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Decrease in catalytic capacity of γ-secretase can facilitate pathogenesis in sporadic and Familial Alzheimer's disease

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ABSTRACT

Background: Alzheimer's disease can be a result of an age-induced disparity between increase in cellular metabolism of Aβ peptides and decrease in maximal activity of a membrane-embedded protease γ-secretase.
Results: We compared activity of WT γ-secretase with the activity of 6 FAD mutants in its presenilin-1 component and 5 FAD mutants in Aβ-part of its APP substrate (Familial Alzheimer's disease). All 11 FAD mutations show linear correlation between the decrease in maximal activity and the clinically observed age-of-onset and age-of-death. Biphasic-inhibitors showed that a higher ratio between physiological Aβ-production and the maximal activity of γ-secretase can be observed in cells that can facilitate pathogenic changes in Aβ-products. For example, Aβ production in cells with WT γ-secretase is at 11% of its maximal activity, with delta-exon-9 mutant at 26%, while with M139V mutant is at 28% of the maximal activity. In the same conditions, G384A mutant is fully saturated and at its maximal activity. Similarly, Aβ production in cells with γ-secretase complex carrying Aph1A$_L$ component is 12% of its maximal activity, while in cells with Aph1B complex is 26% of its maximal activity. Similar to the cell-based studies, clinical studies of biphasic dose–response in plasma samples of 54 healthy individuals showed variable ratios between physiological Aβ production and the maximal activity of γ-secretase.
Conclusions: The increase in the ratio between physiological Aβ production and maximal activity of γ-secretase can be an early sign of pathogenic processes in enzyme-based, cell-based, and clinical studies of sporadic and Familiar Alzheimer's disease.

1. Introduction

Alzheimer's disease is a slowly progressing fatal neurodegenerative disorder that represents the biggest financial burden for the health care providers in developed countries (Holtzman et al., 2011). Impressive efforts in basic and pharmaceutical research have lead to more than a hundred of different therapeutic approaches. Many of them reached clinical trials, including the phase III (Doody et al., 2013; Sambamurti et al., 2011). Sadly, all of those trials led to disappointments and in some cases surprisingly daunting results (Doody et al., 2013; Tong et al., 2012). Most notably we do not understand to what extent the future efforts can be concentrated on the "amyloid hypothesis" or on some of the alternative therapeutic approaches (Hunter and Brayne, 2014). We also lack reliable early diagnostic methods that can facilitate therapeutic approaches before the onset of irreversible neurodegenerative processes (Hunter and Brayne, 2014; Holtzman et al., 2011).

The studies based on "amyloid hypothesis" have explored different evidences that the pathogenesis can be driven by changes in metabolism of Amyloid precursor protein (APP), in particular its C terminal fragment (β-CTF-APP), and the resulting Aβ peptides (Shen and Kelleher, 2007; Hunter and Brayne, 2014; Sambamurti et al., 2011). Contrary to frequent beliefs, the "amyloid hypothesis" is just a fraction of the total research effort. At the time of writing of this manuscript a Pubmed search for "Alzheimer's" disease gives more than 102,234 entries! Only about 61% of all publications on Alzheimer's disease (60,600 entries), can be retrieved using a search that is focused on the "Alzheimer's AND Aβ OR amyloid". Interestingly, only about 6% of all of the Alzheimer's disease publications, or about 5876 entries, could be retrieved with a search focused on "Alzheimer AND gamma-secretase OR beta-secretase". These numbers indicate that a wide range of possible pathogenic processes have been explored, and the main problem could be lack of insights at the key drug-target enzymes (Svedruzic et al., 2012, 2013; Sambamurti et al., 2011; Shen and Kelleher, 2007). Without adequate insights in the catalytic mechanism of γ-secretase, development of the new drug candidates and the early diagnostic methods will remain an expensive guess-work with a high risk of failure (Doody et al., 2013; Tong et al., 2012; Svedruzic et al., 2013).

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FAD mutations (Familial Alzheimer's disease), offer unique opportunities for analysis of pathogenic changes in γ-secretase activity and Aβ production (Chavez-Gutierrez et al., 2012; Pera et al., 2013; Potter et al., 2013; Shen and Kelleher, 2007; Svedruzic et al., 2012; Jonsson et al., 2012; Seidner et al., 2006; Kumar-Singh et al., 2006; Citron et al., 1992). More than 400 mutations have been identified in the last 24 years (www.molgen.ua.ac.be/ADMutations). The mutations affect all steps in APP metabolism however the majority affects the final steps, i.e., presenilin components of γ-secretase, or Aβ part of APP protein, or apo-lipoprotein ApoE (Hunter et al., 2013; Sambamurti et al., 2011; Shen and Kelleher, 2007). There is also a fascinating protective mutation A673T-APP (Jonsson et al., 2012). Different FAD mutations can trigger disease at different age, which indicates that comparative analysis of the underlining mechanism can provide quantitative insights in the pathogenic processes (Kumar-Singh et al., 2006; Seidner et al., 2006). Unfortunately comparative studies of different FAD mutations and WT γ-secretase are still inconclusive. We do not understand why some FAD mutations can increase and some can decrease Aβ production relative to the healthy WT controls (Pera et al., 2013; Potter et al., 2013; Kumar-Singh et al., 2006; Shen and Kelleher, 2007; Citron et al., 1992; Jonsson et al., 2012), or why some of the FAD mutations can both increase and decrease Aβ production depending on the experimental approach (i.e., "gain-of-function" and "loss-of-function" debate (Potter et al., 2013; Kumar-Singh et al., 2006; Shen and Kelleher, 2007)). Finally we do not understand to what extent changes in γ-secretase activity produced by different FAD mutations can be related to the aging processes that lead to sporadic Alzheimer's disease (Hunter et al., 2013; Sambamurti et al., 2011; Kumar-Singh et al., 2006; Shen and Kelleher, 2007; Fukumoto et al., 2004; Kern and Behl, 2009; Kern et al., 2006).

In this study we provide some answers to the presented questions. Different FAD mutants and WT γ-secretase are compared using activity assays that can measure all three parameters that define the enzyme activity in cells (Ferscht, 1998; Svedruzic et al., 2013), namely: the ongoing physiological activity of γ-secretase, the maximal possible activity, and the extent of γ-secretase saturation with its substrate. Similar approaches have been used successfully to describe activation and inhibition of γ-secretase by different drug-candidates (Burton et al., 2008; Svedruzic et al., 2013), or to describe the changes in enzymatic mechanism of γ-secretase that support pathogenic shift in Aβ products and Aβ42/Aβ40 ratio (Kakuda et al., 2006; Svedruzic et al., 2012; Yin et al., 2007).

2. Results

2.1. Correlation between decrease in maximal activity of γ-secretase and "age-of-onset" and "age-of-death" for different FAD mutations

We find that decrease in γ-secretase activity caused by different FAD mutations shows linear correlation with clinically observed "age-of-onset" or "age-of-death" for each mutation (Fig. 1 and Table 1). The presented data come from our previous enzyme-based studies (Svedruzic et al., 2012), and from subsequent enzyme-based studies by a large research group (Chavez-Gutierrez et al., 2012). We combined data from two different studies to maximize statistical significance of the presented analysis, and to show that the presented correlations are not affected by different experimental approaches. The data from different studies can be normalized to the same scale by always setting the WT measurements as 100% activity, so that the corresponding FAD mutants can be presented as a percentage of the WT activity (Table 1).

Two different types of FAD mutations are included in the analysis, the mutations in presenilin 1 core of γ-secretase, and the mutations in Aβ sequence of its APP substrate (Table 1). The FAD mutations in presenilin 1 are shown as the maximal turnover rates (data taken from Fig. 8A in ref. (Svedruzic et al., 2012) and Table 2 in ref. (Chavez-Gutierrez et al., 2012)). Eleven different experiments, with WT gamma secretase and six different FAD mutations in presenilin 1 show linear correlation between maximal activity and clinically observed age-of-onset or age-of-death (Fig. 1, $R^2 = 0.88$ and 0.86 respectively). The linear correlation spans from the most aggressive FAD mutations to the least aggressive mutations and the WT enzyme. Similar linear correlations can be also observed between 5 FAD mutations in Aβ sequence of APP substrate and the WT substrate (Fig. 1, $R^2 = 0.90$ and 0.94, respectively). However for analysis of FAD mutations in the substrate, the different turnover rates represent the readings at the lowest substrate concentrations when substrate dimerization/oligomerization is at the lowest level (Svedruzic et al., 2012). This was necessary since those mutations can affect substrate dimerization (Gorman et al., 2008), and thus γ-secretase's activity in response to increasing substrate concentrations (Svedruzic et al., 2012). The readings at the lowest substrate concentrations are directly proportional to the maximal activity (Ferscht, 1998), and therefore can be used in evaluating the ratios between different maximal activities.

At the end we also show that the correlations can be observed even when all FAD mutations in presenilin 1 and Aβ part of APP substrate are combined together (Fig. 1, $R^2 = 0.9$ and 0.91 respectively). The combined approach strengthens the credibility of the presented analysis and indicates that the presented analysis could be a universal approach for studies of all FAD mutations (www.molgen.ua.ac.be/ADMutations). Following observed correlations when both mutations are combined together the predicted age-of-onset for WT is 51.1, and age-of-death is 58.5 (Fig. 1), so that the duration of the disease is about 8 years. The calculated "age-of-onset" and "age-of-death" are about 20 years earlier than the clinically observed age (Holtzman et al., 2011). Such underestimate can be expected, since the pathogenic processes driven by FAD mutations in young individuals are more aggressive than the pathogenic processes driven by age-induced slow changes in γ-secretase activity and APP metabolism (Kern et al., 2006; Theuns et al., 2003; Fukumoto et al., 2004).

The observed correlations between catalytic activity and "age-of-onset" or "age-of-death" for different FAD mutations cannot be an accidental coincidence. The presented correlations are result of a number of different measurements from different laboratories that used different experimental setup.

2.2. Biphasic inhibitors can be reliable indicators of pathogenic changes in γ-secretase activity in cells

The insights from enzyme-based studies presented in Fig. 1 can be used to analyze pathogenic processes in cells. Measurements of maximal activity of γ-secretase in cell-based assays are more complex than in the enzyme-based assays (Ferscht, 1998; Svedruzic et al., 2013). In enzyme-based assay saturation of γ-secretase with its substrate is an experimentally controlled variable (Svedruzic et al., 2012), while in cell-based assays the saturation of γ-secretase is controlled by the cell physiology (Ferscht, 1998; Svedruzic et al., 2013). Thus, to understand γ-secretase activity in cells, we have to measure the ongoing physiological activity, the maximal possible activity, and the extent of γ-secretase saturation with its β-CTF-APP substrate (Ferscht, 1998; Svedruzic et al., 2012). In earlier studies we showed that all three parameters can be quantitatively measured using biphasic inhibitors of γ-secretase (Svedruzic et al., 2013).

We measured biphasic dose response curves for DAPT using presenilin 1 and 2 double knockout MEF cells that have been transfected with human WT presenilin 1 or ΔE9, M139V and G384A FAD mutations in presenilin 1 (Bentahir et al., 2006). These cells have no modifications in their APP genes, therefore Aβ 1–40 production in the absence of DAPT represents the physiological γ-secretase activity for these cells (Svedruzic et al., 2013). The biphasic profiles show that for WT γ-secretase the physiological activity is 36.3 ± 3 pM Aβ (1–40) secreted while the maximal possible activity is 324 ± 90 pM of Aβ (1–40)

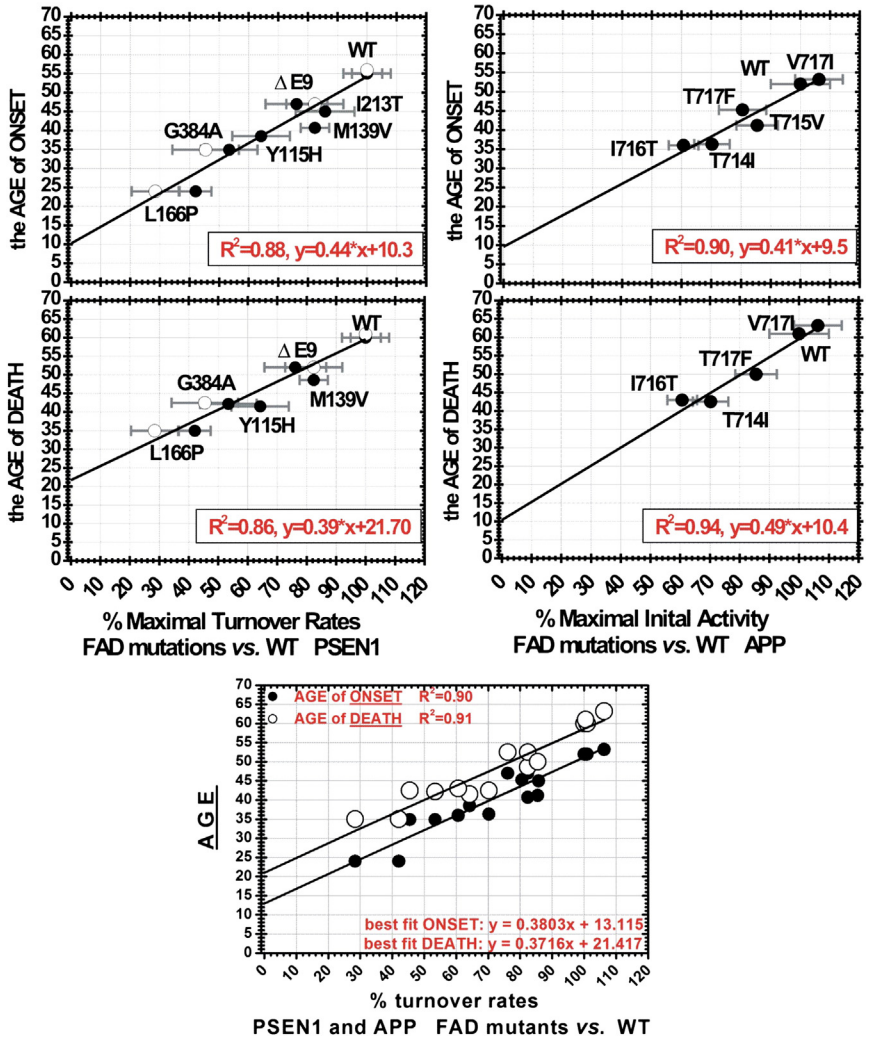

Fig. 1. Decrease in maximal activity of γ-secretase caused by different FAD mutations shows linear correlation with clinically observed age-of-onset and age-of-death for each mutation (Table 1). The correlations are calculated using AICD production rates from two different enzyme-based studies (Chavez-Gutierrez et al., 2012; Svedruzic et al., 2012). The measurements from different studies were normalized to the same scale by setting the WT activity as 100%, so that decrease in the activity caused by different FAD mutants can be analyzed as a percentage of the corresponding WT controls (Table 1). **Left panels**, eleven data points show decrease in maximal activities for six different FAD mutations in presenilin 1 component of γ-secretase (data from refs. (Svedruzic et al., 2012) (open circles) and (Chavez-Gutierrez et al., 2012) (closed circles)). **Right panels**, six different data points show decrease in activity for six different FAD mutations in Aβ part of β-CTF-APP substrate (data from ref. (Chavez-Gutierrez et al., 2012)). **Bottom panel**, all seventeen data points have been included in one analysis. Error bars represent reported errors (Chavez-Gutierrez et al., 2012; Svedruzic et al., 2012). The data for mean age-of-onset and mean age-of-death for different mutations were taken from: www.molgen.ua.ac.be/ADMutations. Statistical significance of the presented correlations is described by the R^2 values, while the corresponding trend lines are described by the best fit linear equations (Motulsky and Christopoulos, 2004).

secreted per 10^6 cells. Thus the percentage ratio between the physiological and the maximal activity is 11% (Table 2), which means that in these cells the WT γ-secretase is only 11% saturated with its β-CTF-APP substrate (Ferscht, 1998; Svedruzic et al., 2013).

In cells with ΔE9 mutant the physiological activity is 58 ± 2 pM Aβ (1–40) secreted while the maximal possible activity is 223 ± 72 pM of Aβ (1–40) secreted per 10^6 cells (Table 2). Thus, in cells with ΔE9 mutant the physiological Aβ 1–40 production is 26% of the maximal activity (Table 2). In cells with M139V mutant the physiological activity is 62 ± 3 pM Aβ (1–40) secreted while the maximal possible activity is 184 ± 82 pM of Aβ (1–40) secreted per 10^6 cells (Table 2). Thus, in cells with M139V mutant the physiological Aβ 1–40 production is about 32% of the maximal activity. Finally in cells with G384A mutant, there is no activation by DAPT, and the inhibition shows a very shallow Hill's coefficient. Such response can be observed in cells with the Swedish APP mutation and in cells with γ-secretase fully saturated with its β-CTF-APP substrate (Burton et al., 2008; Svedruzic et al., 2013). In summary, we show that the cells with FAD mutations in γ-secretase have consistently lower maximal activity and consistently higher saturation with its substrate than the WT controls (Fig. 2 and Table 2). Similar decrease in maximal activity of γ-secretase was observed in earlier cell-based studies of FAD mutations in presenilin 1 (Seidner et al., 2006). Moreover, just as similar studies in the past (Koch et al., 2012; Kumar-Singh et al., 2006; Potter et al., 2013; Shen and Kelleher, 2007), we also find that cells with FAD mutations in γ-secretase can have both increase and decrease in physiological Aβ 1–40 production relative to the WT controls (Fig. 2 bottom right panel). Thus, measurements of ongoing Aβ 1–40 production without measurements of maximal possible activity cannot be a good indicator of pathogenic processes.

The observed changes are not unique for FAD mutations. Similar to the differences between ΔE9 or M139V mutants and the WT enzyme, γ-secretase complex carrying Aph1B subunits shows slightly slower turnover rates than the complex carrying Aph1A$_L$ subunit, but notably higher production of potentially pathogenic Aβ fragments (Serneels et al., 2009). Quantitative analysis of the biphasic profiles in cells

Table 1
Correlations between decrease in maximal activity of γ-secretase and "age-of-onset" and "age-of-death" for different FAD mutations in presenilin 1 and APP substrate.

	Percent WT activity	Age of onset	Age of death
FAD mutation PSEN1			
L166P*	28.4	24	35
L166P	42.0	24	35
G384A*	45.5	34.9	42.5
G384A	53.4	34.9	42.2
Y115H	64.2	38.5	41.5
M139V	82.4	40.7	48.6
dE9*	82.4	47	52.5
dE9	76.1	47	52.5
I213T	85.8	45	
WT*	100.0	52	60
WT	100.0	52	60
FAD mutation APP			
I716T (I45T)	60.7	36	43
T714I (V43A)	70.2	36.3	42.5
V715A (V44A)	80.5	45.3	
V717F (V46I)	85.5	41.2	50
WT	100.0	52	61
V717I (V46A)	106.3	53.2	63.2

The age-of-death and the age-of-onset, were taken from database: www.molgen.ua.ac.be/ADMutations.

* Data recalculated from reference (Svedruzic et al., 2012). All other data are recalculated from Table 1 and from Fig. 4B in reference (Chavez-Gutierrez et al., 2012).

with Aph1A$_L$-γ-secretase complex showed that the physiological activity is 9.4 ± 0.7 pM Aβ (1–40) secreted while the maximal activity is 79.2 ± 9 pM of Aβ (1–40) secreted per 10^6 cells (Table 2). Thus, in cells with Aph1A$_L$-γ-secretase complex the physiological Aβ 1–40 production is about 12% of the maximal activity. With Aph1B-γ-secretase complex the physiological activity is 18.3 ± 0.9 pM of Aβ (1–40) secreted while the maximal possible activity is 69.1 ± 12 pM of Aβ (1–40) secreted per 10^6 cells. Thus, in cells with Aph1B-γ-secretase complex the physiological Aβ 1–40 production is about 26% of the maximal activity.

In conclusion, three different FAD mutants and two different Aph1 forms of γ-secretase, consistently show that a higher ratio between physiological Aβ 1–40 production and the maximal activity can be observed with the slower γ-secretase forms, that are known to facilitate pathogenic changes in Aβ products and Aβ 42/40 ratio (Serneels et al., 2009; Svedruzic et al., 2012). The observed differences in biphasic profiles cannot be an artifact created by variability between the cells. For each γ-secretase forms the ratio between physiological activity and the maximal activity was measured in parallel in 24 well-plates using the same batch of cells, the only variable are different concentrations of the biphasic inhibitors.

2.3. Biphasic inhibitors as a clinical diagnostic tool

The presented insights from enzyme-based and cell-based studies can be used in clinical studies for development of early diagnostic methods. Measurements of the maximal activity of γ-secretase in human brain cells are difficult. However the biphasic dose–response observed in cell-based assays can be also observed in experimental animals and in clinical studies (Burton et al., 2008; Tong et al., 2012; Mitani et al., 2012). Thus, there could be a possibility that similar to the cell-based studies (Fig. 2–3), the biphasic dose–response could be used in clinical studies to estimate the risk for development of the disease. A large number of different compounds targeting γ-secretase could produce biphasic dose response, however to our knowledge a full dataset from clinical trials was published only for Avagacestat (Tong et al., 2012). Clinical trials are expensive and beyond the reach of academic laboratories. Therefore we use the Avagacestat data to show how analysis of biphasic dose response curves can improve current approaches for evaluation of risks for development of Alzheimer's disease (Fig. 4). Fifty-four healthy individuals aged from 28 to 34 years old were treated with different doses of Avagacestat (Tong et al., 2012). For all of the analyzed doses Avagacestat reached stabile levels in plasma about 20 h after administration and remained stable for 144 h (Tong et al., 2012). The Aβ 1–40 levels in plasma samples measured 24 h after administration show a biphasic dose response curves (Fig. 4).

The biphasic curves with Avagacestat are easier for numerical analysis that the curves with DAPT, since the EC50 values for activation and inhibition are separated by about two orders of magnitude (Fig. 4 vs. Figs. 2 and 3). The best fit curve looks remarkably uniform for a dataset that represents a collective response from 54 healthy individuals aged 28 to 34 years old. High scatter is observed primarily at sub-activating drug levels 0–10 ng/ml, while a relatively uniform response is observed above 10 ng/ml in the activation and in the inhibition phase. The best fit residuals show that the scatter at the sub-activating drug-levels is not a result of statistical errors in measurements, but rather a result of variation between individuals in Aβ 1–40 metabolism. Low scatter at the activating concentrations between 10–20 ng/ml Avagacestat indicates that there is very little difference between the different individuals in the maximal Aβ (1–40) production capacity, which is about 210% of the initial average Aβ (1–40) activity.

In conclusion, the biphasic curves from the clinical studies with Avagacestat can be compared to the cell-based studies (Svedruzic et al., 2013). Like the cell-based assays (Svedruzic et al., 2013), the clinical studies with Avagacestat showed variability at sub-activating doses and relatively uniform response at activating and inhibiting doses (Svedruzic et al., 2013). The high scatter at the sub-activating and the activating doses can be caused by variable substrate levels (Svedruzic et al., 2013). Thus, the high scatter at sub-activating doses in clinical trials would indicate variability between healthy individuals in their cellular level of β-CTF-APP substrate, just as earlier reported in clinical studies of β-CTF-APP levels in humans (Pera et al., 2013). The data points that have higher Aβ 1–40 levels at the sub-activating Avagacestat concentration represent individuals that are closest to their maximal activity for Aβ 1–40 production (Kumar-Singh et al., 2006; Potter et al., 2013; Serneels et al., 2009), and therefore those individuals could have higher risk for development of the disease in the future.

Table 2
Best fit parameters for the biphasic activation–inhibition dose–response curves with DAPT (Eq. (1)). The numbers in bold indicate catalytic capacity for different forms of γ-secretase complex.

Data from Figs. 2 and 3	WT	ΔE9	M139V	G384A	Aph1A	Aph1B
Physiological activity[a] PA	36.3 ± 3	58 ± 2	62 ± 3	14.9 ± 0.2	9.4 ± 0.7	18.3 ± 0.9
Maximal activity[a] MA	324 ± 90	223 ± 72	184 ± 96	n.a.	79.2 ± 9	69.1 ± 12
Maximal inhibition[a] MI	−1 ± 0.1	1.9 ± 2	0.8 ± 2	−8 ± 8	0.1 ± 0.2	1.9 ± 1
Ratio between physiological activity and maximal activity activation	**0.11**	**0.26**	**0.32**	**1.00**	**0.12**	**0.26**
EC50, nM	301 ± 90	360 ± 150	490 ± 120	n.a.	79 ± 15	70 ± 12
Activation hill coef[a]	1.3 ± 0.3	1.1 ± 0.3	1.1 ± 0.3	n.a.	1.37 ± 0.3	1.4 ± 0.6
Inhibition IC50, nM[a]	790 ± 150	1260 ± 200	1180 ± 220	7900 ± 5000	794 ± 82	630 ± 80
Inhibition hill's coef[a]	2.1 ± 0.9	2.0 ± 0.4	1.9 ± 0.4	0.78 ± 0.2	2.15 ± 0.6	2.6 ± 0.4

All biphasic profiles were analyzed using nonlinear regression and the equation for biphasic dose–response curve as described in the Methods section and in our earlier studies (Svedruzic et al., 2013).

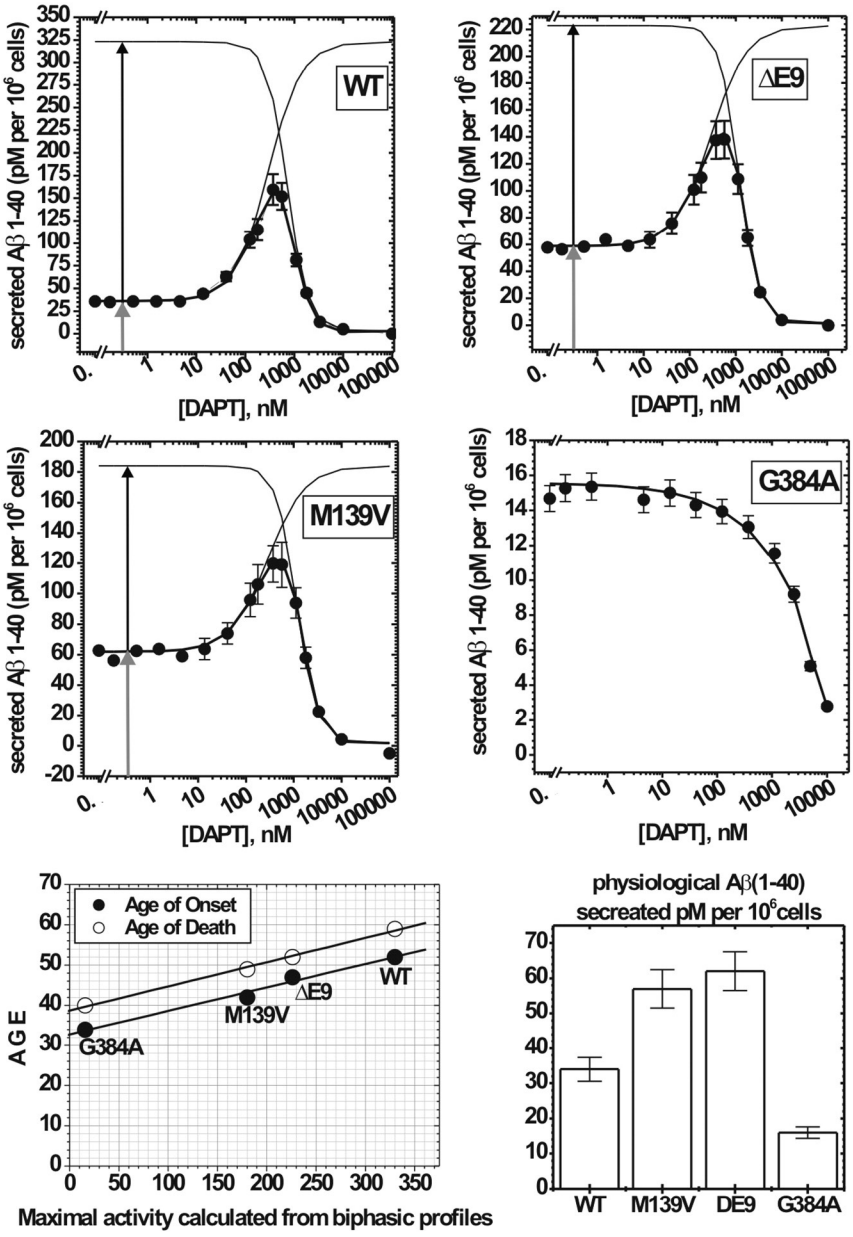

Fig. 2. FAD mutations in presenilin 1 show lower maximal activation with biphasic inhibitors. MEF cells transformed with human WT presenilin 1, or ΔE9, M139V, G384A FAD mutants have been prepared from endogenous presenilin 1 knockout cells (Bentahir et al., 2006). Aβ 1–40 production was measured for each cell line in the presence of increasing concentration of DAPT using sandwich ELISA (Svedruzic et al., 2013). The corresponding dose–response curves were analyzed using Eq. (1) (Svedruzic et al., 2013). The tick lines shows the best-fit curves, while the thin lines represent the corresponding activation and inhibition parts calculated from the best-fit parameters (Table 2). The gray and black arrows represent the ratio between Aβ 1–40 production in absence of DAPT and the calculated maximal activity (Table 2). Aβ 1–40 production in the absence of DAPT represents the physiological γ-secretase activity for each cell line. Thus, the physiological Aβ 1–40 production with WT γ-secretase is about 11% of the maximal activity, with ΔE9 FAD mutant is 26% of the maximal activity and with M139V FAD mutant is at 32% of the maximal activity. No activation is observed with the G384A, and the observed dose response curve shows low Hill's coefficient just like the WT complex at full saturation (Svedruzic et al., 2013). The rations between ongoing Aβ 1–40 production and the maximal activity represents the extent of γ-secretase saturation with its β-CTF-APP substrate in cells (Svedruzic et al., 2013). Thus, we find that FAD mutants in presenilin 1 component of γ-secretase have lower maximal activity and higher degree of saturation with its substrate (bottom left panel and ref. (Serneels et al., 2009; Chavez-Gutierrez et al., 2012; Svedruzic et al., 2012)). The bottom left panel shows that similar to the enzyme-based studies in Fig. 1, the cell-based studies with biphasic inhibitors show correlation between decrease in maximal activity caused by FAD mutations and the mean age-of-onset and mean age-of-death for each mutation (www.molgen.ua.ac.be/ADMutations). Measurements of ongoing Aβ (1–40) production in cells are not a reliable indicator of pathogenic potential of different FAD mutations (the bottom right panel).

3. Discussion

Following the "amyloid hypothesis" different studies have analyzed various Aβ products, most notably Aβ 1–40, Aβ 1–42 and Aβ 42/40 ratio (Sambamurti et al., 2011; Shen and Kelleher, 2007). Unfortunately such approach could give inconsistent results, and by some accounts daunting conclusions that question validity of the "amyloid hypothesis" and the related drug-development efforts (Sambamurti et al., 2011; Shen and Kelleher, 2007). Most notably different pathogenic process and drug-candidates can produce both increase and decrease in Aβ production (Sambamurti et al., 2011; Shen and Kelleher, 2007; Potter et al., 2013). In this study we propose that different doubts about the "amyloid hypothesis" can be resolved by comparing the ongoing physiological activity of γ-secretase with the maximal possible activity (Figs. 1–4). Such measurements can give consistent conclusions in enzyme-based studies (Fig. 1, and ref. (Svedruzic et al., 2012)), in cell-based studies (Figs. 2–3, and ref. (Svedruzic et al., 2013)), in evaluation of different drug candidates (Svedruzic et al., 2013), and possibly in

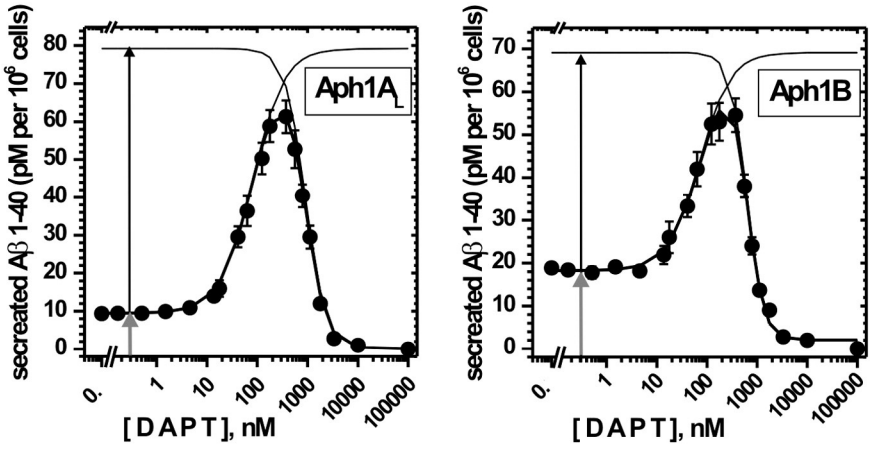

Fig. 3. γ-Secretase complex carrying Aph1B component shows lower maximal activation with biphasic inhibitors than the complex carrying Aph1A component. MEF cells carrying exclusively Aph1A or Aph1B component of γ-secretase have been prepared from endogenous Aph1ABC knockout cells (Serneels et al., 2009). Aβ 1–40 production was measured in the presence of increasing concentration of DAPT using sandwich ELISA (Svedruzic et al., 2013). The resulting dose–response curves were analyzed using Eq. 1 (Table 2). The tick line shows the best fit curves, while the thin lines depict the corresponding activating and the inhibiting parts that were calculated from the best-fit parameters (Svedruzic et al., 2013). The gray and black arrows indicate the ratio between physiological Aβ (1–40) production and the maximal possible activity (Table 2). Thus, the ongoing Aβ (1–40) production activity with Aph1A complex is about 12% of its maximal activity, while the activity with Aph1B form is at about 26% of its maximal activity. These ratios are proportional to the extent of γ-secretase saturation with its substrate (Svedruzic et al., 2013). Thus, once again, we observe that higher degree of saturation with γ-secretase complex that is known to produce higher Aβ42/Aβ40 ratio and the longer hydrophobic Aβ peptides (Serneels et al., 2009). Also, cells carrying Aph1B complex make more Aβ 1–40 than Aph1A form, even though Aph1B form has slower catalytic turnover rates (Serneels et al., 2009).

clinical studies (Fig. 4). Consistent results can be obtained by measuring both AICD and Aβ products of γ-secretase (Figs. 1 to 3).

Analysis of the ratio between ongoing activity and the maximal activity can give consistent results in different studies, since these measurements can include all parameters that control Aβ-production in cells: the physiological rate of Aβ-production, the maximal activity of γ-secretase, and the extent of γ-secretase saturation with its β-CTF-APP substrate (i.e., the Michaelis–Menten principles depicted in Fig. 5). Therefore, such measurements can be directly related to the pathogenic changes in Aβ products and Aβ 42/40 ratio according to differences in the Michaelis–Menten constant for each product (Kakuda et al., 2006; Svedruzic et al., 2013; Yin et al., 2007).

3.1. Sporadic and FAD cases of Alzheimer's disease share the same pathogenic mechanism: decrease in γ-secretase capacity to process its substrate

The results in Figs. 1–3 are consistent with the previous studies on humans, experimental animals, cells, and enzymes which showed that pathogenic events correlate with increase in saturation of γ-secretase with its substrate or decrease in its maximal activity (Fukumoto et al., 2004; Jonsson et al., 2012; Holsinger et al., 2004; Li et al., 2004; Sun et al., 2002; Yang et al., 2003; Guyant-Marechal et al., 2007; Rovelet-Lecrux et al., 2006, 2007; Citron et al., 1992; Cai et al., 1993; German and Eisch, 2004; Marlow et al., 2003; Refolo et al., 1999; Svedruzic et al., 2012, 2013). For example, we find earlier "age-of-onset" and "age-of-death" with the less active (Fig. 1), more saturated FAD mutants (Fig. 2), that have lower capacity to process their β-CTF-APP substrate (Fig. 5). Aging can also facilitate different processes that can decrease maximal activity of γ-secretase secretase or increase saturation of γ-secretase with its β-CTF-APP substrate (Fig. 5, (Kern et al., 2006; Theuns et al., 2003; Fukumoto et al., 2004)). Thus, our results support the idea that the "age-of-onset" and the "age-of-death" for different FAD mutations, are a result of a combined action of mutation-induced and age-induced decrease in γ-secretase's capacity to process its substrate (i.e., pathogenesis is a result of a combined action of decrease in maximal activity of γ-secretase and increase in production of β-CTF-APP substrate (Fig. 5)).

We propose that decrease in γ-secretase capacity to process its substrate can be used for analysis of different pathogenic process in all of sporadic and FAD cases of Alzheimer's disease (Fig. 5 C). For example, all FAD mutations that affect pre-β-secretase steps and β-secretase steps (Fig. 6), can increase saturation of γ-secretase with β-CTF-APP substrate (Fig. 5B). The only mutation that can decrease γ-secretase saturation with its β-CTF-APP substrate is A673T-APP (Fig. 5), and that is a protective mutation (Jonsson et al., 2012). All FAD mutations that affect presenilin 1 component of γ-secretase and Aβ part of its APP substrate (Fig. 6), can decrease maximal activity of γ-secretase (Fig. 5 and Table 1). In sporadic Alzheimer's disease increase in β-CTF-APP substrate can be a result of increase in β-secretase expression due to changes in regulation at RNA level (Boissonneault et al., 2009; Hebert et al., 2008; Li et al., 2004; Wang et al., 2008), or due to increase in β-secretase activity as a result of cholesterol induced co-localization with the APP substrate (Kern et al., 2006). Decrease in γ-secretase activity can be a result of aging induced decrease in γ-secretase expression (Kern et al., 2006; Theuns et al., 2003). Different drug-candidates (Svedruzic et al., 2013; Mitani et al., 2012), and possibly environmental toxins (Hochard et al., 2013), can also decrease maximal activity of γ-secretase. Different pathogenic processes can be also simulated experimentally by decreasing expression of γ-secretase, or by increasing expression of APP substrate (German and Eisch, 2004; Marlow et al., 2003; Refolo et al., 1999).

In summary, decrease in maximal activity of γ-secretase and increase in saturation of γ-secretase with its substrate can be the common pathogenic process in all of sporadic and FAD cases of Alzheimer's disease (Hunter et al., 2013). However we cannot characterize all of the previous studies in one of the two categories, since many of the earlier studies have evaluated pathogenesis only by measuring ongoing Aβ42 and/or Aβ40 production. Such measurements are not sufficient for a complete analysis of the underling molecular mechanism (Fig. 5). Different measurements of the ratio between physiological γ-secretase activity and the maximal activity can be a reliable alternative approach (Fig. 5).

3.2. "Loss-of-function" and "gain-of-function" events in sporadic and FAD cases of Alzheimer's disease

In Figs. 1–3 we also analyze some apparently conflicting observations that are frequently debated in studies of pathogenic processes and Aβ-production (Koch et al., 2012; Kumar-Singh et al., 2006; Potter et al., 2013; Shen and Kelleher, 2007). First, just as similar studies in

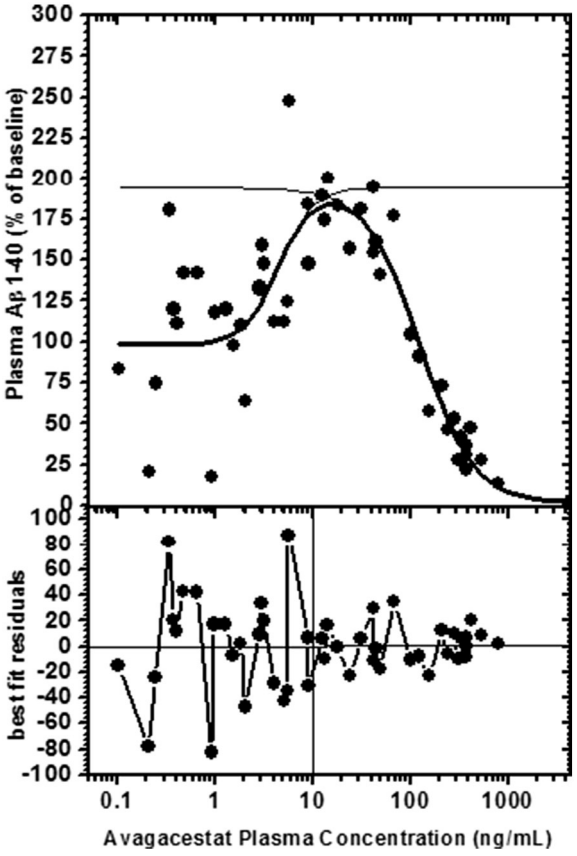

Fig. 4. Biphasic dose response curves can be observed in plasma samples from young healthy individuals treated with different doses of biphasic inhibitor Avagacestat (Tong et al., 2012). The presented data points were read from Fig. 3 from clinical studies by Tong et al. (Tong et al., 2012) using the grid option in Corel-Draw. Fifty-four healthy individuals aged from 28 to 34 years old were treated with different doses of Avagacestat and the corresponding Aβ 1–40 values were measured 24 h after administration (Tong et al., 2012). Thus, each data point represents one individual at the given dose of Agavacestat, while the resulting best fit biphasic profile is a statistical average of 54 healthy individuals (Eq. (1)). The best-fit-residuals shown in the lower panel represent percentage difference between the actual data points and the best fit curve. The residuals show highest scatter at sub-activating drug level, primarily below 1 ng/ml and to a lesser degree in the range between 1–10 ng/ml. Relatively little scatter is observed in the activation and the inhibition phase, i.e., doses higher than 10 ng/ml. Thus, the scatter at the sub-activating drug-levels is not a result of statistical uncertainties in measurements, but rather a result of variation in Aβ 1–40 metabolism between different individuals. The data points that have higher Aβ 1–40 levels at the sub-activating Avagacestat concentration represent individuals with high Aβ metabolism that is closest to the maximal catalytic capacity. Thus those individuals have less catalytic capacity to accommodate to any age-induced increase in Aβ metabolism and therefore could have higher risk for development of Alzheimer's disease in the future (Fukumoto et al., 2004; Kern et al., 2006).

the past (Kumar-Singh et al., 2006; Potter et al., 2013; Shen and Kelleher, 2007), we also observe an apparent paradox that ΔE9 and M139V FAD mutations have higher physiological Aβ production than the WT γ-secretase (Fig. 2), even though both mutants have lower maximal activity than the WT enzyme (Figs. 1 and 2). Second, we also find an apparent paradox that in cells FAD mutations can increase and decrease physiological Aβ production relative to the WT controls (bottom right panel in Fig. 2, and ref. (Koch et al., 2012; Kumar-Singh et al., 2006; Potter et al., 2013; Shen and Kelleher, 2007)). These apparently conflicting observations have been a subject of frequent debates in attempts to describe the pathogenesis by "loss-of-function" and "gain-of-function" hypothesis (Shen and Kelleher, 2007). Our results show that the two hypotheses are not in conflict, but rather represent different manifestations of the same phenomena (Fig. 5). The decrease in maximal activity of γ-secretase caused by ΔE9 and M139V FAD mutations can result in accumulation of unprocessed β-CTF-APP substrate (Figs. 2–3 and ref. (Kumar-Singh et al., 2006; Pera et al., 2013)). Moderate FAD mutations such as ΔE9 and M139V, can respond to increase in β-CTF-APP substrate with compensatory increase in Aβ production (Fig. 5). Some other examples of moderate FAD mutations that have higher physiological Aβ production than the WT enzyme are A79V, H163R, M146L, M139I mutations in presenilin 1 in reference (Potter et al., 2013). The most aggressive FAD mutations such as L166P and G384A have such drastic decrease in the maximal catalytic rates (Fig. 1), that even at full saturation those mutants cannot respond to increase in β-CTF-APP substrate with an increase in Aβ production (Fig. 5A and ref. (Kumar-Singh et al., 2006; Koch et al., 2012)). Similar to different FAD mutations, different inhibitors can also decrease γ-secretase activity and thus result in accumulation of β-CTF-APP substrate and increase in Aβ production (Mitani et al., 2012; Ortega et al., 2013; Tamayev and D'Adamio, 2012; Svedruzic et al., 2013).

The "gain-of-function" hypothesis is based on initial studies which showed that pathogenesis correlates with increase in Aβ metabolism most notably Aβ 1–42, the main component of insoluble Aβ plaques (Shen and Kelleher, 2007). The map of APP metabolism shows that mutations and aging processes that affect β-secretase and pre-β-secretase steps lead to increase in β-CTF-APP production (Fig. 6). This results in increase in Aβ production and the "gain-of-function" mechanism (Fig. 5B). The only exception is A673T mutation, which leads decrease in β-CTF-APP production and acts as a protective mutation (Jonsson et al., 2012). Mutation and aging processes that affect presenilin 1 component of γ-secretase, or Aβ part of its APP substrate, can lead to decrease and increase in Aβ production depending on the experimental set-up (Seidner et al., 2006; Chavez-Gutierrez et al., 2012; Theuns et al., 2003; Kumar-Singh et al., 2006). Thus, those mutations can be characterized as both "loss-of-function" and "gain-of-function" mechanism. The "loss-of-function" mechanism can be observed primarily in experiments in which maximal activity of γ-secretase, or extent of γ-secretase saturation with its substrate, are experimentally controlled variables (Fig. 1 and refs. (Seidner et al., 2006; Chavez-Gutierrez et al., 2012; Theuns et al., 2003; Kumar-Singh et al., 2006)). The "gain-of-function" can be observed primarily in experiments where the extent of γ-secretase saturation with its substrate is a result of a physiological balance between β-CTF-APP production and maximal activity of γ-secretase (Figs. 2 and 3 and ref. (Pera et al., 2013; Potter et al., 2013)).

3.3. Measurements of catalytic capacity of γ-secretase can be used as a diagnostic tool for early evaluation of risk for development of Alzheimer's disease

Presented results show that the current clinical diagnostic methods can be significantly improved if we can compare the ongoing physiological Aβ activity with the maximal possible activity (Figs. 1–4). Such comparisons are easy to do in enzyme-based and cell-based studies (Svedruzic et al., 2012, 2013), but fairly challenging in high-throughput drug-screening assays, in experimental animals, or in clinical studies. Fortunately a good estimate of changes in the maximal activity can be achieved by measuring the activation amplitude with biphasic inhibitors (Svedruzic et al., 2013). Simply the pathogenic processes that can increase Aβ production, or decrease maximal activity of γ-secretase, can also decrease activation of γ-secretase by small-molecule activators (Burton et al., 2008; Svedruzic et al., 2013).

The biphasic inhibitors can be used as diagnostic test in a procedure that is similar to the glucose tolerance test currently used for testing diabetes. First, a plasma sample is taken from the patient to measure the ongoing Aβ activity, then the patient is given a biphasic probe to measure the maximal Aβ activity. A high ratio between the two measurements would indicate a high capacity for processing β-CTF-APP substrate, and thus low risk for development of the disease in future. The proposed approach would require some standardization of different biphasic inhibitors to select the inhibitors with optimal PK/PD properties and a robust quantitative response to the potentially pathogenic

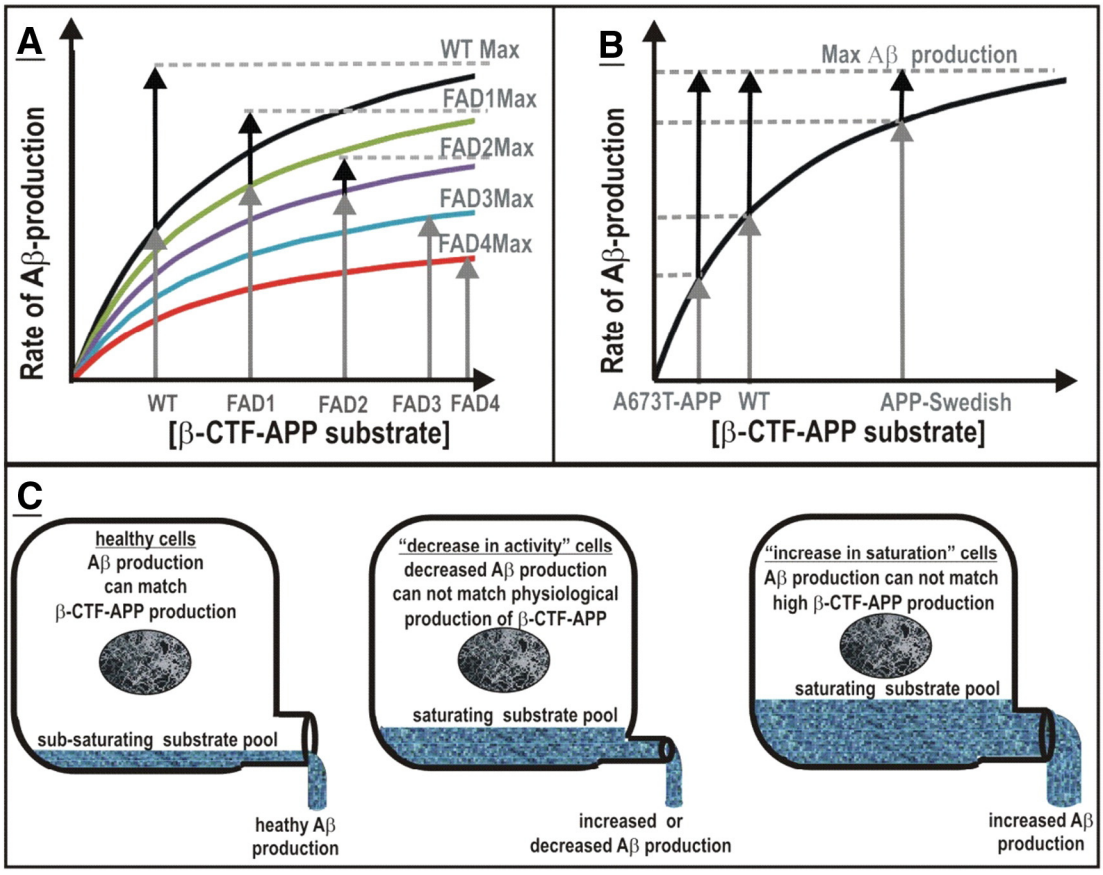

Fig. 5. (A–C) Decrease in catalytic capacity of γ-secretase can support pathogenesis in all of different sporadic and FAD cases of Alzheimer's disease. Gradual saturation of γ-secretase with its substrate leads to hyperbolic changes in the enzyme activity just as with any other enzyme (Ferscht, 1998; Chavez-Gutierrez et al., 2012; Svedruzic et al., 2012, 2013). The hyperbolic curves show that every analysis of γ-secretase activity has to include three factors: the ongoing physiological Aβ-production rates, the maximal activity of γ-secretase, and the extent of γ-secretase saturation with its substrate (Ferscht, 1998; Svedruzic et al., 2012). For cell physiology the hyperbolic curves represent γ-secretase capacity to process its substrate (Ferscht, 1998; Svedruzic et al., 2012). Two molecular events can decrease γ-secretase capacity to process its substrate, decrease in the maximal activity (panel A) or increase in saturation with its substrate (panel B).

(A) The panel depicts experiments which lead to analysis presented in Fig. 1 and in ref. (Chavez-Gutierrez et al., 2012; Svedruzic et al., 2012). These are different "decrease-in-maximal-activity" mutations that affect presenilin 1 component of γ-secretase and Aβ-part of its APP substrate. Similar to Figs. 2 and 3, the arrows illustrate the ratio between measured activity at given β-CTF-APP substrate (lower arrow), and the maximal activity at saturating substrate (Table 2). When compared to the WT enzyme, moderate FAD mutations FAD1 and FAD2 have lower maximal activity (upper arrow), higher Aβ production, and higher saturation with the substrate (lower arrow). Thus relative to the WT, moderate FAD mutations FAD1 and FAD2 have decreased capacity to accommodate to the additional aging induced increase in substrate level (Fukumoto et al., 2004; Kern and Behl, 2009; Kern et al., 2006). The most aggressive FAD mutations have the lowest maximal turnover rates (Fig. 1), the lowest Aβ production and the highest saturation with its substrate (Fig. 2). Thus, the most aggressive FAD mutations FAD3 and FAD4, can trigger pathogenesis at the youngest age since they do not have capacity to accommodate to the aging induced increase in β-CTF-APP substrate (Fukumoto et al., 2004; Kern and Behl, 2009; Kern et al., 2006). Decrease in maximal activity of γ-secretase can be also observed in sporadic Alzheimer's disease with WT proteins as a result of age-induced decrease in activity of γ-secretase genes (Theuns et al., 2003; Kern and Behl, 2009; Kern et al., 2006).

(B) The panel depicts experiments in which WT γ-secretase is exposed to increasing levels of β-CTF-APP substrate. Thus, the panel can depict pathogenesis in sporadic Alzheimer's disease and in FAD mutations that affect pre-β-secretase and pre-β-secretase steps in APP metabolism (Fig. 6). The saturation with β-CTF-APP substrate and the available catalytic capacity can be calculated by comparing measured Aβ production (lower arrows), with the maximal possible Aβ production (upper arrows). For example, APP-Swedish FAD mutation will lead to increase in β-CTF-APP substrate and thus decrease in available capacity to accommodate to any additional aging induced increase in β-CTF-APP substrate (Svedruzic et al., 2013). Opposite situation is observed in case of protective A673T-APP mutation (Jonsson et al., 2012). A673T-APP mutation can decrease β-CTF-APP substrate and thus increase cellular capacity to accommodate to the aging induced increase in β-CTF-APP substrate. In sporadic Alzheimer disease, different age induced changes in cell physiology can increase β-CTF-APP substrate and thus decrease available capacity to accommodate to additional β-CTF-APP substrate (Fukumoto et al., 2004; Kern et al., 2006).

(C) If we depict γ-secretase reaction as a cellular Aβ-drain-pipe, than different "decrease-in-maximal-activity" events (Fig. 5A) can be depicted as a decrease in the pipe diameter, while different "increase-in-saturation" events can be depicted as excessive loads for the pipe (Fig. 5B). In both cases, the end result is decrease in the pipe's capacity to process its loads. Similarly, decrease in catalytic capacity of γ-secretase can result in pathogenic accumulation of β-CTF-APP substrate and toxic Aβ products (Ferscht, 1998; Svedruzic et al., 2012).

changes in the extent of γ-secretase saturation with its substrate (Svedruzic et al., 2013).

The results from clinical studies with Avagacestat suggest that individuals with similar age have similar maximal activity (Fig. 4), and the variability can be observed primarily at the sub-activating doses of Avagacestat (Fig. 4). Therefore the individuals with the lower Aβ activity at sub-activating doses of Avagacestat (Fig. 4) have higher capacity to accommodate to the future age-induced changes in the catalytic capacity of γ-secretase (Fig. 5). Thus, those individuals could have lower risk for development of the disease in the future (Fig. 5B). Different molecular mechanisms have been proposed to describe biphasic inhibition. However the proposed use of biphasic inhibitors as diagnostic tool is not affected by different proposals, since all of the proposed mechanisms indicated that biphasic inhibition depends on the saturation of γ-secretase with its substrate.

3.4. Concluding remarks

Several studies before us showed correlation between age-of-onset or age-of-death and γ-secretase activity (Seidner et al., 2006), or

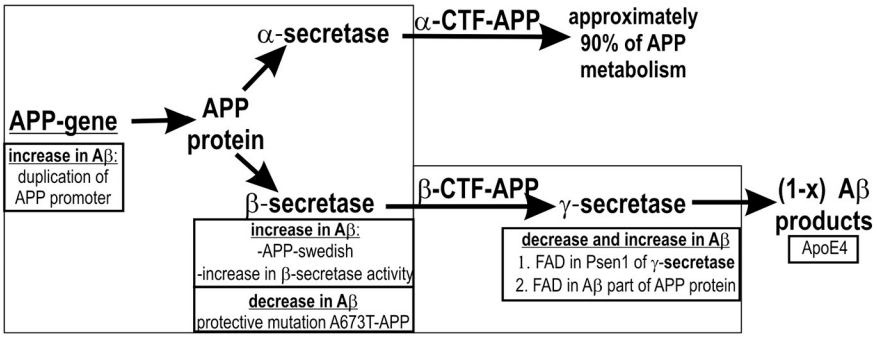

Fig. 6. Based on pathogenic mechanism the key steps in metabolism of amyloid precursor protein (APP) can be separated to two parts. The large rectangle represents pre-γ-secretase steps in APP metabolism (i.e., β-secretase steps and pre-β-secretase steps). These steps can produce pathogenic increase in cellular β-CTF-APP production and Aβ production (Fig. 5B), and thus can be described with the "gain-of-function" hypothesis (Shen and Kelleher, 2007). The most notable examples are duplication of APP gene (Rovelet-Lecrux et al., 2006), FAD mutations in APP protein around the β-secretase cleavage site (Cai et al., 1993; Citron et al., 1992), changes in β-secretase expression at RNA level (Boissonneault et al., 2009; Hebert et al., 2008; Li et al., 2004; Wang et al., 2008), or any other age-induced increase in β-secretase activity (Kern et al., 2006). Notably different is A673T mutation in APP protein, a protective mutation at the β-secretase cleavage site that leads to decrease in production of β-CTF-APP substrate and decrease in Aβ production (Jonsson et al., 2012). The small rectangle represents the steps that affect maximal activity of γ-secretase (Fig. 5A). These steps represent the majority of the FAD mutations, precisely the mutations in presenilin components of γ-secretase or mutations in Aβ part of its β-CTF-APP substrate (Shen and Kelleher, 2007). These steps can be also affected by aging in sporadic Alzheimer's disease, the most notable example are different processes that can decrease activity of γ-secretase genes (Sambamurti et al., 2011; Kern et al., 2006; Theuns et al., 2003).

Aβ42/Aβ40 ratio (Kumar-Singh et al., 2006), or dimerization affinity of its β-CTF-APP substrate (Gorman et al., 2008). This study is the first to show correlations between "age-of-onset" or "age-of-death" and the catalytic capacity of γ-secretase. This allows us to look at the pathogenic processes as specific changes in activity of γ-secretase (Svedruzic et al., 2012) that can be precisely targeted by novel drug-candidates (Svedruzic et al., 2013). For example, decrease in catalytic capacity of γ-secretase can trigger several molecular events that can damage the cell membranes. The first is accumulation and possibly aggregation of unprocessed β-CTF-APP substrate (Mitani et al., 2012; Ortega et al., 2013; Tamayev and D'Adamio, 2012; Gorman et al., 2008; Kumar-Singh et al., 2006; Citron et al., 1992). Several studies suggested that accumulation and aggregation of β-CTF-APP substrate could directly damage the cell membranes (Mitani et al., 2012; Ortega et al., 2013; Tamayev and D'Adamio, 2012). Alternatively, accumulation and aggregation of β-CTF-APP can facilitate binding of multiple substrate molecules to γ-secretase (Svedruzic et al., 2012). The multiple substrate molecules bound to γ-secretase can affect dynamic structural changes that control processive cleavages of the catalytic intermediates and the product dissociation steps (Svedruzic et al., 2012). The end results are pathogenic changes in Aβ 42/40 ratio and other Aβ products (Svedruzic et al., 2012; Yin et al., 2007; Kakuda et al., 2006).

Respect for some of the established rules for enzyme activity studies (Ferscht, 1998; Motulsky and Christopoulos, 2004) can resolve many of the current uncertainties about the "amyloid hypothesis" and the related drug-development efforts (Sambamurti et al., 2011; Svedruzic et al., 2013). Similar enzyme-based approach in studies of DNA methyltransferase lead to description of different regulation mechanisms almost 10 years before the initial ideas become the mainstream (Svedruzic, 2008; Svedruzic and Reich, 2005a,2005b; Di Ruscio et al., 2013).

4. Materials and methods

4.1. Materials

DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-*S*-phenylglycine *t*-butyl ester) was purchased from Calbiochem. MEF cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. We are grateful to the scientist from Katholieke Universiteit Leuven, Vlaams Instituut voor Biotechnologie for MEF cells double knockout for endogenous presenilin 1 transduced with human WT, ΔE9, M139V, G384A presenilin 1, and Aph1A$_L$ or Aph1B subunits of γ-secretase (Bentahir et al., 2006; Serneels et al., 2009).

4.2. Secretion of Aβ1-40 in MEF cells in the presence of increasing concentration of DAPT

The measurements of the biphasic inhibition with DAPT and the corresponding data analysis have been described in detail in our earlier studies (Svedruzic et al., 2013). Briefly, different concentrations of DAPT were prepared in DMSO, and added to the cells so that the final DMSO concentration in the culture was 0.1% (v/v). DMSO vehicle represents 0 nM DAPT. The cells were incubated with DAPT at given concentrations for 18 h.

4.3. Sandwich ELISA for quantitative detection of Aβ 1–40

Sandwich ELISA kits for quantitative detection of human Aβ 1–40 peptides with highly selective monoclonal antibodies in a flexible 96 well format were purchased from Milipore (cat. #. TK40HS, The Genetics company Switzerland). The assay linear response is in the range from 6–125 pM of Aβ 1–40. The assays were performed by closely following the manufacturer's instructions. To assure the most representative Aβ 1–40 samples, the samples were used immediately after collection following the manufacturer's suggestion and our earlier reported experimental experiences (Svedruzic et al., 2012). Each well was filled with 50 μl of the antibody conjugate solution and 50 μl of sample. The Aβ 1–40 standards were supplied by the manufacturer and prepared in parallel with other samples. All of the prepared wells were wrapped in aluminum foil and incubated overnight at 4 °C with gentle mixing. The next day each well was washed five times with 300 μl of wash solution. After each 20 minute wash, the wash solution was poured out and the wells were dried by tapping the plates on an absorbing paper. Washed wells were filled with 100 μl of the enzyme conjugate solution, covered, and incubated for 30 min at room temperature with shaking. The washing procedure was repeated once again to remove excess of the enzyme-conjugate. Next 100 μl of the substrate solution was added in each well in dark, and kept for 30 min covered at room temperature. The reaction was quenched by adding 50 μl of stop solution to each well, and within 15 min the signal intensity was read by measuring absorption at 450 nm.

4.4. Data analysis

All experimental results were analyzed using MicroCal Origin 7.0 program. All biphasic profiles were analyzed using nonlinear regression and the equation for biphasic dose–response curve that was described in detail in our earlier studies (Svedruzic et al., 2013):

$$S(x) = PA + \frac{(MA-IA)}{\left(1+10^{(EC50-X)\,p}\right)} + \frac{(MA-MI)}{\left(1+10^{(X-EC50)\,q}\right)} \quad (1)$$

where, $S(x)$ represents measured activity at inhibitor concentration x. *PA* is the physiological Aβ 1–40 production activity at inhibitor concentration zero, *MA* is the calculated maximal activity, and *MI* is maximal inhibition. *EC50* and *IC50* represent activation and inhibition constant, respectively, while p and q represent the corresponding Hill's coefficients (Motulsky and Christopoulos, 2004).

To facilitate numerical analysis in nonlinear regression we used logarithmic values of inhibitor concentrations (Motulsky and Christopoulos, 2004). The final best-fit results are shown in units of concentration in the tables and graphs. All results are reported as the best fit value ± standard error (Motulsky and Christopoulos, 2004). The reported standard errors were calculated using nonlinear regression with all six free fit parameters (Table 2). An order of magnitude lower standard errors can be obtained with less free fit parameters (Svedruzic et al., 2013). Also to obtain sharper best fit values we took a large number of independent data points that are distributed over the full range of the best-fit curve (i.e., to maximize the resolution of each parameter (Motulsky and Christopoulos, 2004)). The low scatter from the best fit curves indicates that the measurements of biphasic dose–response curves have a low random error.

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