

Comparison of Pharmacological Modulation of APP Metabolism in Primary Chicken Telencephalic Neurons and in a Human Neuroglioma Cell Line

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Abstract Sequential cleavage of amyloid precursor protein (APP) by β - and γ -secretases and the formation of A β peptides are pivotal for Alzheimer's disease. Therefore, a large number of drugs has been developed targeting APP metabolism. However, many pharmacological compounds have been identified in vitro in immortalized APP over-expressing cell lines rather than in primary neurons. Here, we compared the effect of already characterized secretase inhibitors and modulators on A β formation in primary chicken telencephalic neurons and in a human neuroglioma cell line (H4) ectopically expressing human APP with the Swedish double mutation. Primary chicken neurons replicated the effects of a β -secretase inhibitor (β -secretase inhibitor IV), two γ -secretase inhibitors (DAPM, DAPT), two non-steroidal-anti-inflammatory drugs (sulindac sulfide, CW), and of the calpain inhibitor calpeptin. With the exception of the two γ -secretase inhibitors, all tested compounds were more efficacious in primary chicken telencephalic neurons than in the immortalized H4 cell line. Moreover, H4 cells failed to reproduce the effect of calpeptin. Hence, primary chicken telencephalic neurons represent a suitable cell culture model for testing drugs interfering with APP processing and are overall more

sensitive to pharmacological interference than immortalized H4 cells ectopically expressing mutant human APP.

Keywords Alzheimer's disease · A β secretion · Culture model · Secretase · Drug testing

Introduction

Alzheimer's disease (AD) is the most abundant age-related form of dementia. Insoluble amyloid plaques consisting of fibrillar A β 42 aggregates are one of the hallmarks of Alzheimer's pathology (Hardy and Selkoe 2002).

A β peptides are generated by β - and γ -secretase-mediated cleavage of the amyloid precursor protein (APP), whereas α -secretase activity produces only non-amyloidogenic APP fragments and prevents A β production (Haass 2004). The N-terminus of the A β domain is cleaved by β -secretase (BACE-1), a step which is essential for the formation of all A β peptides including A β 38, A β 40, and A β 42 (Cole and Vassar 2008). The γ -secretase is a protein complex consisting of four subunits, presenilin 1 (PS1) or PS2, APH-1a or APH-1b, PEN-2, and nicastrin, and cuts APP at the intramembranous C-terminal end of the A β domain (Wolfe 2006). A β 42 has the biophysical property to aggregate fast and to form soluble oligomers as well as fibrillar structures, in contrast to A β 40 and other A β peptides (Dahlgren et al. 2002; Walsh and Selkoe 2007). Indeed, soluble A β 42 oligomers were identified as major pathological source causing neuronal toxicity and cognitive impairment (Haass and Selkoe 2007). In humans, A β 42 levels are elevated in the cerebral cortex in both sporadic and familiar forms of AD. Mutations either directly in the APP gene, such as the Swedish double mutation at K595N/M596L in human

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APP (Citron et al. 1992; Mullan 1992; Mullan et al. 1992; Citron et al. 1995) or in presenilin genes (De Strooper 2007) elevate total A β 42 content by increasing β - or γ -secretase-mediated APP cleavage. Thus, A β 42 formation plays a pivotal role in AD.

In recent years, numerous pharmacological substances have been developed that either inhibit or modulate β - and γ -secretase activity to reduce A β production (Ghosh et al. 2008; Imbimbo 2008; Wolfe 2008; Silvestri 2009). However, many in vitro screens aiming to identify substances that alter APP metabolism have been performed in neuronal or non-neuronal cell lines highly overexpressing human wildtype or mutant APP. In contrast to immortalized cell lines, primary neurons produce endogenous levels of APP, undergo differentiation, and form functional synapses and thus exhibit a more physiological system for drug testing.

Primary chicken neurons fulfill the criteria that are required for analyzing APP processing. First, chicken embryos express the long APP751 and APP695 variants during development, which share 93% sequence identity with human APP (Carrodeguas et al. 2005; Sarasa and Pesini 2009). The human and chick A β 42 sequences are identical in contrast to rodent A β domains, which differ in several amino acids from the human sequence. In addition, chick embryos express the essential enzyme machinery required for both amyloidogenic and non-amyloidogenic processing of APP including α -, β -, and γ -secretases (Carrodeguas et al. 2005; Sarasa and Pesini 2009). Indeed, primary cultures of chicken telencephalic neurons secrete detectable levels of A β 1-38, A β 1-40, A β 1-42, and soluble APP into their cell supernatant (Esselmann et al. 2004; Leuchtenberger et al. 2009).

In this study, we examined primary chicken telencephalic neurons and a human neuroglioma cell line overexpressing human APP695 with the Swedish double mutation as cell culture models for testing compounds that interfere with APP processing. Therefore, substances previously described to inhibit or modulate APP cleavage were administered, and effects on A β 38, A β 40, and A β 42 formation were evaluated.

Material and Methods

Materials

The following compounds were included in the study: CW (3,5-bis(4-nitrophenoxy)benzoic acid), DAPM (*N*-(*N*-3,5-difluorophenacetyl)-*L*-alanyl-*S*-phenylglycine methyl ester), DAPT (*N*-[*N*-(3,5-difluorophenacetyl)-*L*-alanyl]-*S*-phenylglycine *t*-butyl ester), calpeptin (benzyloxycarbonylleucyl-norleucinal), sulindac sulfide

((*Z*)-5-fluoro-2-methyl-1-[*p*-(methylthio)benzylidene]indene-3-acetic acid), and β -secretase inhibitor IV (all Calbiochem). All compounds were dissolved in dimethyl sulfoxide. The amount of vehicle was kept constant for all concentrations of a respective compound.

For primary chicken telencephalic cultures, Nu Serum was purchased from Becton Dickinson (BD). All other cell medium components, unless indicated, were purchased from Lonza.

Culture of Primary Embryonic Chicken Telencephalic Neurons

Preparation of chicken telencephalic neurons was performed according to Pettmann et al. 1979 with modifications. Embryonic day 8 (E8) embryos from fertilized Lohman Brown hybrid chicken eggs were isolated, and telencephalic neurons were mechanically dissociated using a sterile nylon sieve with pore size 100 μ M (BD Falcon). Neurons were maintained in Dulbecco's Modified Eagles Medium containing 4.5 g glucose/l, 5% Nu Serum, 0.01% gentamicin, and 2 mM L-glutamine.

For the A β secretion assay, chicken neurons were plated on poly-D-lysine pre-coated 24-well plates (BD Biocoat) at a cell density of 1.8×10^6 neurons per 24 well. After 48 h, culture medium was exchanged, and neurons were incubated for 24 h with different concentrations of compounds in a total volume of 300 μ l culture medium. For the MTT viability assay, neurons were plated on poly-D-lysine pre-coated 96-well plates (BD Biocoat) at a cell density of 3×10^5 neurons per 96 well in a total volume of 160 μ l culture medium.

Culture of H4 Cells Ectopically Expressing Human APP_{K595N/M596L}

A human neuroglioma H4 cell line stably transfected with the pAG3 vector containing human APP695 with the Swedish double mutation K595N/M596L was used as cell line model (Haugabook et al. 2001). The pAG3 vector is a modified pcDNA3 plasmid and contains the hygromycin-B resistance cassette with the transgene under the control of a fused CMV and chicken β -actin promoter. H4 cells were plated on 96-well plates (BD) at a cell density of 1×10^4 cells per 96 well in 160 μ l OptiMEM (Invitrogen) containing 10% fetal bovine serum, 1% penicillin/streptomycin, 200 μ g/ml hygromycin-B (Roth), and 2.5 μ g/ml blasticidin-S (BioChemika). After 48 h, culture medium was exchanged and neurons were incubated for 24 h with different concentrations of compounds in a total volume of 60 μ l culture medium. All cultures were kept at 37°C and 5% CO₂. Viability of cultures was determined by the MTT assay (Gutmann et al. 2002).

Detection of A β Peptides

After treatment, cell supernatants were snap frozen at -80°C and defrosted prior to A β measurement. At least three separate wells were analyzed per experiment for each condition.

Cell supernatants were examined for A β 38, A β 40, and A β 42 content with a commercially available A β -Triplex kit from Mesoscale Discovery (MSD). Conditioned media of primary chicken telencephalic cultures were measured undiluted, whereas supernatants of H4 cells were diluted 1:10 with culture medium prior to measurement. For capture, A β 38, A β 40, and A β 42 specific antibodies binding the C-terminus of the corresponding A β peptide (provided by MSD) were used. Bound A β peptides were detected with a monoclonal 6E10 antibody and quantified by electrochemiluminescence. A β levels in cell supernatants were evaluated in comparison to an A β peptide standard in picogram or nanogram per milliliter. Vehicle-treated control cultures were set as 100%. All data of compound-treated cultures are displayed in relation to vehicle-treated controls as mean \pm standard error of mean. The total A β content was calculated as sum of A β 38, A β 40, and A β 42.

Data Analysis

GraphPad Prism (GraphPad Software, Inc., San Diego, CA) was used to graph and analyze data.

Results

A β Peptides are Detectable in Conditioned Media of Primary Chick Cultures and H4 Cells Expressing Human APP_{K595N/M596L}

Chicken embryos express endogenous APP in the developing telencephalon and also the required enzymes for A β formation (Carrodeguas et al. 2005). Hence, we first determined the amount of A β 38, A β 40, and A β 42 being released into the cell supernatant by primary E8 chicken telencephalic neurons and H4 cells stably expressing human APP_{K595N/M596L}.

Primary chicken neurons were cultured in 24-well plates at high cell density for 48 h. For determining A β production, 300 μl of fresh medium were applied for 24 h. H4 cells overexpressing human APP_{K595N/M596L} were plated in 96-well plates and grown to 80–90% confluency. Cells were then incubated with 60 μl of fresh medium for 24 h. Conditioned media of cultures were then analyzed for all three A β analytes using a Triplex immunosorbent assay.

Both culture systems released A β 38, A β 40, and A β 42 peptides at detectable levels (Fig. 1a, b). H4 cells overexpressing human APP_{K595N/M596L} secreted 1.7 ± 0.1 ng/ml A β 38, 24 ± 1.0 ng/ml A β 40, and 1.9 ± 0.1 ng/ml A β 42 (27.8 ± 1.0 ng/ml total A β), whereas primary telencephalic neurons produced 0.11 ± 0.01 ng/ml A β 38, 1.37 ± 0.06 ng/ml A β 40, and 0.17 ± 0.01 ng/ml A β 42 (1.64 ± 0.08 ng/ml total A β). The A β content in cell free culture medium was negligible (data not shown). Thus, the H4 cell line produced at least tenfold more A β peptides than primary telencephalic chicken neurons.

However, the relative abundance of different A β peptides was comparable between cultures of primary neurons and the H4 cell line. The main A β species detected in conditioned media was A β 40, which comprised more than 80% of all analyzed A β forms. A β 38 and A β 42 were produced considerably less than A β 40 but both in similar quantities at approximately 7–10%. Importantly, the relative

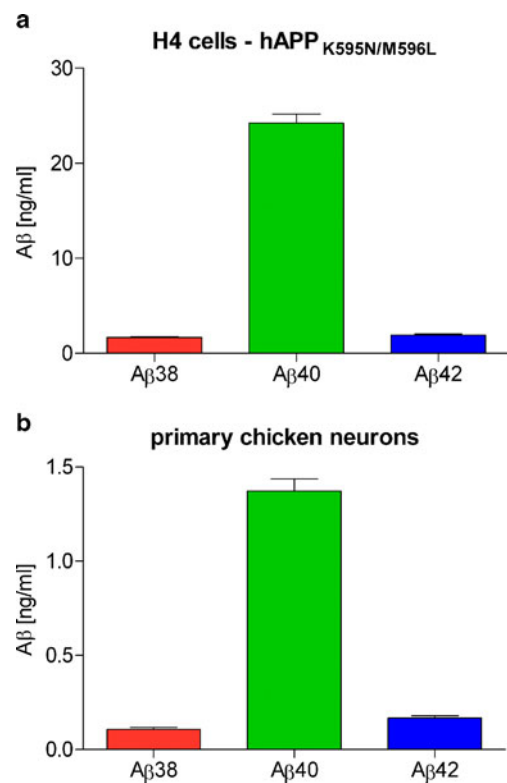


Fig. 1 Immortalized cell line produces more A β peptides than primary neurons in vitro. **a** The H4 neuroglioma cell line overexpressing human APP_{K595N/M596L} was assessed for ectopic A β production by an immunosorbent assay. The H4 cell line generated more A β 38, A β 40, and A β 42 peptides in a 24-h time period than primary neurons. A β 40 was the predominant A β peptide. **b** Supernatants of primary telencephalic chicken neurons were examined for endogenous A β production by an immunosorbent assay. Primary neurons secreted detectable amounts of A β 38, A β 40, and A β 42 peptides in a 24-h time period. A β 40 was the predominant A β peptide. Graphs represent the individual A β content as mean \pm standard error of mean in nanogram per milliliter

abundance of A β peptides in both culture systems correlates with results from previously described primary chicken telencephalic cultures (Esselmann et al. 2004). Hence, primary chicken telencephalic neurons and H4 cells stably transfected with human APP_{K595N/M596L} produced sufficient amounts of A β 38, A β 40, and A β 42 peptides and could be used for further analysis.

To compare the effect of drugs modulating or inhibiting the generation of A β 38, A β 40, and A β 42 peptides in cultures of primary chick neurons and in the H4 neuroglioma cell line, we examined already characterized β - and γ -secretase inhibitors, non-steroidal anti-inflammatory drugs (NSAIDs), and a calpain inhibitor. Almost all of the selected compounds were demonstrated to alter APP processing but not total APP protein levels (Dovey et al. 2001; Weggen et al. 2001; Mathews et al. 2002; Na et al. 2007). Hence, supernatants of compound-treated cultures were analyzed for their A β 38, A β 40, and A β 42 content by an immunosorbent assay assuming that total APP levels remained unaffected. Viability of cultures was assessed in a colorimetric MTT assay. The respective IC₅₀ and E_{max} of each compound are summarized in Table 1.

BACE-1 Inhibition is More Potent in Primary Neurons than in the H4 Cell Line Expressing Human APP with the Swedish Double Mutation

First, we examined the β -secretase inhibitor IV, a commercially available BACE-1 inhibitor, which has been previously shown to inhibit BACE-1 in a HEK cell culture model overexpressing APP_{NFEV} at an IC₅₀ of 15 nM (Stachel et al. 2004). When the β -secretase inhibitor IV

was applied on the H4 cell line, a concentration-dependent decrease in A β secretion (IC₅₀ A β 38=92.3 nM, IC₅₀ A β 40=124.7 nM, IC₅₀ A β 42=215.6 nM) was evident (Fig. 2a), but the reduction occurred at distinctly higher concentrations than in the HEK cell culture model (Stachel et al. 2004). In contrast, the A β 38, A β 40, and A β 42 content declined in primary neuronal cultures already at 5 nM (Fig. 2b), and A β 42 was reduced by more than 50% at an inhibitor concentration of 25 nM, indicating that the extent of inhibition in telencephalic neurons complies with the reported IC₅₀ in HEK cells (Stachel et al. 2004). Unexpectedly, A β 40 and A β 42 production was not completely inhibited by the BACE-1 inhibitor (Table 1). The effect of the β -secretase inhibitor on A β cleavage was not due to compound toxicity as no reduction in cell viability was observed (data not shown). Altogether, BACE-1 inhibition was approximately tenfold more potent in primary cells than in the human neuroglioma cell line expressing human APP_{K595N/M596L}.

γ -Secretase Inhibition is More Pronounced in the H4 Neuroglioma Cell Line

Next, we addressed the effect of two γ -secretase inhibitors, DAPM and DAPT, in our two culture models. DAPM was shown to reduce secretion of A β monomers in CHO cells expressing human APP751_{Val1717Phe} at an IC₅₀ of approximately 100 nM by a highly sensitive immunoprecipitation and western blotting method (Walsh et al. 2002). DAPT was reported to abolish A β 42 formation in primary human neuronal cultures at an IC₅₀ of 200 nM (Dovey et al. 2001).

Table 1 Effects of pharmacological modulators on A β formation

		A β 38		A β 40		A β 42	
		IC ₅₀	E _{max}	IC ₅₀	E _{max}	IC ₅₀	E _{max}
Beta secretase inhibitor IV	H4 cells—hAPP _{K595N/N596L}	92 nM	88%	125 nM	79%	216 nM	86%
	Primary chicken neurons	3.7 nM	91%	4.7 nM	76%	4.8 nM	70%
DAPM	H4 cells—hAPP _{K595N/N596L}	20 nM	100%	23 nM	100%	26 nM	100%
	Primary chicken neurons	62 nM	100%	100 nM	100%	107 nM	100%
DAPT	H4 cells—hAPP _{K595N/N596L}	50 nM	100%	61 nM	100%	64 nM	100%
	Primary chicken neurons	71 nM	100%	93 nM	100%	101 nM	100%
Sulindac sulfide	H4 cells—hAPP _{K595N/N596L}	—	—	—	—	76 μ M	100%
	Primary chicken neurons	—	—	—	—	33 μ M	100%
CW	H4 cells—hAPP _{K595N/N596L}	—	—	—	—	41 μ M	100%
	Primary chicken neurons	—	—	—	—	17 μ M	100%
Calpeptin	H4 cells—hAPP _{K595N/N596L}	4.0 μ M	100%	7.4 μ M	100%	—	—
	Primary chicken neurons	5.0 μ M	100%	—	—	—	—

The IC₅₀ and E_{max} values for A β 38, A β 40, and A β 42 generation in H4 cells overexpressing human APP_{K595N/M596L} and in primary telencephalic chicken neurons are summarized for each individual compound

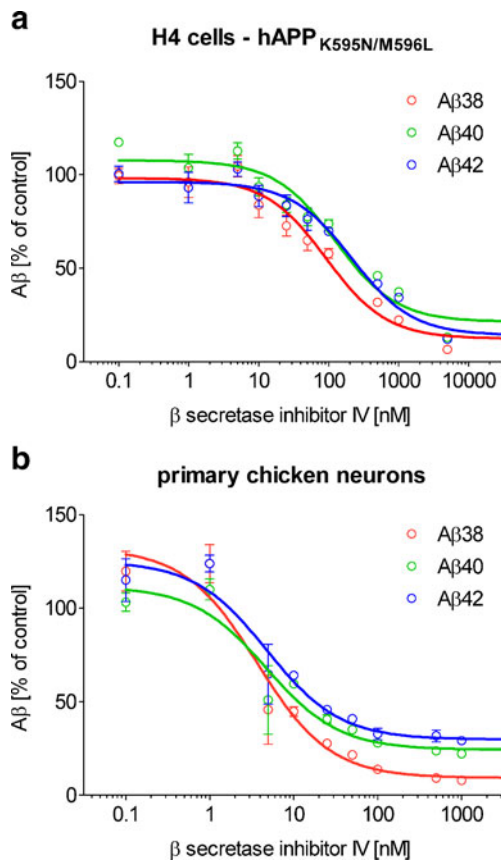


Fig. 2 BACE-1 inhibition is more efficacious in primary neurons. H4 cells overexpressing human APP_{K595N/M596L} and primary telencephalic chicken neurons were treated with different concentrations of the β -secretase inhibitor IV for 24 h. A β content of cell supernatants was determined by an immunosorbent assay. **a** In H4 cells, levels of A β 38, A β 40, and A β 42 peptides decreased in a concentration-dependent manner. **b** In primary telencephalic neurons, A β 38, A β 40, and A β 42 levels declined already dramatically at low nanomolar concentrations of the BACE-1 inhibitor, in contrast to H4 cells. Graphs represent the individual A β content in percent of vehicle-treated cultures as mean \pm standard error of mean

DAPM reduced secretion of all three A β species in H4 cells (IC_{50} A β 38=19.9 nM, IC_{50} A β 40=23.1 nM, IC_{50} A β 42=25.9 nM) but conversely caused an increase of all three A β forms at low nanomolar concentrations (Fig. 3a). In cultures of primary telencephalic neurons, DAPM also decreased formation of A β peptides (IC_{50} A β 38=61.9 nM, IC_{50} A β 40=99.7 nM, IC_{50} A β 42=106.9 nM) but at higher concentrations than in H4 cultures. Also, no increase in A β secretion was observed in primary cultures at any concentration of the γ -secretase inhibitor (Fig. 3b). Hence, the effect of DAPM was slightly more potent in H4 cells overexpressing human APP_{K595N/M596L} than in primary telencephalic neurons. DAPM had no effect on cell viability in both culture systems (data not shown).

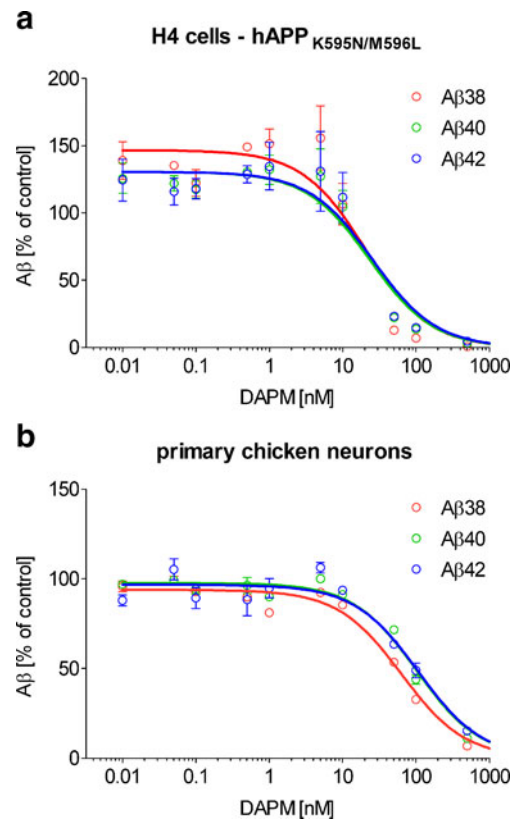


Fig. 3 The γ -secretase inhibitor DAPM reduces A β formation in both culture models. H4 cells overexpressing human APP_{K595N/M596L} and primary telencephalic chicken neurons were treated with different concentrations of the γ -secretase inhibitor DAPM for 24 h. A β content of cell supernatants was determined by an immunosorbent assay. **a** Levels of A β 38, A β 40, and A β 42 peptides declined dramatically starting at a concentration of 50 nM DAPM in the H4 cell line. **b** A β 38, A β 40, and A β 42 content decreased at a concentration of 50 nM DAPM in primary neurons but not as pronounced as in the H4 cell line. Graphs represent the individual A β content in percent of vehicle-treated cultures as mean \pm standard error of mean

The second γ -secretase inhibitor, DAPT, inhibited APP cleavage in H4 cells (IC_{50} A β 38=50.3 nM, IC_{50} A β 40=61.4 nM, IC_{50} A β 42=63.8 nM) but also increased the formation of all three A β species at 10 nM (Fig. 4a). Similarly, A β 38, A β 40, and A β 42 peptide levels decreased in supernatants of primary neurons (IC_{50} A β 38=71.2 nM, IC_{50} A β 40=92.8 nM, IC_{50} A β 42=100.8 nM), but no dramatic increase in A β levels was observed at low nanomolar concentrations of the compound in contrast to H4 cells (Fig. 4b). Viability of primary neurons and H4 cells was not affected by DAPT treatment (data not shown). As in DAPM treated cultures, the effect of DAPT was slightly more pronounced in H4 cells stably expressing human APP_{K595N/M596L} than in primary telencephalic neurons. However, the IC_{50} of DAPT and DAPM for

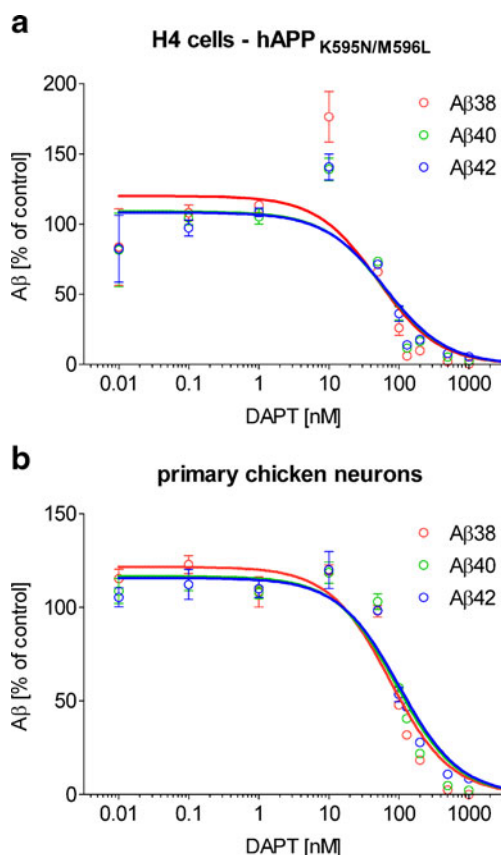


Fig. 4 The γ -secretase inhibitor DAPT reduces A β formation in primary and immortalized cells. H4 cells overexpressing human APP_{K595N/M596L} and primary telencephalic chicken neurons were treated with different concentrations of the γ -secretase inhibitor DAPT for 24 h. A β content of cell supernatants was determined by an immunosorbent assay. **a** In the H4 cell line, A β 38, A β 40, and A β 42 levels started to be reduced at a concentration of 50 nM DAPT. **b** In primary telencephalic neurons, A β peptides started to diminish at a concentration of 100 nM DAPT. Graphs represent the individual A β content in percent of vehicle-treated cultures as mean \pm standard error of mean

A β 42 cleavage was not higher in primary neurons when compared to the published efficacies of these inhibitors. Hence, both culture systems reproduced the reported effects of the selected γ -secretase inhibitors.

Two NSAIDs Modulate Processing of A β Peptides in Primary Neurons at Lower Concentrations than in the H4 Cell Line

A subset of non-steroidal anti-inflammatory drugs (NSAIDs) has been shown to interfere with APP processing by modulating γ -secretase-mediated APP cleavage independent of their impact on cyclooxygenase-1 (COX1) and COX2 activity (Weggen et al. 2001). Here, we investigated the effect of sulindac sulfide and CW in our cell culture models. The non-selective COX-inhibitor sulindac sulfide has previously

been reported to reduce A β 42 levels by $\geq 50\%$ at concentrations of 80–100 μ M in several cell lines overexpressing human wildtype or mutant APP (Weggen et al. 2001). Concomitant with the decrease in A β 42 content, an increase in A β 38 was detected in supernatants of treated cultures (Weggen et al. 2001). In CHO cells expressing human APP containing the Swedish mutation, 40 μ M sulindac sulfide reduced A β 42 secretion to $\sim 45\%$ (Weggen et al. 2003). The second NSAID we examined was the cell-permeable benzoate CW, which was identified in a computer-based structural similarity search for NSAIDs. CW has been demonstrated to reduce A β 42 levels at a concentration of 100 μ M (Okochi et al. 2006).

In H4 cells expressing human APP_{K595N/M596L}, sulindac sulfide enhanced generation of A β 38 peptides at low micromolar concentration (Fig. 5a). A decrease in A β 42 levels appeared at a concentration of 50 μ M with A β 42 levels being reduced to $75.0\pm 7.1\%$. The A β 42 content declined further to $39.3\pm 2.0\%$ at 100 μ M, whereas A β 40 production remained still unaltered. At 150 μ M, sulindac sulfide started to display already some minor cell toxicity in the viability assay (data not shown). Thus, the concentration of sulindac sulfide necessary to diminish A β 42 levels by 50% was between 75 and 100 μ M. The different extent of sulindac sulfide-mediated modulation of A β 38 and A β 42 levels was not unexpected. Analyses of different PS1 mutations associated with familial Alzheimer's disease (FAD) revealed that sulindac sulfide can modulate A β 38 and A β 42 formation independently (Page et al. 2008).

In contrast to H4 cells, secretion of A β 42 peptides was already reduced at lower micromolar concentrations of sulindac sulfide in primary neuronal cultures (Fig. 5b). A β 38 content started to increase at 25 μ M to approximately $149.7\pm 6.4\%$, whereas A β 42 levels decreased concomitantly to $54.7\pm 6.4\%$. At 50 μ M, A β 38 levels did not rise any further, but A β 42 continued to drop to $25.3\pm 2.1\%$, and also A β 40 levels started to decline. At concentrations beginning with 75 μ M, the NSAID started to become toxic in primary cultures (data not shown). Hence, the concentration of sulindac sulfide required to reduce A β 42 levels in primary chick cultures by 50% is at ~ 33 μ M. This also complies with the efficacy of sulindac sulfide described in HEK cell (Weggen et al. 2003), whereas sulindac sulfide was surprisingly less efficacious in the stable H4 cell line expressing human APP_{K595N/M596L}.

CW started to elevate A β 38 and moderately reduce A β 42 levels in H4 cells at a concentration of 50 μ M (Fig. 6a). At 100 μ M, A β 38 increased to $287.5\pm 31.2\%$, whereas A β 42 was further reduced to $16.0\pm 1.8\%$ compared to vehicle-treated cultures. At 150 and 200 μ M, A β 38 and A β 40 secretion started also to decline, although no cell toxicity was observed in the MTT viability assay at these concentrations (data not shown). Therefore, CW

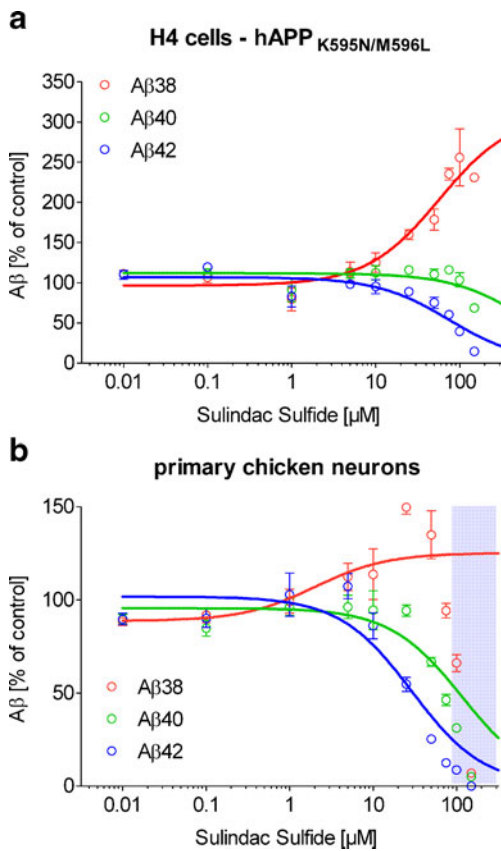


Fig. 5 Sulindac sulfide modulates A β processing more efficaciously in primary neurons than in the H4 cell line. H4 cells overexpressing human APP_{K595N/M596L} and primary telencephalic chicken neurons were treated with different concentrations of the NSAID sulindac sulfide for 24 h. A β content of cell supernatants was determined by an immunosorbent assay. **a** In the H4 cell line, A β 42 levels started to decline at a concentration of 50 μ M. **b** In primary telencephalic neurons, A β 42 levels dropped already at a concentration of 25 μ M. The blue bar indicates toxic concentrations. Graphs represent the individual A β content in percent of vehicle-treated cultures as mean \pm standard error of mean

decreased A β 42 levels in H4 cells by 50% at concentrations between 50 and 100 μ M.

In cultures of primary telencephalic neurons, moderate changes in A β 38 and A β 42 peptide were already detected at 5 μ M CW (Fig. 6b). At 10 μ M, an increase of 127.9 \pm 0.9% A β 38 was observed, whereas A β 42 levels declined to 63.6 \pm 2.4%. The effect of CW was further potentiated at 50 μ M, with A β 38 levels rising to 159.3 \pm 8.0% and A β 42 falling to 29.9 \pm 1.2%. Therefore, CW diminished A β 42 levels by 50% at \sim 17 μ M in primary neurons. The A β 40 content remained unchanged up to 50 μ M compared to vehicle-treated cells. However, starting from 100 μ M, levels of all A β species declined progressively with increasing concentration of the NSAID. The reduction of A β species at the two highest concentrations was due to neuronal toxicity of the compound as CW affected cell viability at 150 and 200 μ M but not at lower concentrations

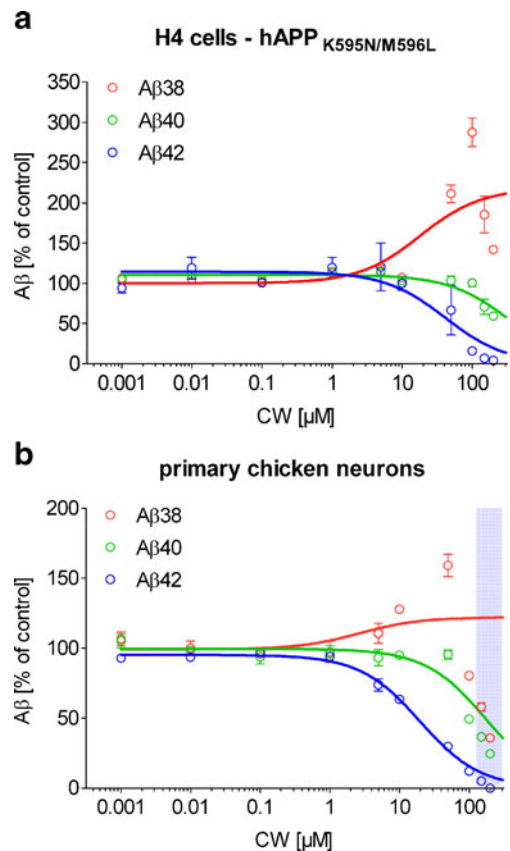


Fig. 6 Compound CW modulates A β 42 formation at lower concentrations in primary neurons than in the H4 cell line. Different concentrations of the non-steroidal anti-inflammatory drug CW were administered in cultures of H4 cells overexpressing human APP_{K595N/M596L} and primary telencephalic chicken neurons. Cell supernatants were assessed for A β content by an immunosorbent assay. **a** In the H4 cell line, A β 42 levels started to diminish at a concentration of 50 μ M. **b** In primary telencephalic neurons, A β 42 levels started to decrease already at a concentration of 5 μ M. The blue bar indicates toxic concentrations. Graphs represent the individual A β content in percent of vehicle-treated cultures as mean \pm standard error of mean

(data not shown). As sulindac sulfide, CW was also more efficacious in modulating APP processing in primary telencephalic neurons than in the H4 cell line ectopically expressing human mutant APP.

Interfering with Calpain Signaling Affects A β 42 Formation Only in Primary Chicken Neurons

An unregulated intracellular increase of Ca²⁺ and elevated activity of the Ca²⁺-dependent calpain proteases has been linked with inducing cell death (Harwood et al. 2005; Golstein and Kroemer 2007). Further, calpain inhibitors have been demonstrated to counteract excitotoxic insults (Hara and Snyder 2007). But besides their role in cell death, calpains have also been implicated in APP processing since inhibition of calpain activity was demonstrated to increase

A β 42 production in cell cultures (Klafki et al. 1996; Zhang et al. 1999; Mathews et al. 2002). The cell-permeable calpain inhibitor calpeptin has been reported to elevate secretion of A β 42 but not of A β 40 at a concentration of 10 μ M in a murine cell line ectopically expressing human wildtype APP695 and also in untransfected murine cells (Mathews et al. 2002).

Calpeptin did not evoke any dramatic changes in H4 cells expressing mutant human APP up to a concentration of 1 μ M (Fig. 7a). However, at 10 μ M, A β 38 (12.9 \pm 2.6%) and A β 40 (35.5 \pm 2.2%) peptide levels decreased sharply, whereas A β 42 (90.3 \pm 6.0%) remained almost on the same level as in vehicle control cultures.

Similarly, no effect of calpeptin was observed in cultures of primary chicken neurons up to a concentration of 1 μ M (Fig. 7b). In contrast to H4 cells, calpeptin increased A β 42 levels to 259.3 \pm 7.3% at a concentration of 10 μ M, whereas A β 40 remained almost unchanged at 89.9 \pm 3.8%. The A β 38

content in supernatants was reduced to 31.1 \pm 1.8%. No toxicity of calpeptin was observed in primary and H4 cell cultures (data not shown). Hence, primary telencephalic neurons but not H4 cells expressing human APP_{K595N/M596L} increased A β 42 generation at a calpeptin concentration of 10 μ M. Therefore, primary chicken neurons but not H4 cells reproduced the previously reported effect of calpeptin on APP processing (Mathews et al. 2002).

Discussion

Increased formation of toxic A β 42 peptides is characteristic for sporadic and familial forms of Alzheimer's disease. Therefore, compounds targeting β - and γ -secretase activities were developed in recent years to render formation of aberrant A β 42. Most in vitro screens identifying such compounds were performed in immortalized cells expressing mutant human APP. Here, we compared primary chicken telencephalic neurons with a human neuroglioma cell line overexpressing human APP harboring the Swedish double mutation to decipher differences in their response to known modulators of APP processing.

We chose chicken telencephalic rather than rodent neurons as source for primary cultures since chick A β 42 is identical to the human sequence and primary chicken telencephalic neurons were previously demonstrated to secrete detectable amounts of A β 1-37, A β 1-38, A β 1-39, A β 1-40, and A β 1-42 in vitro by using an immunoprecipitation and Western Blot protocol (Esselmann et al. 2004). In accordance, we confirmed formation of A β 38, A β 40, and A β 42 peptides in cell supernatants of primary chicken telencephalic neurons using an electrochemiluminescence based immunosorbent assay. Similar to human cerebrospinal fluid (Lewczuk et al. 2004), A β 40 was the predominant A β species produced by primary chicken neurons and the neuroglioma cell line. H4 cells overexpressing human mutant APP secreted at least tenfold more A β peptides than chicken neurons, but the A β 42/A β 40 ratio was comparable in supernatants of H4 cells and primary telencephalic neurons.

Since both culture systems produced desirable levels of A β peptides, we administered compounds known to inhibit or modulate APP cleavage and determined production of A β 38, A β 40, and A β 42 peptides.

In cultures treated with the β -secretase inhibitor IV, we observed a more severe inhibition of β -secretase activity in primary neurons than in the H4 cell line expressing human APP harboring the Swedish double mutation. This appears particularly interesting as this mutation has been associated with accelerated APP cleavage by β -secretase. Other groups also demonstrated that BACE-1 inhibition is approximately tenfold less powerful in cells expressing

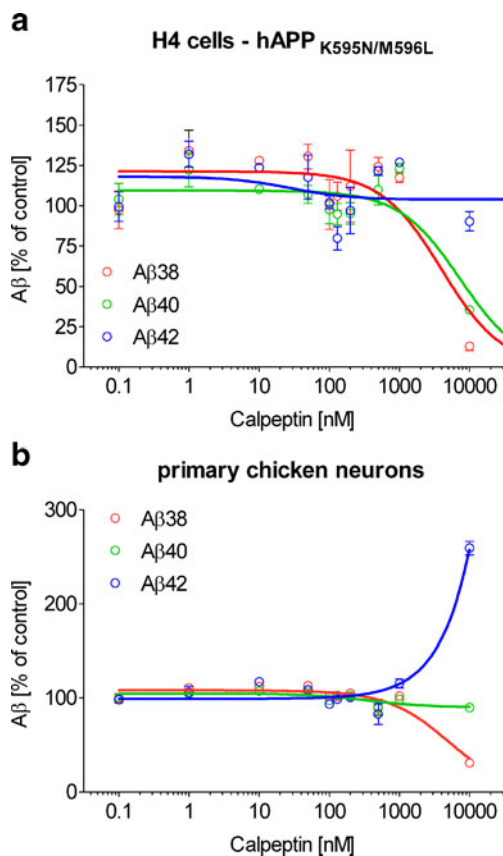


Fig. 7 Calpain inhibition increases A β 42 formation in primary but not immortalized cells. H4 cells overexpressing human APP_{K595N/M596L} and primary telencephalic chicken neurons were treated with different concentrations of calpeptin for 24 h. A β content of cell supernatants was determined by an immunosorbent assay. **a** No modulation of A β 42 levels was observed in H4 cells. **b** In contrast, A β 42 content was increased at 10 μ M in primary neuronal cultures. Graphs represent the individual A β content in percent of vehicle-treated cultures as mean \pm standard error of mean

human APP_{K595N/M596L} compared to cells overexpressing wildtype APP (Hussain et al. 2007; Yamakawa et al. 2010). Recently, the difference in β -secretase inhibition has been linked to different subcellular localization of wildtype APP and APP_{K595N/M596L} (Koo and Squazzo 1994; Haass et al. 1995; Yamakawa et al. 2010). Similarly, the FAD-linked mutations APP_{V717F}, PS1_{M146L}, and PS1_{L166P} which all affect γ -secretase cleavage, are less sensitive to γ -secretase inhibitors (Xia et al. 2000; Czirr et al. 2007). Moreover, cells transfected with APP_{V717F} or different PS1 mutations display altered secretion of A β peptides in response to sulindac sulfide treatment compared to cells transfected with the respective wildtype protein (Weggen et al. 2003; Czirr et al. 2007; Page et al. 2008). Thus, secretase inhibitors and modulators exhibit different potencies and effects in cellular assays depending on the presence or absence of FAD-linked mutations. Since the vast majority of patients who suffer from sporadic AD encode wildtype and not mutant *APP* or *PS1*, screening compounds in cells expressing wildtype APP and PS1 reflects more on human pathology and also defines the efficacy of a substance more precisely.

Both γ -secretase inhibitors, DAPM and DAPT, evoked a dose-dependent decrease in A β 38, A β 40, and A β 42 levels, but the reduction was more pronounced in the neuroglioma cell line than in primary telencephalic neurons. Indeed, DAPT has been demonstrated to inhibit A β formation more severely in a HEK293 cell line expressing human APP751 containing the Swedish mutation than in primary neuronal cultures with DAPT exhibiting a five- to tenfold lower potency in human and mouse primary cultures (Dovey et al. 2001). Similarly to DAPT, a selective tyrosine kinase inhibitor, which has been suggested to indirectly regulate γ -secretase activity, reduced A β production faster in N2a cells producing Swedish mutant APP than in primary cortical neurons (Netzer et al. 2003). As β -secretase cleavage is a prerequisite for γ -secretase-mediated APP processing, APP containing the Swedish mutation simply increases the intracellular concentration of γ -secretase substrate and thus provides a higher and more favorable substrate/enzyme ratio than in primary neurons (Citron et al. 1992; Yang et al. 2003). Subsequently, an enzymatic reaction at a high substrate/enzyme ratio enables a more efficient inhibition of APP cleavage by secretase inhibitors. A comparison of γ -secretase inhibitors in cell lines expressing either wildtype or APP containing the Swedish mutation would address the influence of Swedish mutant APP on γ -secretase inhibition. When DAPT was administered *in vivo*, the actual concentration of DAPT in brain tissue (~200 nM and higher) that reduced total A β content was in excess of the IC₅₀ in primary cultures (IC₅₀ A β total=115 nM) (Dovey et al. 2001). Thus, *in vivo* DAPT

data indicate that primary neurons are more likely to define the potency of a γ -secretase inhibitor for *in vivo* administration than cell lines. Beside the reduction in A β levels, we also observed an increase in A β 38, A β 40, and A β 42 formation at DAPM and DAPT concentrations below their respective IC₅₀. Such an effect has already been described for a difluoroketoamide-based γ -secretase inhibitor, which also increased A β 42 levels at sub-inhibitory concentrations (Durkin et al. 1999).

In contrast, modulation of γ -secretase activity by NSAIDs appeared more severe in primary cultures. A comparison of N2a cells expressing human APP bearing the Swedish mutation and primary rat cortical neurons also indicated a stronger reduction of A β 40 and A β 42 levels in primary cultures at a concentration of 25 μ M (Gasparini et al. 2004), although we only observed a reduction in A β 40 levels at very high sub-lethal and lethal concentrations. The reduced potency of NSAIDs in the neuroglioma cell line is, unlike BACE-1 inhibitors, independent of the APP Swedish double mutation. CHO cells transfected with APP_{K595N/M596L} secrete the same levels of A β 42 after treatment with sulindac sulfide as cells expressing wildtype APP (Weggen et al. 2003). However, primary neurons and cell lines differ in their APP content. Whereas primary neurons produce endogenous APP at physiological levels, cell lines highly over-express ectopic APP suggesting that the modulating activity of NSAIDs is dependent on the amount and availability of APP substrate. Such a scenario would assume that sulindac sulfide and CW directly interact with APP. Indeed, a study using labeled γ -secretase modulators (GSM) revealed a direct interaction of GSMs with residues of the beta-amyloid peptide rather than with the core proteins of the γ -secretase complex (Kukar et al. 2008). Hence, total APP levels can be critical for the potency of NSAIDs. Besides their higher sensitivity for APP modulation, primary neurons are also more prone to cell toxicity mediated by NSAIDs and give, in addition to APP processing, valuable information on the neurotoxicity of substances.

Further, we analyzed the calpain inhibitor calpeptin in our culture models. Interestingly, we replicated the A β 42 increasing effect of calpeptin in primary cultures but not in the H4 cell line although the modulating activity of calpeptin was initially demonstrated in a murine fibroblast-like cell line and in murine and human neuroblastoma cells (Mathews et al. 2002). Hence, the lack of effect of calpeptin in H4 cells can be attributed at least in parts to the nature of the H4 neuroglioma cell line. This finding highlights the problem that most data on APP processing has been generated in a variety of different cell lines and hence a comparison between cellular assays proves difficult. Some cell lines originate from non-

neuronal cell types, whereas others are from different species. Also, the expression of various FAD-linked mutations in cell lines does not allow a comprehensive comparison of secretase inhibitors and modulators. Therefore, the use of a unifying cell culture system such as primary neurons would be of advantage to allow a direct comparison of APP modifying substances.

In conclusion, primary telencephalic chicken neurons are a well-suited cell culture model for testing drugs targeting A β formation. Primary chicken neurons produce endogenous levels of wildtype APP and PS1 and secrete A β peptides which are identical in their sequence to human A β and thus reflect the molecular condition of sporadic Alzheimer's disease. In addition, primary telencephalic neurons are overall more sensitive to pharmacological intervention than immortalized cell lines with the exception of γ -secretase inhibitors and in sum define the potency and mechanism of drugs more accurately regarding their in vivo efficacy and toxicity. For drug discovery, primary neurons expressing wildtype APP are a suitable system to model sporadic Alzheimer's disease. In contrast, cell lines producing proteins containing FAD-linked mutations exhibit a model for testing drugs targeting familial forms of AD.

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