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APP Processing in Human Pluripotent Stem Cell-Derived Neurons Is Resistant to NSAID-Based γ-Secretase Modulation

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SUMMARY

Increasing evidence suggests that elevated Aβ42 fractions in the brain cause Alzheimer's disease (AD). Although γ-secretase modulators (GSMs), including a set of nonsteroidal anti-inflammatory drugs (NSAIDs), were found to lower Aβ42 in various model systems, NSAID-based GSMs proved to be surprisingly inefficient in human clinical trials. Reasoning that the nonhuman and nonneuronal cells typically used in pharmaceutical compound validation might not adequately reflect the drug responses of human neurons, we used human pluripotent stem cell-derived neurons from AD patients and unaffected donors to explore the efficacy of NSAID-based γ-secretase modulation. We found that pharmaceutically relevant concentrations of these GSMs that are clearly efficacious in conventional nonneuronal cell models fail to elicit any effect on Aβ42/Aß40 ratios in human neurons. Our work reveals resistance of human neurons to NSAID-based γ-secretase modulation, highlighting the need to validate compound efficacy directly in the human cell type affected by the respective disease.

INTRODUCTION

Alzheimer's disease (AD) is a common and fatal neurodegenerative disorder. Currently, no effective drugs that can stop, slow, or prevent disease progression are available. Deposition of amyloid plaques consisting of aggregated Aβ peptides in the brain is a hallmark of the disease (Selkoe, 2001). The amyloid cascade hypothesis presumes that the accumulation and oligomerization of Aβ peptides trigger a complex pathological cascade resulting in synaptic dysfunction, tau hyperphosphorylation, and eventually progressive neurodegeneration and dementia (Selkoe et al., 2012). Aβ is a proteolytic derivative of the transmembrane amyloid precursor protein (APP), which is sequentially cleaved by β- and γ-secretases in the amyloidogenic processing pathway (Haass et al., 2012). Intramembranous γ-secretase cleavage of the C-terminal fragments of APP (APP-CTF), which represent the immediate precursors of Aβ, results in multiple length variants of Aβ (Haass et al., 2012). Longer Aβ variants such as Aβ42 and Aβ43 are more prone to aggregation and thus are considered more pathogenic than shorter ones such as Aβ38 and Aβ40 (Karran et al., 2011). Today, the peptide ratio of Aβ42 to Aβ40 in the cerebrospinal fluid (CSF) represents the most sensitive and specific primary biomarker for AD and inversely correlates with the age of disease onset in both sporadic (Blennow et al., 2012) and familial (Kumar-Singh et al., 2006) forms of AD. Mutations in APP or in the γ-secretase subunits presenilin-1 (PS1) and PS2 are the main cause of autosomal-inherited early-onset forms of AD and commonly lead to increased Aβ42/Aß40 ratios and/or overall elevated levels of Aβ. These observations suggest that misprocessing of APP with a consecutive increase of Aβ42/Aß40 ratios is characteristic of and, most probably, causative for sporadic and familial AD (Wiltfang et al., 2001). Based on this hypothesis, several antiamyloidogenic drugs, including compounds that inhibit β- and γ-secretase activity, have been developed (Ghosh et al., 2012; Imbimbo and Giardina, 2011). Interestingly, a subset of nonsteroidal anti-inflammatory drugs (NSAIDs) were identified to act as γ-secretase modulators (GSMs) that specifically lower the production of Aβ42 in favor of shorter Aβ isoforms by targeting γ-secretase PS1 or its substrate APP (Jumpertz et al., 2012; Kukar et al., 2008; Weggen et al., 2001). Unfortunately, and despite solid preclinical data acquired using transgenic animals and APP-transgenic cell lines, NSAIDs such as flurbiprofen and indometacin were not effective in delaying disease progression in

mild-to-moderate AD patients in phase 2 and phase 3 clinical trials (de Jong et al., 2008; Eriksen et al., 2003; Green et al., 2009; Imbimbo and Giardina, 2011; Vellas, 2010). The reasons for these negative outcomes are speculative and have been in part attributed to inappropriate study design, as symptomatic AD patients were treated when the disease may have already been irreversibly advanced (Golde et al., 2011). Also, it remains unclear whether the trialed GSMs indeed lowered Aβ42 levels in the human brain, leaving the important question as to whether γ-secretase modulation is a valid approach in AD therapy unresolved. Further, insufficient brain penetration of the tested compounds, as well as a general failure of the amyloid cascade hypothesis, has been considered (Golde et al., 2011). Remarkably, the efficacy of GSMs in human neurons as the primary target cell type has never been directly explored. Recent advances in neural differentiation of human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) enable the derivation of authentic neuronal cultures to dissect the pathological mechanisms relevant to AD and drug testing (Israel et al., 2012; Koch et al., 2012; Mattis and Svendsen, 2011; Mertens et al., 2013). Here, we used this approach to determine the efficacy of NSAIDs previously employed in clinical GSM trials in human neurons derived from iPSCs of patients with familial AD and unaffected controls (Ctrl; Figure 1A).

RESULTS AND DISCUSSION

Neurons Derived from Familial AD Patients Show Elevated Aβ42/Aβ40 Ratios

To elucidate APP processing in human neurons from various genetic backgrounds, we took advantage of our recently described and highly standardized pluripotent stem cell-derived, long-term self-renewing neural stem cells (lt-NES cells), which consistently give rise to cultures containing >70% functional human neurons (Falk et al., 2012; Koch et al., 2009). We generated iPSCs from two patients with familial AD (two clones each). Patient AD-1 (AD-1a and AD-1b) carries an A79V substitution in one allele of the PS1 gene, which results in autosomal-dominant AD (Larner and Doran, 2006). Patient AD-2 (AD-2a and AD-2b) carries a K724N mutation in the intracytosolic fragment of APP (for clinical details, see Table S1 available online; Theuns et al., 2006). Characterization of the established iPSC lines revealed sustained silencing of the reprogramming transgenes, a normal karyotype, expression of pluripotency markers, and formation of teratomas upon in vivo transplantation (Figures S1A–S1D). The four AD-patient-derived lines were subsequently differentiated into lt-NES cells according to established protocols (Falk et al., 2012; Koch et al., 2009). We further included lt-NES cells derived from reprogrammed fibroblasts from three unaffected individuals (Ctrl-1: clone a and b; Ctrl-2: clone a and b; Ctrl-3: clone a) (Falk et al., 2012; Koch et al., 2011) and from the hESC lines I3 and I6 (hES-1 and hES-2) (Koch et al., 2009). All Ctrl and AD lt-NES cell lines expressed the rosette-associated neuroectodermal markers PLZF, Nestin, DACH1, SOX2, and apically accentuated ZO-1 (Figure 1B). Familial AD mutations were confirmed by sequencing genomic DNA from patient-derived lt-NES cells (Figure S1E). Following differentiation for 4 weeks, 75%–85% of the cultures consisted of postmitotic neurons that expressed β-III tubulin and MAP2ab, while <10% of differentiated cells were positive for the glial marker glial fibrillary acidic protein (GFAP; Figure 1B). We further detected a consistent neuronal expression of PS1, APP, and phosphorylated Tau protein (PHF1 antibody; Figure 1B). Similarly to Ctrl neurons, which have been described previously (Falk et al., 2012; Koch et al., 2009), the AD lt-NES cell-derived neurons developed mature functional properties, including the generation of action potentials upon depolarization and the establishment of spontaneously active synaptic circuitries (Figure 1C). The neuron-specific APP_{695} variant and β- and γ-secretase-associated genes were expressed at comparable levels in the neuronal cultures (Figure S1F). We detected no apparent variations in neuronal differentiation efficiency, neuronal morphology, basic electrophysiological function, or marker expression in Ctrl and AD lt-NES cell-derived neurons.

Mutations in PS1 and APP are known to result in elevated Aβ42/Aß40 ratios in the CSF of familial AD patients (Borchelt et al., 1996; Kumar-Singh et al., 2006). Hence, we determined the levels of secreted Aβ40 and Aβ42 in conditioned media of the generated Ctrl and AD neurons by ELISA and calculated the Aβ42/Aß40 ratio. No significant difference in the Aβ42/Aß40 ratio between hESC-derived (0.092 ± 0.017) and Ctrl-iPSC-derived (0.096 ± 0.008) neurons was detected (Figure 2A). In contrast, AD-1 and AD-2 neurons showed 37% and 89% increases in Aβ42/Aß40 ratios to 0.126 ± 0.001 and 0.174 ± 0.018, respectively (Figure 2A). Interestingly, the increased Aβ42/Aß40 ratio in AD-1a neurons was solely attributable to decreased secretion of total Aβ40 by 26%, with Aβ42 levels remaining comparable to those generated by hESC- and Ctrl-iPSC-derived neurons (Figure 2B). AD-2a neurons also exhibited a decrease in total Aβ40 secretion by 31%, but in addition showed a 33% increase in Aβ42 secretion, resulting in an overall higher increase in the Aβ42/Aß40 ratio (Figure 2B). Thus, elevated Aβ42/Aß40 ratios in PS1(A79V) mutant neurons are likely due to a partial loss of function in γ-secretase function, while neurons from the patient with the APP(K724N) mutation showed decreased Aβ40 production in combination with a gain of function in Aβ42 secretion (Koch et al., 2012).

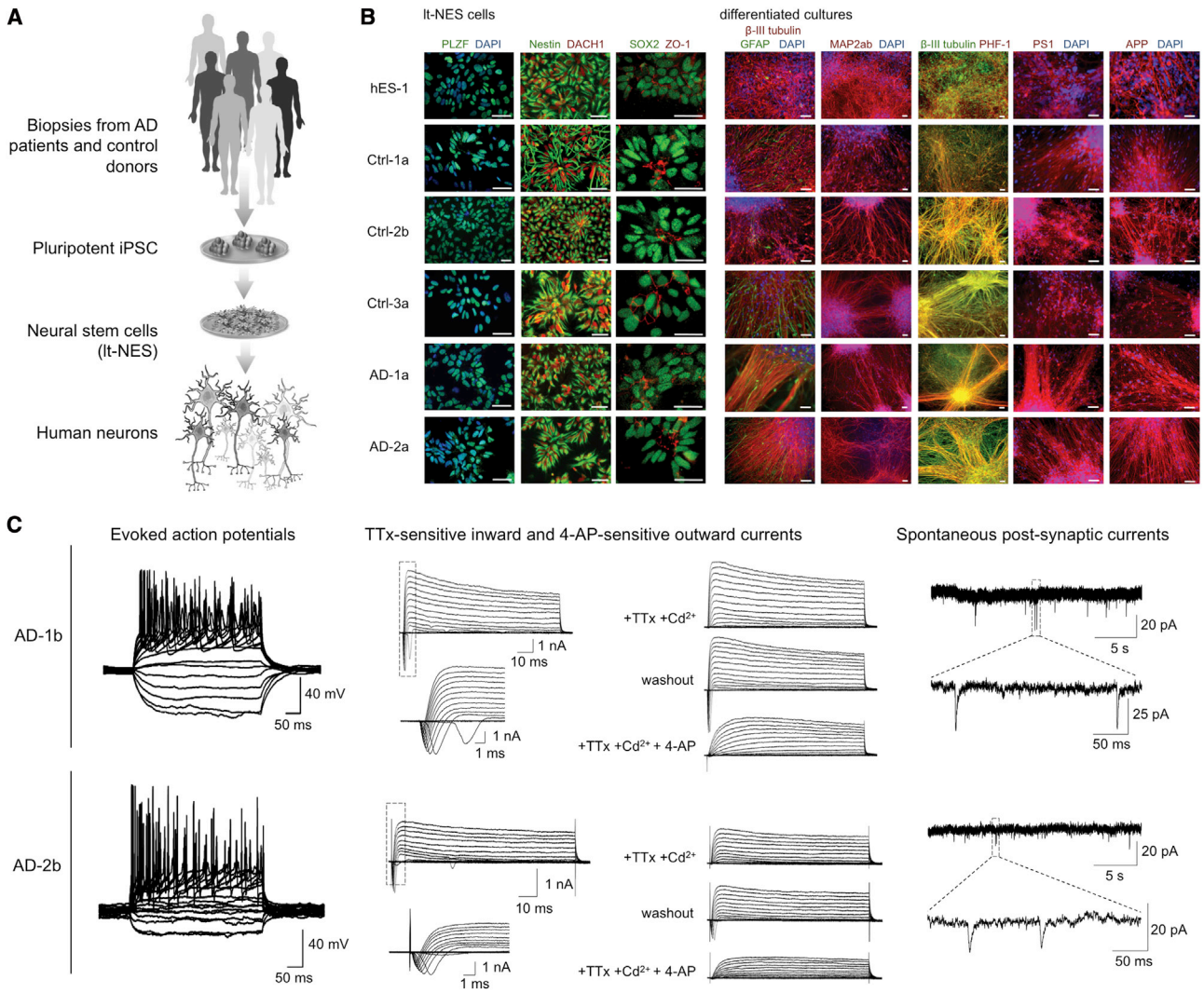

Figure 1. Human Neural Stem Cells and Neurons from Ctrl and AD-Patient-Specific iPSCs

(A) Experimental design: iPSCs were generated from dermal fibroblasts (Takahashi et al., 2007) and differentiated into lt-NES cell lines (n = 11 [5 Ctrl-iPSC derived, 4 AD-iPSC derived, and 2 hESC derived]). Lt-NES cells were differentiated for 4 weeks into mature neuronal cultures and subjected to GSM treatment and Aβ ELISA measurements in the conditioned media.

(B) Immunocytochemical characterization of lt-NES cells and differentiated neuronal cultures. Lt-NES were stained for the neuroectodermal stem cell markers Nestin, PLZF, DACH1, SOX2, and ZO-1. Differentiated cultures were analyzed for expression of β-III tubulin, GFAP, MAP2ab, phosphorylated Tau (PHF1), PS1, and APP. Scale bars, 20 μm.

(C) Electrophysiological characterization of AD neurons. Representative traces depict multiple evoked action potentials during current-clamp recording (n = 6) as well as whole-cell currents showing TTx/Cd^{2+}-sensitive Na^{+} channel-mediated inward currents and 4-AP-sensitive K^{+} channel-mediated outward currents in response to depolarizing voltage steps (n = 7). Spontaneous postsynaptic currents in neurons demonstrate functional synapses (n = 6).

See also Figure S1.

Interestingly, this might reflect the fact that although both mutations typically result in early-onset familial AD (age of onset: 52–59 years), PS1(A79V) is considered a "weak" mutation with a slowly progressing pathogenesis, whereas patients carrying the APP(K724N) mutation typically suffer from a rapid pathogenesis (Larner and Doran, 2006).

NSAID-Based GSMs Can Lower Aβ42/Aβ40 Ratios at High Concentrations

To explore how known NSAID-based GSMs impact endogenous γ-secretase activity and thus influence the generation of Aβ42 and Aβ40 in human neurons, we first applied a set of ten candidate compounds to hESC-derived

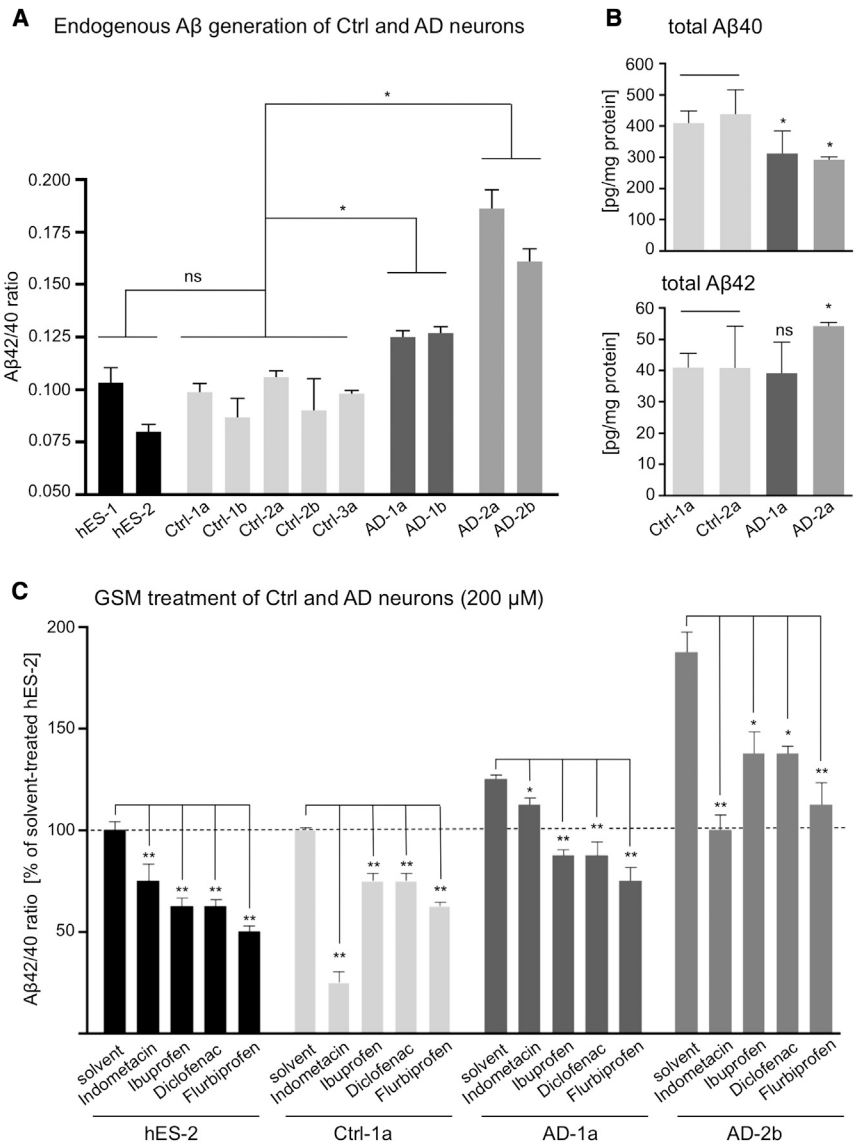

Figure 2. AD Patient iPSC-Derived Neurons Show Elevated Aβ42/Aβ40 Ratios that Can Be Lowered by High Concentrations of NSAID-Based GSMs

(A) Endogenous secretion of Aβ40 and Aβ42 was analyzed by ELISA in conditioned media of differentiated neuronal cultures, and Aβ42/Aß40 ratios were calculated.

(B) The total levels of Aβ40 and Aβ42 were measured and normalized to the total cellular protein amount of the respective cultures.

(C) High-concentration screen: Aβ42/Aß40 ratios of differentiated neuronal cultures treated for 36 hr with 200 μM indometacin, ibuprofen, diclofenac, or flurbiprofen. Bar graphs show mean + SD; significance values determined by ANOVA: *$p \leq 0.01$; **$p \leq 0.001$.

ELISA measurements were performed at least as biological triplicates.

See also Figure S2.

neurons (Figures S2A and S2B). Secreted Aβ40 and Aβ42 levels were determined by ELISA after 36 hr of treatment. We found that indometacin, ibuprofen, diclofenac, and flurbiprofen significantly reduced the Aβ42/Aß40 ratio at 200 μM, with indometacin and flurbiprofen exhibiting the strongest effects (Figure S2A). Direct γ-secretase inhibition by N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester (DAPT; 10 μM) or inhibition of the γ-secretase activating protein (GSAP) by imatinib (10 μM) resulted in a strong decrease in both Aβ variants. Treatment with SC-560 (200 μM) specifically inhibited Aβ40 secretion, thereby leading to a 3-fold increased Aβ42/Aß40 ratio. Aspirin (250 μM) and naproxen (200 μM), as well as the ROCK (Rho-associated coiled coil forming protein serine/threonine kinase) inhibitor Y-27632 (5 μM), had no detectable effects on the neurons (Figures S2A and S2B). We next tested the four identified NSAID-based GSMs on an extended set of Ctrl- and AD-patient-derived neurons. Ibuprofen and diclofenac (200 μM) reduced the Aβ42/Aß40 ratio by 25.0%–36.5% in all Ctrl- and AD-patient-derived neuronal cultures (Figure 2C). Flurbiprofen (200 μM) proved to be slightly more potent, as it consistently reduced the ratio by 37.5%–49.9% in all lines. Notably, the decrease in the Aβ42/Aβ40 ratio induced by 200 μM indometacin varied markedly between the cell lines, ranging between 10.0% ± 3.1% (AD-1a) and 75.0% ± 5.4% (Ctrl-1a). From these data, we conclude that neuronal cultures from AD-patient-derived iPSCs show pathologically altered Aβ generation, and that high concentrations of NSAID-based GSMs

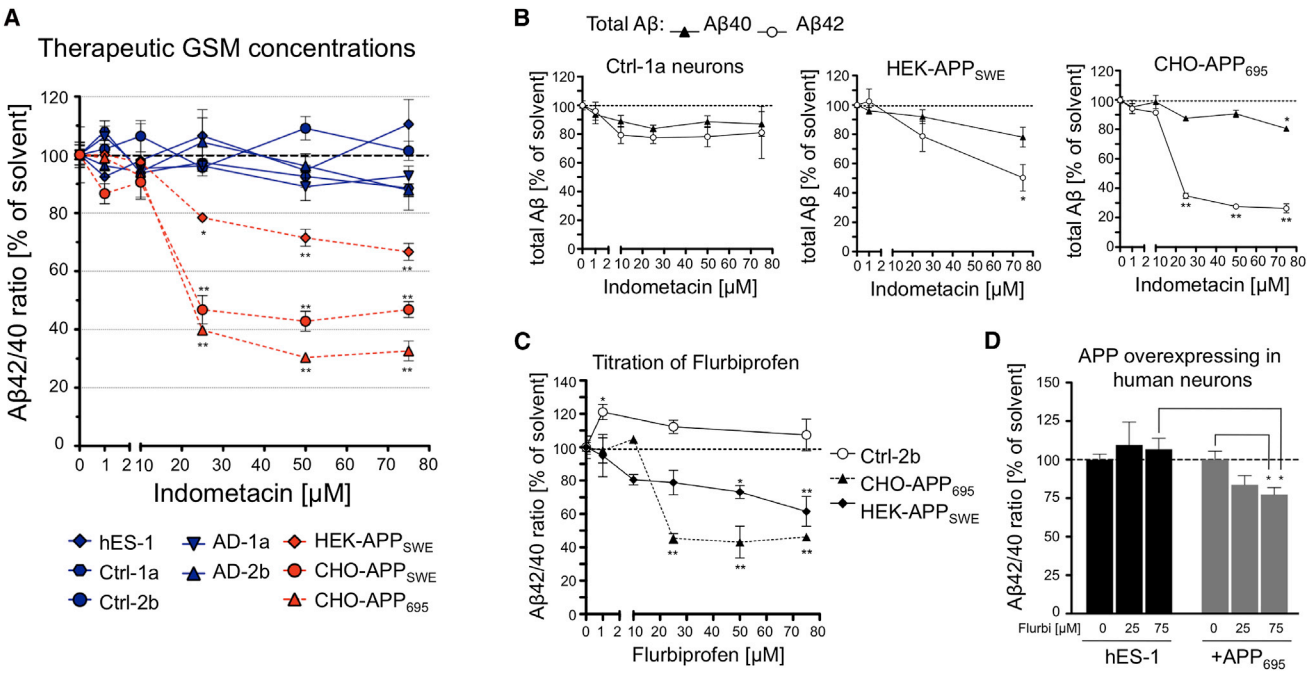

Figure 3. Human Neurons from Ctrl- and AD-Patient-Derived iPSCs Are Resistant to Therapeutically Relevant Concentrations of NSAID-Based GSMs that Failed in Clinical Trials

(A) Effect on the Aβ42/Aß40 ratio of GSM concentrations that can be reached in the human CSF (Bannwarth et al., 1990; Kumpulainen et al., 2010; Ritschel, 1999). Ctrl and AD neurons, as well as CHO-APP_{695}, CHO-APP_{SWE}, and HEK-APP_{SWE} cells, were treated with increasing concentrations of indometacin and Aβ42/Aß40 ratios were measured.

(B) Total Aβ40 and Aβ42 levels in conditioned media of human neurons (Ctrl-1) and HEK-APP_{SWE} and CHO-APP_{695} cells treated with different concentrations of indometacin.

(C) Aβ42/Aß40 ratios in human neurons (Ctrl-2) and HEK-APP_{SWE} and CHO-APP_{695} cells in response to flurbiprofen. Data in (A)–(C) are depicted as mean ± SEM.

(D) Aβ42/Aß40 ratios of APP_{695}-overexpressing hESC-derived neurons treated with flurbiprofen. All ELISA measurements were performed at least as biological triplicates. Bar graph shows mean + SD. Significance values determined by ANOVA: *$p \leq 0.01$; **$p \leq 0.001$.

See also Figure S3.

effectively lower Aβ42/Aß40 ratios in Ctrl- and AD-patient-derived neurons.

Human Neurons Do Not Respond to Therapeutically Relevant Concentrations of GSMs

Based on pharmacokinetic studies in humans, therapeutic dosages of most NSAIDs result in CSF concentrations in the low micromolar range (Ritschel, 1999). For example, maximum CSF levels can be expected to not exceed 30 μM for indometacin (Bannwarth et al., 1990; Ritschel, 1999) and 2 μM for flurbiprofen (Galasko et al., 2007; Kumpulainen et al., 2010). Such low micromolar concentrations were sufficient to decrease Aβ42/Aß40 ratios in human APP-transgenic rodent cells (e.g., Chinese hamster ovary [CHO] cells), human nonneural cell lines (e.g., human embryonic kidney [HEK] cells), and the brains of transgenic mice. For example, 25 μM indometacin reduced the Aβ42/Aβ40 ratio by ~50% in CHO-APP cells and 1.3 μM flurbiprofen reduced Aβ42 levels by ~80% in Tg2576 mice (Eriksen et al., 2003; Weggen et al., 2001). Based on these promising preclinical results, indometacin and flurbiprofen were tested for their efficiency to delay cognitive decline in human clinical trials. Yet, these GSMs failed to show any significant effect on the course of the disease compared with placebo controls (de Jong et al., 2008; Green et al., 2009).

To evaluate the potency of these clinically failed NSAID-based GSMs in modulating endogenous human neuronal Aβ generation at concentrations that can realistically be achieved in the human brain, we treated differentiated neuronal cultures from Ctrl and AD patients with increasing concentrations of indometacin, and measured Aβ40 and Aβ42 levels by ELISA in conditioned medium after 36 hr (Figure 3A). As controls, CHO cells overexpressing human APP_{695}, APP_{SWE} (containing the K670N/M671L double mutation), or HEK-APP_{SWE} (Citron et al.,

1992) cells were treated in parallel. As expected, as little as 25 μM indometacin sufficed to reduce the Aβ42/Aβ40 ratio in CHO-APP cells by >50%, and by 22% in HEK-APP_{SWE} cells. Surprisingly, and in contrast to the APP-transgenic cell lines, concentrations of up to 75 μM indometacin had no significant effect on the ratio of Aß42 to Aß40 generated from endogenous APP in human neurons from all five genetic backgrounds, including both AD patients (Figure 3A; for separate graphs, see Figure S3A). Whereas the total levels of Aβ42 from CHO-APP_{695} and HEK-APP_{SWE} cells selectively decreased in a concentration-dependent manner, total secretion of Aβ40 and Aβ42 remained unaffected in the human neuronal cultures (Figure 3B). Similarly, human neurons also proved largely resistant to clinically relevant concentrations of flurbiprofen, while CHO-APP_{695} and HEK-APP_{SWE} cells displayed a strong dose-dependent response (Figure 3C; also see Figure S3B). To exclude interference of culture-medium ingredients, we tested hESC- and iPSC-derived neurons in parallel with HEK-APP_{695} cells in the identical serum-containing medium. Under these conditions as well, human neurons exhibited a specific resistance to GSM treatment (Figure S3C). In order to detect a possible GSM-inactivating activity of human neurons, we preincubated drug-containing medium (indometacin or flurbiprofen; both 75 μM) on human neurons or CHO_{WT} cells before transferring it to CHO-APP_{SWE}. We found that preincubation on neurons or CHO_{WT} did not reduce the drugs' ability to decrease the Aß42/Aß40 ratio in CHO-APP_{SWE} cells (Figure S3D).

We next asked whether artificially high protein levels of APP/APP-CTFs might provoke an abnormally amplified GSM effect that is not observed at endogenous expression levels. To test this, we generated transgenic lt-NES cells that conditionally overexpress APP_{695} when treated with doxycycline (tetOn). Transgenic APP_{695} overexpression was induced following 4 weeks of differentiation. Interestingly, overexpressing hES-1-APP_{695} neurons significantly responded to 75 μM flurbiprofen, whereas nontransgenic hES-1 neurons remained inert (Figure 3D). However, the effect was mild compared with that detected in CHO-APP_{695} cells and was not observed when the APP overexpressing neurons were treated with 25 μM or 75 μM indometacin (Figure S3E). A plausible explanation might be that the APP protein levels achieved in transgenic human neurons are still significantly lower than those in CHO cells and thus are only partially sufficient to evoke the pronounced effect seen in tumor cell lines (Figure S3F). Although increased GSM responses can be triggered by exaggerated APP levels in neurons, the actual cause of the low GSM responsiveness of human neurons might be complex. For example, it could involve specific subcellular localizations and posttranslational modifications of the γ-secretase/APP complex and/or interactors that may be specifically regulated in human neurons, such as γ-secretase activating protein (GSAP) and CD147 (Rajendran et al., 2010). Species-specific effects may also contribute to this phenomenon, as human HEK cells consistently showed weaker responses to GSMs as compared with rodent CHO cells.

Taken together, our experiments reveal a cell-type-specific resistance of human neurons to pharmacologically relevant concentrations of known NSAID-based GSMs that stands in sharp contrast to widely used conventional model and drug-screening systems. Thus, it appears to be conceivable that data generated using APP-overexpressing cell lines and APP-transgenic mouse models have led to an overestimation of GSM efficacy in human neurons, a hypothesis that is supported by the clinical failure of this class of compounds. Extrapolating from our data, we conclude that much higher CSF concentrations of these NSAID-based GSMs would have been required to elicit beneficial effects in the human CNS. Our findings also strongly underline the importance and necessity of assessing compound efficacy in the appropriate human target cell type. Although in the past this route has been restricted due to the lack of primary tissue, the availability of hESCs and iPSCs now provides unprecedented opportunities for drug screening and preclinical compound validation in cell type-, disease-, and even patient-specific cell culture models.

EXPERIMENTAL PROCEDURES

Culture and Differentiation of lt-NES Cells

Lt-NES cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12, 2 mM L-glutamine, 1.6 g/l glucose, 0.1 mg/ml penicillin/streptomycin, N2 supplement (high transferrin; PAA), B27 (1 μl/ml; Life Technologies), and fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF; both 10 ng/ml; R&D Systems) on tissue culture plates coated with poly-L-ornithine/laminin (both Sigma), and passaged every 3–4 days. Neuronal differentiation was induced by withdrawal of FGF2 and EGF in differentiation media (Neurobasal medium supplemented with B27 [1:50; Life Technologies] and DMEM/F12 supplemented with N2 mixed at a 1:1 ratio and containing 300 ng/ml cyclic AMP) that was exchanged every second day (Koch et al., 2009).

The study was approved by the local ethics committee.

Treatment of Cells with GSMs and Other Small Molecules

Cells were pretreated for 12 hr with medium containing the respective compound. Then the drug-containing medium was replaced and, 24 hr later, subjected to ELISA measurements. Stock solutions were as follows: ibuprofen (200 mM; Alexis Biochemicals), diclofenac (200 mM), naproxen (200 mM), flurbiprofen (200 mM), aspirin (250 mM), indometacin (50 mM; Cayman Chemical), SC-560 (200 mM), imatinib (10 mM; Novartis; all in ethanol),

DAPT (5 mM; Sigma Aldrich; DMSO), and ROCK inhibitor (25 mM; Sigma Aldrich; DMSO).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2013.10.011.

AUTHOR CONTRIBUTIONS

J.M. and P.K. conceived and designed the study; collected, analyzed, and interpreted data; and wrote the manuscript. K.S., P.W., J.L., and J.C.K. collected, analyzed, and interpreted data. R.V., M.V., and P.v.D. provided material. J.W. and O.B. interpreted data, provided financial support, and wrote the manuscript.

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