Reduced Aβ secretion by human neurons under conditions of strongly increased BACE activity

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Abstract

The initial step in the amyloidogenic cascade of amyloid precursor protein (APP) processing is catalyzed by beta-site APP-cleaving enzyme (BACE), and this protease has increased activities in affected areas of Alzheimer’s disease brains. We hypothesized that altered APP processing, because of augmented BACE activity, would affect the actions of direct and indirect BACE inhibitors. We therefore compared post-mitotic human neurons (LUHMES) with their BACE-overexpressing counterparts (BLUHMES). Although β-cleavage of APP was strongly increased in BLUHMES, they produced less full-length and truncated amyloid beta (Aβ) than LUHMES. Moreover, low concentrations of BACE inhibitors decreased cellular BACE activity as expected, but increased Aβ1-40 levels. Several other approaches to modulate BACE activity led to a similar, apparently paradoxical, behavior. For instance, reduction in intracellular acidification by bepridil increased Aβ production in parallel with decreased BACE activity. In contrast to BLUHMES, the respective control cells (LUHMES or BLUHMES with catalytically inactive BACE) showed conventional pharmacological responses. Other non-canonical neurochemical responses (so-called ‘rebound effects’) are well-documented for the Aβ pathway, especially for γ-secretase: a partial block of its activity leads to an increased Aβ secretion by some cell types. We therefore compared LUHMES and BLUHMES regarding rebound effects of γ-secretase inhibitors and found an Aβ rise in LUHMES but not in BLUHMES. Thus, different cellular factors are responsible for the γ-secretase- versus BACE-related Aβ rebound. We conclude that increased BACE activity, possibly accompanied by an altered cellular localization pattern, can dramatically influence Aβ generation in human neurons and affect pharmacological responses to secretase inhibitors.

Keywords: Alzheimer’s, APP, BACE, neuropharmacology, γ-secretase.
BACE and APP mature by post-translational modifications. These include simple glycosylation in the endoplasmic reticulum (ER) and further addition of complex carbohydrates as well as removal of the BACE propeptide in the Golgi apparatus (Weidemann et al. 1989; Pahlsson et al. 1992; Bennett et al. 2000; Capell et al. 2000; Benjannet et al. 2001; Thimakaran and Koo 2008). Both proteins are transported to the cell surface, then undergo rapid endocytosis and are either recycled back or targeted to endosomes/lysosomes (Haass et al. 1992; Koo et al. 1996; Huse et al. 2000; Walter et al. 2001). APP cleavage by BACE predominantly takes place in acidic intracellular organelles because of the low pH optimum of BACE activity (Yan et al. 2001; De Strooper et al. 2010). It is not entirely clear how C99/C89 is subsequently transported from its generation site to the compartment in which γ-secretase resides.

BACE-overexpressing cell lines or mice often express ectopic APP to raise the levels of detectable Aβ. Only few studies have addressed the effects of augmented cellular BACE activity alone or of increased BACE-APP ratios on the generation of Aβ. Some cell culture studies indicate that levels of full-length Aβ increased (Lee et al. 2003a) or remained almost unchanged (Huse et al. 2002; Liu et al. 2002) when BACE was elevated. In mice, the situation seems to depend on the degree of BACE over-expression. Full-length Aβ was increased when BACE levels were moderately raised in APPxBACE tg mice (Bodendorf et al. 2002; Chiocco et al. 2004), but Aβ levels and plaque numbers decreased at high BACE levels (Lee et al. 2005; Rockenstein et al. 2005).

As a divergent change in BACE activity and Aβ secretion may have important pharmacological and clinical consequences, we investigated how BACE levels affected the processing of endogenous, non-mutated APP in human neurons. As a model system, we used LUHMES cells, which allow the generation of fully post-mitotic neurons with electrical activity, an elaborate neurite network, and many other typical neuronal features (Scholz et al. 2011; Stiegl et al. 2011) after the shutdown of the tetracycline-controlled γ-myc transgene in precursor cells (Lotharius et al. 2005). Differentiated LUHMES express all major AD-related proteins, and they generate and release APP cleavage products such as sAPPα/β and Aβ (Scholz et al. 2013). In this study, BACE-overexpressing cells and control cell lines were generated to study the effect of moderately and strongly elevated BACE activity on full-length and truncated Aβ. Moreover, the pharmacological impact of increased BACE levels on the action of β- and γ-secretase inhibitors was tested. Our findings suggest that the extent to which BACE activity is raised in human neurons may have a profound influence on Aβ generation and on the pharmacology of BACE inhibitors.

Materials and methods

No animals or animal cell lines were used in this study, and no institutional approval or pre-registration was required.

Reagents

The isoprenylated BACE inhibitor IPAD (Cat#565794, Stachel et al. 2004; Volbracht et al. 2009), OMP99-(Cat#496000), DAPT (Cat#565770), and GM6001 (Cat#364205) were purchased from Merck/Cabiochem (Darmstadt, Germany). LY450139 and the aminooquinazoline BACE inhibitor aminooquinazoline derivative (Baxter et al. 2007) were synthesized following the schemes provided by Lilly (Indianapolis, IN, USA) and Johnson & Johnson (Beere, Belgium), respectively. N-glycosidase F (PNGase, Cat#P0704) and endoglycosidase H (EndoH, Cat#P0702) were purchased from New England BioLabs (Ipswich, MA, USA). Baflonycin A1 (Cat#B1793), bepridil (Cat#B5016), brefeldin A (Cat#B6542), and hygromycin B (Cat#3274) were from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Wild-type (wt) and transgenic LUHMES cells (ATCC Cat#CRL-2927, RRID:CVCL_B056) were grown and differentiated exactly as described previously (Schildknecht et al. 2009, 2013; Scholz et al. 2011, 2013). This cell line is not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee (ICLAC). Identity of the LUHMES cells was authenticated by analysis as described previously (Pallocca et al. 2017). Briefly, DNA samples were prepared using a commercial kit (Purogene Cell Kit, Cat#58767, Qiagen, Hilden, Germany). The GlobalFiler™ PCR Amplification Kit (Cat#447613, ThermoFisher, Waltham, MA, USA) was then used to determine the cell-specific profile for 16 different genomic loci using an Applied Biosystems GeneMapper Device. Short Tandem Repeat repeats, amelogenin (X), CSF1PO (13,14), D5S818 (11,13), D13S317 (9,11), D7S820 (11,13), D16S539 (11,12), vWA (14,17), and TH01 (7,9,3) showed 100% identity with the ATCC profile. For details on the cell culture, see supplementary methods.

Cell viability

Viability of cells during differentiation and pharmacological experiments was regularly controlled using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay as described previously (Volbracht et al. 2009). For details, see supplementary methods.

DNA constructs and lentivirus production

A 3.2 kb DNA fragment, containing cytomegalovirus (CMV)-promoter-driven human wild-type BACE1 (isoform A, NM_012 104), linked to a hygromycin B resistance gene via an internal ribosome entry site sequence, was excised from a pCI (Promega, Madison, WI, USA) mammalian expression vector (Volbracht et al. 2009). The pHcCBHW construct, used in our study, was obtained by inserting this fragment into the multiple cloning site of the lentiviral transfer vector pHcCXW (Leander Johansen et al. 2005; GenBank accession no. AY468486). The pHcCB-D289A-HW vector, bearing an active site mutant form of BACE (aspartic acid residue 289 mutated to alanine), was derived from pHcCBHWW by site-directed mutagenesis (Jin et al. 2010).

Lentivirus production was carried out as already described by us earlier (Vergo et al. 2007; Scholz et al. 2011). In brief, 293FT cells (Cat#R70007, Invitrogen, Carlsbad, CA, USA), grown in LUHMES proliferation medium, were transiently transfected by lipofection
with a three-plasmid vector system. This system consisted of pMD2.G (Cat#12259, http://www.addgene.com) as envelope vector, pBRS.91 (Zufferey et al. 1997) as packaging vector, and either pHscEw [containing enhanced green fluorescent protein (GFP)] (Leander Johansen et al. 2005), pHsCBHw, or pHsCB-D289A-HW as transfer vector. After 48 h, the 293FT supernatant was filtered, and virus particles were precipitated using PEG-ir solution according to the manufacturer’s instructions (Cat#LV825A-1, SBI, Mountain View, CA, USA). The virus pellet was dissolved in a small volume of fresh LUHMES proliferation medium and stored at −80°C.

**Generation of stable poly- and monoclonal cell lines**

Undifferentiated LUHMES were transduced by incubation of the cells for 12 h with the virus-containing medium. A pure pool of GFP-overexpressing LUHMES (GLUHMES) was obtained without the necessity of antibiotic selection after expansion of the cell cultures, and subsequent fluorescence activated cell sorting (FACSAria II, BD, Heidelberg, Germany). Pure, polyclonal pools of LUHMES, overexpressing either BACE (BLUHMES) or inactive BACE with the D289A mutation (D289A_BLUHMES) were obtained in blinded manner. For details, see supplementary methods.

**Immunocytochemistry**

BLUHMES and LUHMES were fixed on various days of differentiation with 4% paraformaldehyde, and after permeabilization and blocking steps, primary antibodies were added overnight at 4°C (Fig. S1). Following incubation with secondary antibodies, samples were imaged with an Olympus IX 81 inverted epi-fluorescence microscope (Hamburg, Germany) and evaluated in blinded manner. For details, see supplementary methods.

**Quantitative reverse-transcription PCR**

Quantitative PCR (qPCR) analysis was performed as described earlier (Scholz et al. 2011). Briefly, 1 µg of isolated total RNA was reverse transcribed and the cDNA was amplified using Platinum® SYBR® Green qPCR SuperMix (Cat#1733038, Invitrogen) and gene-specific primer pairs (Eurofins MWG Operon, Ebersberg, Germany) (Fig. S1). All qPCRs were run in a Biorad Light Cycler (Biorad, München, Germany). For details, see supplementary methods.

**Protein analysis**

Cells were lysed in radioimmunoprecipitation assay buffer. For endoglycosidase digestion, 20 µg of protein were incubated overnight at 37°C with PNGase, EndoH (New England BioLabs), or only buffer. After protein separation by 10% Tris-glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transfer of the protein bands to nitrocellulose membranes, primary antibodies were added overnight at 4°C (Fig. S1). Following incubation with horseradish peroxidase-conjugated secondary antibodies, blots were visualized in a FUSION-SL 4.2 MP chemiluminescence system (Peqlab, Erlangen, Germany) and evaluated/quantified in blinded manner. For details, see supplementary methods.

**Aβ**

Secreted Aβ(1–40), Aβ(1–42), Aβ(1–x), and sAPPβ were measured in the conditioned supernatant of LUHMES cells, 24 h after medium change. The peptides were directly detected by robust, validated (Volbracht et al. 2009; Scholz et al. 2013) Aβ(1–40)-specific (Cat#KBB3482, Invitrogen), Aβ(1–42)-specific (Cat#SIG-38950, Covance, Princeton, NJ, USA), or Aβ(1–x)-specific (Cat#JP27729, IBL International, Hamburg, Germany) sandwich enzyme-linked immunosorbent assays (ELISA) kits according to the manufacturer’s instructions. The Aβ(1–40) ELISA detects Aβ(1–40) and variants lacking parts of its N-terminus. The Aβ(1–x) ELISA applies a detection antibody directed against amino acids 11–28 of human Aβ and can detect C-terminally truncated Aβ variants bigger than Aβ(1–16), therefore including Aβ(1–34), Aβ(1–40), and Aβ(1–42). Concentrations were normalized to the protein concentration in the respective cell lysates if not indicated otherwise. For western blotting of sAPPβ, supernatants were concentrated 20-fold with Amicon centrifugal filter units (Cat#UFC905096, Millipore, Billerica, MA, USA), and normalized volumes (25–30 µL, depending on protein amount/well) of the concentrated supernatants were then subjected to 8% Tris-glycine sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

**BACE activity measurement**

The β-secretase activity was measured with a kit (Cat#565785, Calbiochem), following the included instructions, and with following adaptations: for each data point, 2 x 10⁶ cells were lysed in 100 µL of extraction buffer. The protein content was determined, and 10 µg of total protein were used for each measurement. The assay was based on the cleavage of an APP-derived β-secretase target peptide, coupled to 5-((2-aminoethyl)laminonaphthalene-1-sulfonic acid) (EDANS) as Förster resonance energy transfer (FRET) donor at its N-terminus, and 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL) as FRET acceptor at its C-terminus (Fukumoto et al. 2002). Enzyme activity was detected by determination of the fluorescence change (excitation wavelength: 340 nm; emission wavelength: 500 nm) at 40 min after the addition of fluorogenic substrate to the sample. The ideal time point was determined experimentally. Under these assay conditions, a standard probe containing 1 ng of recombinant BACE1 resulted in a fluorescence increase by 22 relative units. All samples were measured in the absence or presence of the specific β-secretase inhibitor IPAD (200 nM). This compound treatment blocked >99% of BACE1 activity, and the specific β-secretase activity was calculated as the difference of the fluorescence change in each sample in the presence and absence of IPAD. For each experiment,
triplicate samples were prepared and measured, and the mean of these values was considered as one data point.

**Statistics**
All experiments were independently repeated 2–6 times with at least two biological replicates per experiment, and data are displayed as means ± SEM or means ± SD as indicated. Statistical analyses were performed by Student’s t-test or one-way ANOVA with Dunnett’s or Bonferroni’s post hoc tests as appropriate, using the GraphPad Prism software (San Diego, CA, USA). Means were considered as being significantly different if p < 0.05. IC50 values were derived from curves obtained by sigmoidal dose-response curve fitting (GraphPad Prism). No randomization methods or predefined criteria for outliers were applied. Normality of data was assessed using GraphPad Prism’s Shapiro–Wilk test.

**Results**
**Neuronal differentiation and BACE over-expression of BLUHMES**
Results from several studies suggest that an elevation in BACE level or activity might initiate or accelerate AD pathogenesis (Vassar et al. 2009). In order to study the effects of increased BACE levels in human neurons, we generated LUHMES cells stably overexpressing BACE (BLUHMES). As control, we also generated GFP-overexpressing LUHMES (GLUHMES), and LUHMES that over-expressed enzymatically inactive BACE (see details in materials and methods). For most studies, a polyclonal cell pool was used to avoid selection effects, but in some experiments a monoclonal BLUHMES line was used (see below). Initially, we tested whether BLUHMES displayed proper neuronal differentiation and BACE over-expression. A qPCR analysis of several pre- and post-synaptic markers during differentiation of LUHMES and BLUHMES showed that they were up-regulated in a very similar manner in both cell types (Fig. 1A). Moreover, immunostaining of, for example, the neuronal post-mitotic protein NeuN confirmed that BACE over-expression did not alter the differentiation process of the cells (Fig. 1B). Detection of BACE by western blot, and quantification of the respective bands from cells differentiated for 5 days [d5, fully mature neurons (Scholz et al. 2011, 2013)] revealed an approximately 50-fold over-expression of BACE in BLUHMES compared to LUHMES (Fig. 1C). We additionally evaluated BACE expression and localization in the cellular context by immunostaining. BACE fluorescence intensity slightly varied from cell to cell, as expected from a polyclonal cell culture. Overall, BACE staining in d5 BLUHMES somata was much brighter than in d5 LUHMES; the staining in the neurites was similar in both cell types (Fig. 1D). In summary, these data demonstrate that we generated a reasonably homogeneous, stable cell pool, which can be normally differentiated into fully post-mitotic neurons, strongly overexpressing BACE.

**BACE expression during differentiation of BLUHMES**
Prior to assessing APP processing by BACE and γ-secretase in BLUHMES, we characterized the over-expression of BACE in BLUHMES in more detail and compared it to LUHMES: we determined BACE mRNA and protein levels during differentiation of the cells and observed that there was a surprising difference between proliferating (d0) and differentiating BLUHMES (Fig. 1A and B). The level of BACE transcripts was only slightly higher in d0 BLUHMES than in d0 LUHMES and comparable to differentiated LUHMES (Fig. 1A). After initiation of differentiation, BACE expression was strongly enhanced and then remained at almost steady levels from d4 onward. At this time point, BACE mRNA levels in BLUHMES were approx. 60-fold higher than in differentiated LUHMES. Importantly, BACE mRNA expression was also considerably stronger than APP and presenilin 1 (PSEN1) mRNA expression, which remained unaffected by BACE over-expression (Fig. S3A). We corroborated this result by western blot analysis of APP and BACE expression at various stages of differentiation. In line with the mRNA data, we detected a relatively faint BACE band in undifferentiated BLUHMES and a progressive, strong up-regulation of BACE expression until d6 (Fig. 1B). Accordingly, we detected a drastic rise of BACE activity in BLUHMES cells after the second day of differentiation, whereas an increase in BACE activity from d2 to d5 in differentiating LUHMES was rather modest (Fig. 1C). APP protein up-regulation in BLUHMES was similar to the one in LUHMES; however, considering APP maturation, there was a notable reduction in the band intensity of mature APP in BLUHMES on d6 (Fig. S3B). This may be the result of extensive cleavage of this APP form in BLUHMES, and this finding is in accordance with other BACE-overexpressing systems (Lee et al. 2005). Finally, we performed further immunostainings of BACE and APP in d5 BLUHMES and LUHMES to investigate the subcellular distribution of BACE. Using two different antibodies against the N- and C-terminus of BACE, we found that the protein accumulated inside the cell bodies at sites with features of the Golgi apparatus (Fig. 1D and not shown). These sites were also detected in d5 LUHMES, but here, both antibodies also stained BACE in the cell bodies and neurites. Costaining of BACE with early-endosome-associated protein 1 revealed a certain extent of overlap in BLUHMES as well as in d5 LUHMES. However, the extent of overlap varied from cell to cell and no obvious overall difference between BLUHMES and LUHMES was observed (Fig. 1D). When we immunostained APP and its sAPP cleavage products (APP-N antibody), we found them distributed throughout the whole cell and in the neurites without differences between BLUHMES and LUHMES (Fig. S3C and D). An antibody, raised against the C-terminus of APP and therefore detecting CTFs besides full-length APP, strongly labeled certain sites within the cell...
bodies similar to the staining patterns of BACE, indicating that APP processing by BACE took place at these sites (Fig. S3D).

Taken together, we found that BACE over-expression in BLUHMES was up-regulated during differentiation in spite of an apparently ‘constitutive’ CMV promoter. Therefore, BLUHMES cells represent a model system that allowed us to study the effects of a progressive shift of the BACE-APP ratio toward the protease side, as has also been reported for the development of idiopathic AD. Notably, such a shift is different from many other model systems, in which usually the substrate (APP) level is increased.

**BACE localization and maturation in differentiated BLUHMES**

Based on our immunostaining results, we hypothesized that BACE in BLUHMES was localized primarily to a (post-/trans-) Golgi compartment. This should be mirrored biochemically in its maturation state, that is, the extent of N-linked glycosylations and the presence or absence of the propeptide. Coinmunostaining of BACE and trans-Golgi network protein 46 (TGN46) in BLUHMES showed that a high proportion of BACE was indeed localized to the labeled Golgi compartment (Fig. 2a). The remaining part most probably resided in the cis or intermediate Golgi. To test the specificity of the staining results, we treated the cells with brefeldin A (BrefA), which inhibits the protein transport from endoplasmic reticulum (ER) to Golgi and leads to accumulation of immature, only ER-modified proteins. As expected, BrefA led to a redistribution of both BACE and
TGN46 into a defined ER-staining pattern (Fig. 2a). Western blot analysis of BACE showed that BrefA treatment of BLUHMES resulted in a band with lower molecular weight, consistent with the immature form of BACE with ER-acquired N-glycosylation (Fig. 2b). To further confirm that the fully N-glycosylated Golgi form of BACE was present in differentiated BLUHMES, we treated the protein samples with N-glycosidase F (PNGase) or endoglycosidase H (EndoH). PNGase hydrolyzes nearly all types of N-glycan chains from proteins, whereas EndoH cleaves only high mannose structures attached in the ER. BACE detection on western blot revealed that PNGase completely removed the N-linked oligosaccharides from BACE in BLUHMES, leading to a reduction in molecular weight by approx. 20 kDa, whereas treatment with EndoH had no effect (Fig. 2b). Moreover, PNGase digestion of BrefA-treated BLUHMES samples resulted in a shift of the BACE band to a molecular weight of approx. 50 kDa, which was a bit higher than the band of the deglycosylated mature BACE protein (Fig. 2b). This suggested that BACE in BrefA-treated BLUHMES still carried the propeptide. In agreement with this hypothesis, the respective bands were also recognized by an antibody against the BACE propeptide (proBACE), whereas almost no proBACE was detected in control BLUHMES (Fig. 2c). These data demonstrate that overexpressed BACE in differentiated BLUHMES is primarily located in the (trans-) Golgi network and is fully mature regarding N-linked glycosylation. Therefore, initial processing of APP by BACE (to form C99/89) is likely to take place in this area, too.

Dependence of APP processing on BACE levels during cell differentiation

We next investigated the processing of endogenous APP by ectopic BACE (BLUHMES vs. LUHMES) and endogenous γ-secretase. As expected from APP and BACE expression levels, the amount of sAPPβ in the supernatant was already higher in early differentiated BLUHMES than in mature LUHMES, and it strongly increased further during differentiation (Fig. 3a). Accordingly, levels of sAPPα, originating from an alternative, Aβ-preventing cleavage of APP by α-secretase instead of β-secretase, were low in BLUHMES compared to LUHMES culture supernatants throughout differentiation (Fig. 3a). To exclude an unspecific effect due to possible cell heterogeneity, some of the major APP processing findings were re-investigated in clonally derived BLUHMES lines, yielding similar results. A full quantification was performed with BLUHMES clonal line #7 (BLc7): direct determination of sAPPβ levels in the supernatant of BLc7 by ELISA showed a strong production already in undifferentiated cells and a further increase until d6 (not shown). Quantification of western blots and ELISA data revealed that sAPPβ amounts generated by differentiated BLUHMES (pool) or BLc7 (clone), were 25- or 100-fold
higher, respectively, compared to LUHMES (Fig. 3a and not shown). In order to see whether this extensive cleavage of APP was reflected in γ-cleavage product levels, we quantified Aβ1-40 in the supernatant of BLUHMES and of different control cells during differentiation. LUHMES, GLUHMES, and D289A_BLUHMES (LUHMES overexpressing an inactive form of BACE) displayed a very similar behavior regarding Aβ production, namely increasing levels during the course of differentiation (Fig. 3b), fully in line with our earlier detailed characterization (Scholz et al. 2013).

In contrast to this, BLUHMES generated high amounts of Aβ1-40 in the immature state (d0-d2), followed by a progressive decline in Aβ release during differentiation (Fig. 3b). This effect was even more extreme in monoclonal BLC7 cells (with particularly high BACE expression; see methods), in which Aβ1-40 was extraordinarily highly produced in undifferentiated cells and hardly detectable from d4 onward (Fig. 3c). Since it has been reported that BACE-overexpressing cells preferably generate truncated (e.g., Aβ11-40) instead of full-length Aβ, we analyzed the same supernatants by an Aβx-40 ELISA, detecting Aβ independently of its N-terminus (Fig. 3c). Levels of Aβx-40 were slightly higher than Aβ1-40 levels in differentiating BLC7, indicating that minor amounts of truncated Aβ were produced in addition to Aβ1-40. However, Aβx-40 amounts clearly decreased below detection limit on d6 just as observed for Aβ1-40 (Fig. 3c). Moreover, we did not find signs of Aβ1-40 retention inside the cells or formation of aggregates as assessed by ELISA measurement of cell lysates and Thioflavin S staining (not shown).

One biochemical explanation could be that Aβ1-40 is further degraded by BACE to Aβ1-34, or other C-terminally truncated species, which are not detected in the Aβ1-40 ELISA (Fluhrer et al. 2003; Caillava et al. 2014). We therefore analyzed cell supernatants with an ELISA detecting C-terminally truncated Aβ (but also Aβ1-40/42). Both LUHMES and GLUHMES increased the production substantially (about 10-fold) during their neuronal differentiation. In contrast to this, the production of Aβ1-40 by BLC7 was high in the undifferentiated state and did not increase with differentiation. Notably, Aβ1-40 levels were overall lower than those of LUHMES or GLUHMES (Fig. 3d). From this result, we concluded that C-terminally truncated Aβ species (possibly including Aβ1-34) were indeed produced in
substantial amounts by all our cell lines. However, the correlation with the level of BACE activity was less clear than previously reported (Fluhler et al. 2003), as for instance 75% of Aβ in GLUHMES with low BACE activity comprised of variants other than Aβ1-40, while it was only 50% in undifferentiated BLc7 cells with moderately increased BACE levels. Moreover, the consideration of C-terminally truncated Aβ species did not explain the lack of an overall enhanced Aβ generation in the cells with very high BACE activity.

As reported earlier (Scholz et al. 2013), LUHMES produced too low amounts of C99 and Aβ1-42 to be detected by any of several methods we used, and the same was true for BLUHMES. As alternative approach to investigate whether APP might be handled and distributed differently in LUHMES/BLUHMES, we quantified the C-terminal phosphorylation of APP at threonine 668 (Thr668). This post-translational modification, known to be associated with amyloidogenic processing and axonal transport of APP (Ando et al. 1999; Iijima et al. 2000; Akiyama et al. 2005) was reduced to 36 ± 7% in BLUHMES relative to LUHMES (n = 3, Fig. S5A). Moreover, we examined the mRNA expression levels of different receptors and adaptors that affect intracellular localization and processing of APP. While Golgi-associated, gamma adaptin ear containing, ADP-ribosylation factor-binding (ARF) protein 1 (GGA1), GGA3, sorting nexin-6, and sortilin related receptor 1 differed by maximally 20% between LUHMES and BLUHMES, sortilin related VPS10 domain containing receptor 1 (SORCS1) expression in BLUHMES was 400 ± 80% compared to LUHMES (p < 0.05, Fig. S5B).

In summary, these data indicate that although increased levels of BACE on one hand correlated with a higher cellular β-secretase activity (sAPPβ production), this activity on the other hand did not correlate with the production of Aβ. This unexpected finding may be because of altered intracellular sorting of proteases and their substrates, but it was not as a result of the high levels of BACE protein per se, because cells expressing similar levels of BACE but in an inactive form showed normal Aβ production (Fig. 3b). Our main interest was to examine next whether the altered processing of APP would affect the pharmacology of secretase inhibitors.

**Modification of Aβ generation by BACE inhibitors in BLUHMES versus LUHMES**

The important finding, that mice with genetic deletion of BACE did not generate any Aβ (Cai et al. 2001; Luo et al. 2001), has placed BACE into the center of interest as a target for AD therapeutics. Therefore, numerous small-molecule inhibitors of BACE are under development (Volbracht et al. 2009; De Strooper et al. 2010). We used such inhibitors to examine which effect either partial or complete reduction in BACE activity would have in LUHMES/BLUHMES cells. First, we treated the cells with the cell permeable BACE inhibitor IPAD. As expected, Aβ secretion was lowered in d5 BLUHMES with a highly reproducible dose–response curve and low IC50 value (Fig. 4a). In d1 BLUHMES, BACE...
inhibition was still achieved by IPAD, although at slightly higher concentrations with a 2.5-fold IC50 (Fig. 4a), possibly because of the higher amounts of BACE. In d5 BLUHMES, at the approximate IC50 concentrations derived from d1 BLUHMES cultures, we surprisingly detected a peak-shaped, strong increase in Aβ1-40 generation, instead of an inhibition (Fig. 4a). Western blot analysis of α-/β-cleavage fragments of APP in the supernatant of treated and untreated BLUHMES showed that the Aβ rise was connected to a clear decrease in sAPPβ and a slight increase in sAPPα (Fig. 4b).

As it has been demonstrated that BACE inhibition can lead to an increased generation of Aβ5-40 at the expense of Aβ1-40 (Mattsson et al. 2012), we quantified Aβx-40 in the supernatants of IPAD-treated BLUHMES cells and detected essentially the same amounts as with the Aβ1-40 ELISA (Fig. S6). This indicates that in our system, N-terminally truncated species did not considerably contribute to the observed rise in Aβ after partial BACE inhibition. The findings with IPAD were fully reproduced with aminoquinazoline derivative, another potent BACE inhibitor (Fig. 4c).

In order to investigate whether similar effects were observable under blockage of cell surface BACE, we incubated the cells with the highly potent (Ki = 1.6 nM) (Gosh et al. 2000), but cell impermeable peptidomimetic inhibitor OM99-2. Inhibition was only achieved at high concentrations in d5 LUHMES cells and did not completely block Aβ production (Fig. 4d). In d1 BLUHMES, even at the highest non-toxic concentration, a maximum of ~ 50% Aβ reduction was obtained and in d5 BLUHMES, the inhibitor had no effect at all (Fig. 4d). These findings may be explained by only a minor amount of plasma membrane BACE being involved in Aβ generation in differentiated BLUHMES (in line with its localization to the Golgi, as described above). From these data and previous ones, we concluded that an increase in BACE expression levels, and therefore BACE activity, beyond a certain threshold leads to a decreased production of Aβ, and that under such conditions, BACE inhibitors may show paradoxical effects, that is, an elevation of Aβ levels.

Modification of Aβ generation in BLUHMES versus LUHMES by α-/γ- secretase inhibition

We next tested how Aβ generation in BLUHMES would be affected by inhibitors of α- or γ-secretase. We compared the Aβ1-40 production of d5 LUHMES, d1 and d5 BLUHMES; inhibition of α-secretase in d5 LUHMES by GM6001 led to an increase in Aβ generation, as expected. By contrast, the inhibitor had no effect on the Aβ levels released by d5 BLUHMES (Fig. 5a). This is in line with the notion that

![Fig. 5 α- and γ-secretase inhibition in BLUHMES versus LUHMES.](image-url)

LUHMES (L) and BLUHMES (BL or BLc7) cells were treated on the indicated days of differentiation after a medium change with non-toxic concentrations of different secretase inhibitors or solvent (Ctrl.). After 24 h, the conditioned supernatants were analyzed for the concentration of secreted Aβ1-40 (A,B + D) or Aβ1-x (C) by ELISA. (a) Cells were treated with the cell permeable α-secretase inhibitor GM6001 (IC50 = 8 μM in d5 L). (b) Cells were treated with the cell permeable γ-secretase inhibitors LY450139 or DAPT. LY450139: IC50 = 40 nM in d1 BL, 190 nM in d5 L, and 160 nM in d5 BL. DAPT: IC50 = 620 nM in d5 L and 450 nM in d5 BL. Data are means ± SD (n = 3 biological replicates). *p < 0.05 (L ctrl. vs. BLc7 ctrl.); ***p < 0.001 (ctrl. vs. treated). (c) Cells were treated with 5 μM of DAPT. Data are means ± SD (n = 3 independent samples). (d) Treatment of d5 cells with low concentrations of DAPT and LY450139. Data are means ± SD (n = 3 independent samples) and were analyzed by one-way ANOVA with Dunnett’s post hoc test, **p < 0.01, ctrl. versus treated (different conc.).
Exposure of LUHMES to the γ-secretase inhibitor LY450139 resulted in the expected reduction in Aβ levels (Fig. 5b). Likewise, in d1 BLUHMES, Aβ production was blocked by LY450139 in a concentration-dependent manner (IC50 = 40 nM). In d5 BLUHMES, exposed to LY450139 at concentrations > 20 nM, the generation of Aβ was not detectable anymore at all (Fig. 5b). All these predictable pharmacological findings for Aβ1-40 were fully corroborated when d5 LUHMES or BLUHMES were exposed to the γ-secretase inhibitor DAPT (Fig. 5b), which also abolished the production of Aβ1-x by both LUHMES and BLUHMES clone 7 (Fig. 5c).

As we were particularly interested in the pharmacological response under conditions of slight to moderate secretase inhibition, we closely explored the low concentration range for the two γ-secretase inhibitors. Under such conditions, we observed a peak-shaped increase in Aβ1-40 (Fig. 5d) for d5 LUHMES but not for d5 BLUHMES. The LY450139-/DAPT-induced Aβ rise observed here is a phenomenon well-described in the literature for cell systems with relatively low levels of C99 (Lanz et al. 2006; Burton et al. 2008; Wolfe 2012).

Modulation of Aβ production independently of secretase inhibitors and cell differentiation

In order to see to which extent the findings with secretase inhibitors could be generalized, we investigated whether Aβ production was altered in similar ways by indirect modulations of BACE activity. We incubated both d5 LUHMES and BLUHMES with substances, known to elevate the pH in intracellular acidic compartments. Since BACE has an acidic pH optimum, this is expected to lead to a decrease in Aβ production by reduced β-cleavage (Schrader-Fischer and Paganetti 1996). Accordingly, we found bafilomycin, an inhibitor of the vacuolar-type H+-ATPase, to be very potent (EC50 = 2.2 nM) regarding the lowering of Aβ1-40, observed in d5 BLUHMES (Fig. 6a). Similarly, the endosome modifier bepridil (Mitterreiter et al. 2010) led to a reproducible reduction in Aβ generation with an EC50 value of 2 μM (Fig. 6b). Analogous to the response under pharmacological BACE inhibition, the effect in d5 BLUHMES cells was exactly the opposite, meaning that bafilomycin or bepridil treatment resulted in an increase in Aβ production (Fig. 6a and b), paralleled by a reduction in the amounts of sAPPβ produced (not shown). When measuring Aβ1-x instead of only intact Aβ1-40 treatment of LUHMES with a relatively high concentration of bepridil led to a strong reduction in those Aβ species, whereas hardly any effect was detectable on the Aβ1-x generation by BLUHMES clone 7 (Fig. 7S). In the next step, we sought to reproduce the effect of decreased Aβ1-40 observed in d5 BLUHMES, in d1 BLUHMES. To this end, LUHMES and BLUHMES were kept in slightly acidified medium (pH ~ 6.7) in order to enhance BACE activity. In d1 LUHMES, Aβ generation was not

α- and β-secretases are competing for APP to some extent in LUHMES, but not at all in BLUHMES (with respect to Aβ generation).
significantly affected, whereas in d1 BLUHMES (normally releasing high amounts of Aβ), acification resulted in a significant reduction in Aβ (Fig. 6c).

Opposite results were obtained in d5 cells kept at low pH: in LUHMES, Aβ production was increased, while no effect was observed on d5 BLUHMES cells (Fig. 6c). Western blot analysis of supernatants and cell lysates revealed that the incubation with acidic medium led to an increase in BACE expression and β-cleavage both in BLUHMES and LUHMES cells (Fig. 6d and not shown). These data fully corroborate the findings obtained with secretase inhibitors by an independent approach. Taken together, the data suggest that the different effects on Aβ production (decrease and peak), detected in d1 versus d5 BLUHMES, were not a primary consequence of the differentiation status of the cells, but were rather linked to the expression level and activity of BACE. Here, we showed that BACE level/activity could be either altered using cells of different age or by several other approaches. Most likely, such different amounts of active BACE affected the quantity of substrate (C99) for γ-secretase in one of the cell compartments relevant for Aβ generation (Fig. 7).

Discussion

During the development of AD, localization and ratios of the principal components of the Aβ pathway may change. In already diseased brains of sporadic AD cases, the BACE level/activity is thought to be raised without an overall change in APP or γ-secretase levels (Fukumoto et al. 2002; Yang et al. 2003). In contrast to this, the most widely used cell and mouse models over-express APP to facilitate measurements of Aβ and the formation of plaques (Duyckaerts et al. 2008). BACE over-expression always leads to an enhanced cleavage of APP (formation of sAPPβ or C99), but literature reports differ considerably concerning the consequences on Aβ generation (Bodendorf et al. 2002; Huse et al. 2002; Liu et al. 2002; Lee et al. 2003a, 2005; Chiocco et al. 2004; Rockenstein et al. 2005). To further investigate this matter and the related pharmacology in human neurons, we established here a stable LUHMES cell line (BLUHMES) with ectopic BACE expression, but unchanged endogenous levels of APP and γ-secretase. We showed that the cells (polyclonal pool or monoclonal cell line) normally differentiated into post-mitotic neurons and that neither APP nor PSEN1 expression were affected by BACE over-expression.

The levels of BACE in differentiated BLUHMES were much higher than in LUHMES. The over-expression ratio used here may be beyond the normal regulation level in health and disease. However, it needs to be considered that LUHMES have a relatively low BACE expression compared to human cortical neurons (based on our mRNA expression data). In our over-expression model, we observed that BACE mRNA levels augmented during differentiation of the neurons, paralleled by increasing amounts of BACE protein and of the direct cleavage product sAPPβ. The strong enhancement of CMV promoter activity during neuronal differentiation is a typical feature of LUHMES cells and is observed in our laboratory for a large panel of proteins. Here, we took advantage of this effect to investigate the influence of increasing BACE levels on Aβ generation.

Similar to the results of in vivo studies (Lee et al. 2005), we found that generation of Aβ1-40 was high in young BLUHMES with moderately elevated BACE activity and then decreased with increasing levels of BACE in more
mature BLUHMES. In this context, it was important to demonstrate that APP indeed underwent efficient cleavage by BACE. As direct cleavage product, we measured here sAPPβ, and we assume that the amount of this N-terminal APP fragment correlated with the amount of CTFs (such as C99) that were not quantifiable in our system. Following the β-secretase cut, the intracellular fate of APP (e.g., degradation or further processing into Aβ) is highly complex, and was found here to differ between LUHMES and BLUHMES. Notably, the LUHMES/BLUHMES model system displayed an interesting behavior observed earlier as paradoxical Aβ rise.

This effect, also called ‘Aβ rebound’ (Li et al. 2012) due to partial inhibition of γ-secretase, has been frequently reported in the literature, and the conditions favoring it have been investigated. One important factor seems to be the level of C99 generated in an experimental system, with low level systems (e.g., expressing wt APP) showing the phenomenon, and high level systems (e.g., expressing APP with the Swedish mutation that is a preferential substrate of BACE) not doing so (Burton et al. 2008). These findings are well in line with our results obtained for the partial inhibition of γ-secretase in d5 LUHMES and BLUHMES cells, and together with the sAPPβ quantification strengthen our assumption that d5 LUHMES are a low and BLUHMES a high C99 level system (Fig. 7).

The key finding of this study was that partial inhibition of BACE (by pharmacological inhibitors or agents raising the pH) led to an increase in Aβ production by BLUHMES cells. These data strongly suggest that the extent of Aβ generation is not always directly linked to the cleavage activity of BACE, and therefore to the amounts of the produced fragments. Mouse studies have clearly shown that the amount of BACE in the brain and Aβ production are not linearly correlated (Georgievska et al. 2015), and both genetic and pharmacologic approaches have been used to show that reduction in BACE activity may not always lower Aβ levels and plaque load (Devi and Ohno 2013; Devi et al. 2015). In view of such findings, the data produced here may help to interpret results from clinical trials and mouse models: researchers have argued that partial BACE inhibition would lead to unwanted (augmenting) effects on Aβ. As the amount of BACE can determine the downstream pathway of APP processing to be amyloidogenic or not (Fig. 7), the large heterogeneity in human brain concerning the levels of BACE and APP may require some more detailed investigation.

We want to clearly acknowledge that the BLUHMES model with its relatively high ectopic BACE expression might be built on biological conditions that are not fully representative of human brain. However, the data presented here warrant further studies into whether there are not at least some groups of neurons in the brain that would behave like BLUHMES. This could be particularly true for a diseased brain, for which at least some puzzling evidence has been provided that BACE inhibitors lose their pharmacological efficacy over time (Devi and Ohno 2013; Devi et al. 2015). Moreover, at least some Aβ species seem to increase in cerebrospinal fluids upon BACE inhibition (Mattsson et al. 2012).

As LUHMES are a complex cell model that can be influenced by differentiation processes taking place during the assessment of APP metabolism, we investigated in one set of experiments whether our observations were independent of cell differentiation, but solely linked to BACE level/activity. Therefore, we performed acidification experiments, in which the pH of the medium was lowered to ~ 6.7. This resulted in an increase in BACE expression and activity already in young BLUHMES, connected to the same Aβ lowering effect as observed before in differentiated BLUHMES. Further investigations will be needed to determine the intracellular effects of an elevated medium pH on BACE expression and activity.

Another important point of investigation was the determination of N- and C-terminally truncated Aβ species (in particular, Aβ1-40 and Aβ1-34), since their generation has been often described for BACE-overexpressing systems (Huse et al. 2002; Liu et al. 2002; Lee et al. 2003a). Especially neuronal in vitro cultures may produce high amounts of Aβ1-34, and this species is also abundant in the cerebrospinal fluid (Fluhrer et al. 2003; Mattsson et al. 2012). Importantly, we did not detect considerable amounts of N-terminally truncated Aβ in the supernatant of BLUHMES cells, even after partial inhibition of BACE. This also held true for the BLc7 clone, which displayed a particularly extreme decline from very high to non-detectable Aβ1-40 in dependence on BACE levels. As N-terminal truncation did not offer an explanation for paradoxical effects observed in BLUHMES, we addressed C-terminally truncated Aβ species. The levels of Aβ1-34 that we detected in the supernatant of differentiated BLc7 cells were lower compared with LUHMES or GLUHMES control cells, and the increase in BACE during differentiation of BLUHMES did not lead to more Aβ1-34 formation, but rather to a decline. There is obviously the possibility that there are multiple, potentially competing degradation pathways for full-length and truncated Aβ variants. Neprilysin has been shown to degrade both Aβ1-40 and Aβ1-34 either prior to or after their secretion (Hama et al. 2001; Caillaud et al. 2014), and BACE inhibition in vivo decreases most known truncated and non-truncated Aβ variants, but it strongly increases Aβ5.
Such species may be formed independently of known secretases, but we showed here at least for Aβ1-40 and Aβ1-44 that their generation strictly depends on γ-secretase and thus likely follows the canonical pathway.

Besides the hypothetical channeling of Aβ in BLUHMES to a rapid degradation pathway that makes it undetectable, we see two possible explanations for the paradoxical behavior of BLUHMES. The first one is that the CTFs, produced by BACE, encounter γ-secretase but cannot be properly processed by it. It has been argued already earlier that γ-secretase activity might be impaired in the presence of very high levels of C99 (Vassar et al. 1999). This could be explained by the finding that APP possesses a substrate inhibitory domain within the C99 fragment, which blocks γ-secretase activity (i.e., reduces secreted Aβ) in a concentration-dependent manner (Tian et al. 2010). However, there is also evidence suggesting that γ-secretase activity is little compromised by high C99 levels: Notch processing by γ-secretase was similar in wild-type mice and BACE-overexpressing mice (Rockenstein et al. 2005). Alternatively to a ‘competitive’ or allosteric inhibition of γ-secretase, the structures required for γ-processing and Aβ formation may be damaged. A recent publication showed that in cells with high BACE activity, the retromer complex was affected and APP processing was defective (Mecozzi et al. 2014). Moreover, in cells with extensive β-cleavage of APP, large amounts of CTFs accumulated in rab5-positive early endosomes and ultimately rendered them dysfunctional (Kim et al. 2016). Although not explicitly stated by the authors, it can be seen as likely that further processing of the CTFs by γ-secretase was impaired under those conditions as well.

The second possible cause for the reduced generation of Aβ in BLUHMES is that APP and BACE localization, and therefore APP trafficking and processing, are altered at high BACE levels (Fig. 7). This could lead to a shift in the cellular site of β-cleavage and to an accumulation of CTFs in compartments in which γ-secretes is not present as fully active enzyme complex. It appears that Aβ production from wt APP takes place primarily in endosomes and at synapses (Koo and Squazzo 1994; Perez et al. 1999; Pasternak et al. 2004), and the Aβ rise at γ-inhibition, which was in fact absent in BLUHMES cells, was reported to occur only when Aβ is produced in the endosomal trafficking pathway (Burton et al. 2008). Moreover, it was suggested that C99 has to be transported at least to the cell surface to be efficiently processed by γ-secretase (Kaether et al. 2006). Importantly, several studies underscore the prevalence of PSEN1 in the degradative limb of the endosomal sorting compartments, that is, late endosomes, multivesicular bodies, and lysosomes (Kaether et al. 2002; Chyung et al. 2005; De Strooper and Annaert 2010). A role of γ-secretase at synapses is also suggested by its cleavage of substrates other than APP-CTFs (Restituito et al. 2011). On the other hand, BACE is known to be localized to the trans-Golgi compartment and endosomes. This has been confirmed in several neuronal and non-neuronal cells with endogenous and ectopically over-expressed BACE (Huse et al. 2000; Walter et al. 2001; Yan et al. 2001). It is also in accordance with our findings from BACE immunocytochemistry and maturation studies. In the BACExAPP mouse models, β-cleavage of APP was found to be enhanced in the trans-Golgi network (TGN) (Lee et al. 2005). The authors hypothesize that this increased processing in the TGN prevented full-length APP from being transported to the axons, because fully glycosylated APP forms were diminished. Our findings point into a similar direction, since the mature form of APP was reduced in BLUHMES compared to LUHMES, and C-terminal detection of APP (fragments) yielded a particularly strong signal in the cell bodies. According to Lee et al. (2005), the fact that APP and C99, phosphorylated at Thr668, were hardly detectable in BACExAPP mice, was another argument in favor of this theory (Lee et al. 2005). Indeed, it is known that this post-translational modification of APP is specifically detected in neurites but not cell bodies of mature neurons (Iijima et al. 2000), and that it is involved in the regulation of APP processing (Lee et al. 2003b; Pastorino et al. 2006). Taken together with our finding that SORCS1 was increased in BLUHMES – over-expression of SORCS1 was previously shown to reduce Aβ levels, probably by influencing γ-secretase activity (Lane et al. 2010; Reitz et al. 2011) – it is possible that a shift of the APP β-cleavage site from the endocytic to the secretory compartments could preclude Aβ formation by preventing the further transport of APP and/or the CTFs, thereby altering their fate (Fig. 7). Under such circumstances, reduction in the β-cleavage by partial inhibition of BACE would restore to a certain extent, the normal trafficking routes and lead to increased generation of Aβ, which is in fact what we see in BLUHMES.

In summary, our study has demonstrated that the relative amounts and intracellular distribution of the key components of the amyloidogenic pathway (secretases and their substrates) can have a dramatic effect on the pharmacology of secretase inhibitors, not just by slightly altering potency or efficacy, but by inducing entirely unexpected responses. Although it is unclear whether the model system used here is of strong clinical relevance, we believe that the findings are important for guiding the design of future neurochemical studies in the field. The levels, activities, and subcellular localizations of the enzymatic components of the APP processing pathway may need to be more extensively characterized for different neuronal types in AD brains. This knowledge would need to be compared with similar information from the multiple models used to test future drugs designed to affect the amyloidogenic pathway of APP processing, and a systematic study of Aβ generation and effects of secretase inhibitors over a wide range of APP/BACE ratios would for sure be of high interest.
Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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Disclosure statement

Marcel Leist is an editor for Journal of Neurochemistry. The authors declare no conflict of interest. This manuscript is based on the PhD thesis prepared by D. Scholz (Scholz, 2011. http://nbn-resolving.de/urn:nbn:de:bsz:352-173910).

Supporting information

Additional Supporting Information may be found online in the supporting information section at the end of the article. Figure S1. Antibodies used for immunodetection (ICC and WB) and primers used for qPCR.

Figure S2. Basic characterization and neuronal differentiation of BLUHMES versus LUHMES.

Figure S3. APP expression in BLUHMES versus LUHMES.

Figure S4. Focus on apparent sAPP sizes generated by BLUHMES.

Figure S5. APP phosphorylation and expression of trafficking-related genes in BLUHMES versus LUHMES.

Figure S6. Generation of Aβ1-40 versus Aβx-40 by BLUHMES upon partial inhibition of BACE.

Figure S7. Effect of bepridil treatment on Aβ1-x generation by BLUHMES versus LUHMES.

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Multiplicy attenuated lentiviral vector achieves efficient gene