of central Aβ reduction (Lanz et al., 2004). Furthermore, the use of the rat as a preclinical model allows an effective means of measurement of efficacy and toxicity to be combined in one animal model thereby reducing the number of animals used and allowing therapeutic windows to be determined before clinical studies begin.

Acknowledgements

We would like to thank Jonathan Rose and Helen Sheppard for expert DMPK support, and Dirk Beher for critical reading of the manuscript.

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JPET Fast Forward. Published on March 2, 2005 as DOI: 10.1124/jpet.104.081174
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Legends for Figures.

Figure 1:

a) Logged standard curve for detecting endogenous Aβ(40) levels in the brain and CSF of the male rat using an immunoassay. ■ represent synthetic Aβ(1-40) peptide standards. ▼represent position with respect to vehicle-treated samples. Insert- linear plotted standard curve demonstrating number of ECL counts attained. b) Bar chart of ECL counts attained for vehicle-treated brain samples versus APP-KO brain samples showing large signal to noise ratio,

Figure 2:

Dose response curves for LY-411575 on Aβ(40) levels in the a) brain and b) CSF of the male rat, 4 hours after a single dose. Columns represent average Aβ(40) levels + S.E.M. Numbers per group are 6. Veh=vehicle.

Figure 3:

Time course of the effects of LY-411575 on Aβ(40) levels in the brain (■ dotted line) and CSF (○ solid line) of the male rat after a dose of a) 3mg/kg and b) 10mg/kg. Animals were given a single dose and then culled at 1, 2, 4, 8, 16 and 24 hours afterwards. Data points represent average Aβ(40) levels normalised to respective vehicle levels $\pm$ S.E.M. Numbers per group are 3-5.

Figure 4:

Correlation between the brain and CSF Aβ(40) levels (nM) in the rat. Points represent Aβ(40) levels from individual animals combined from the dose response and time course of figures 1 and 2. A robust significant correlation was demonstrated between CSF and brain Aβ(40) levels including all data (r^2=0.69, $p<0.001$) and excluding points where a time lag was seen between

CSF and brain Aβ(40) levels (open circles- r^2=0.79, $p<0.001$). Linear regression calculated using Prism 3.03 stats package.

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Tables

Table 1: Comparison of Aβ(40) levels in rat and transgenic mice.

Species/strain	Brain, nM	CSF, nM
Rat	0.7	0.3
Tg2576 mouse[§]	24	na
Tg2576 mouse[$]	25	13
PDAPP mouse[1]	na	4.5
TgCRND8 mouse[2]	460	na

§-generated from in-house data (DEA extracted brain) and $-literature values Lanz et al., (2004) 3-5 month old (GnHCL extracted cortex). 1 -calculated from DeMattos et al., (2002) 3 month old. 2- calculated from Wong et al., (2004) 6 week old (GnHCL extracted cortex). na- not assayed.

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Table 2: Comparison of rat and transgenic mice ID_{50} values

Species/genotype	Brain, mg/kg	CSF, mg/kg
Rat	1.3	1.3
Tg2576 mouse*	2.5	1.5
TgCRND8 mouse§	1.7	na
PDAPP mouse	na	na

**-calculated from Lanz et al., 2004 at 3h. § –calculated from Wong et al., 2004 after 15 days of dosing 6h after the last dose. na- not assayed*

Figure 1

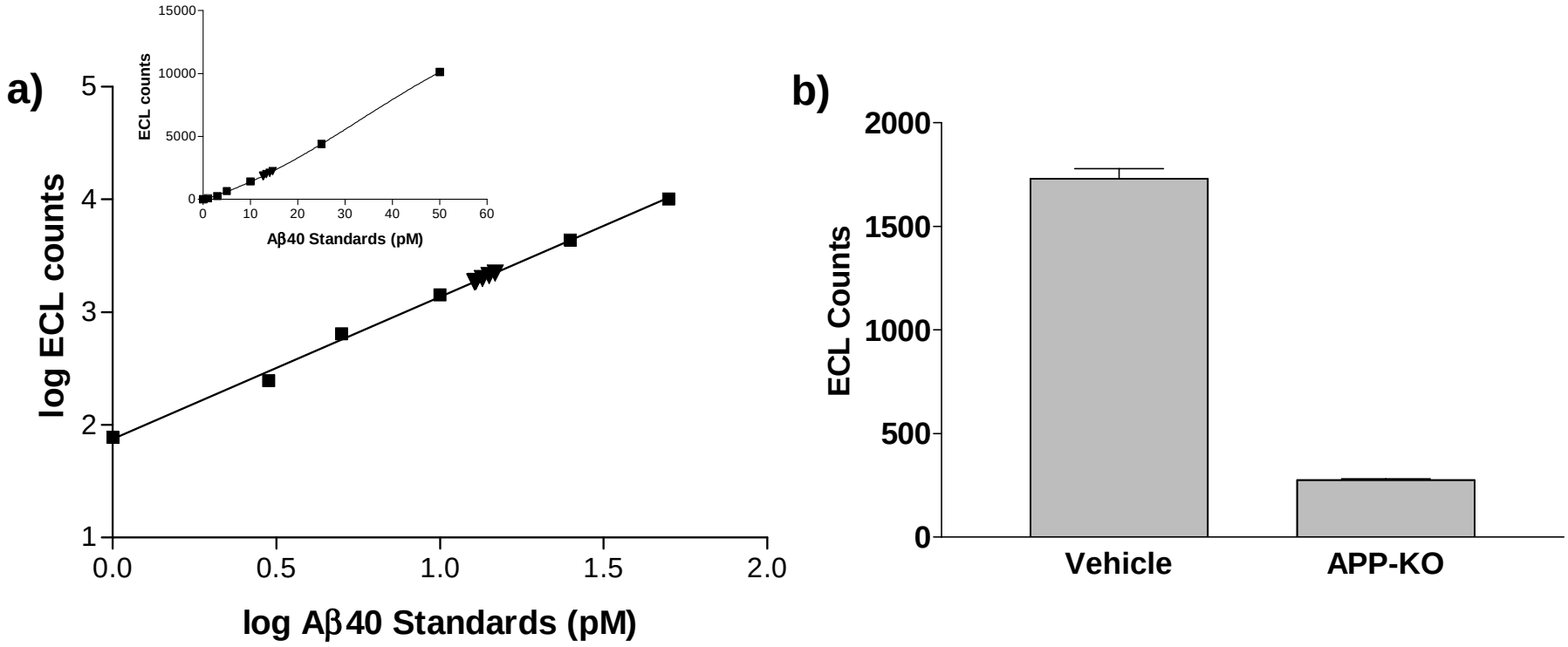

Figure 2

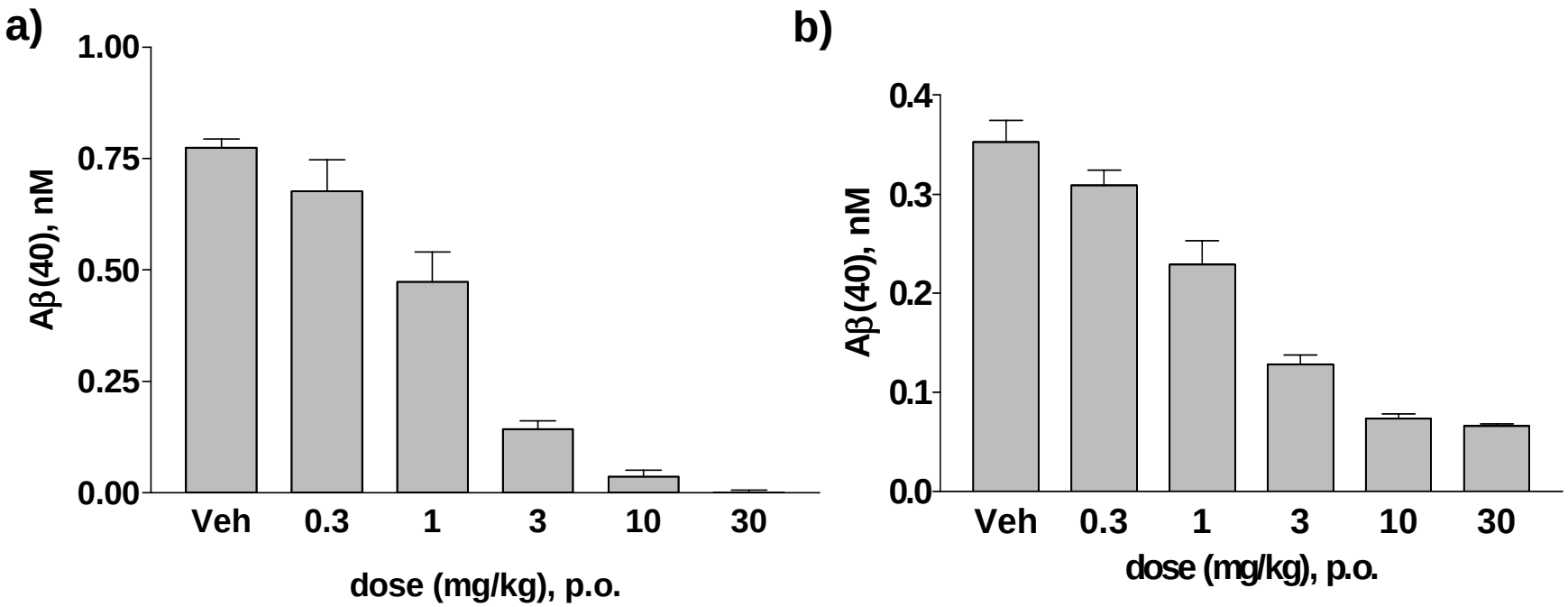

Figure 3

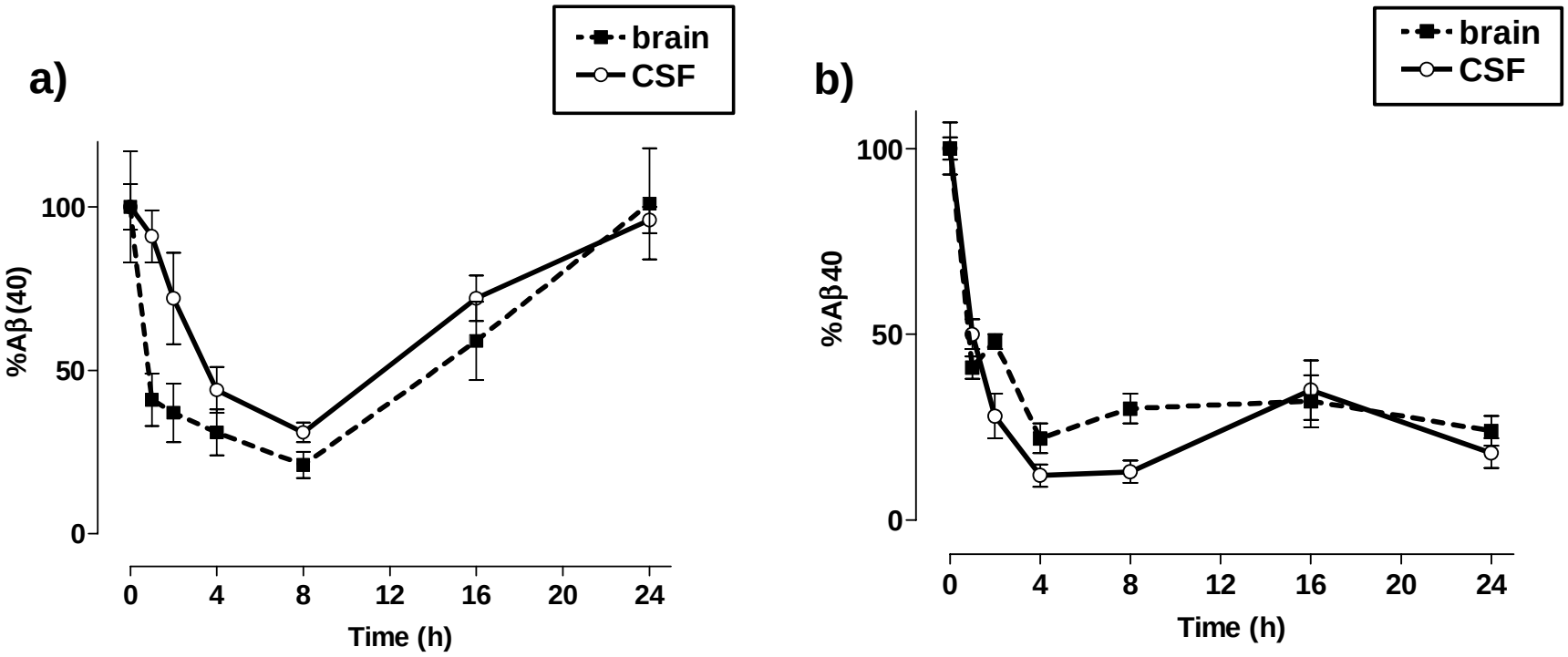

Figure 4