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## Phenotypic Assays For $\beta$ -Amyloid In Mouse Embryonic Stem Cell-derived Neurons

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### SUMMARY

Given the complex nature of Alzheimer's disease (AD), a cell-based model that recapitulates physiological properties of the target neuronal population carries significant value in discovering improved drug candidates and chemical probes for uncovering disease mechanisms. We established phenotypic neuronal assays for biogenesis and synaptic action of amyloid  $\beta$ -peptide (A $\beta$ ) based on embryonic stem (ES) cell-derived neurons (ESNs). ESNs enriched with pyramidal neurons were robust, scalable and amenable to a small molecule screening assay, overcoming apparent limitations of neuronal models derived from human pluripotent cells. Small molecule screening of clinical compounds identified four compounds capable of reducing A $\beta$  levels in ESNs derived from the Tg2576 mouse model of AD. Our approach is therefore highly suitable for phenotypic screening in AD drug discovery and has the potential to identify therapeutic candidates with improved efficacy and safety potential.

### INTRODUCTION

Elevation and accumulation of amyloid  $\beta$ -peptide (A $\beta$ ) in brain are early and necessary steps in pathogenesis of Alzheimer's disease (AD), preceding all known clinical and pathological phenotypes of the disease, including memory and cognitive decline, by at least a decade (Hardy and Selkoe, 2002; O'Brien and Wong, 2011; Zheng and Koo, 2011). A $\beta$  is liberated from  $\beta$ -amyloid precursor protein (APP) by membrane-bound proteases,  $\beta$ -secretase (BACE1) and  $\gamma$ -secretase (Hardy and Selkoe, 2002; O'Brien and Wong, 2011; Zheng and Koo, 2011). The majority of current therapeutic strategies have focused on developing potent small molecule inhibitors for targeting well-defined enzymatic targets, including the secretase enzymes. Despite initial promise, the majority of these approaches suffered major setbacks in late stage clinical trials (Mangialasche, 2010). Therefore, model systems and biomarkers that can better predict the efficacy and safety of therapeutic candidates in human clinical trials have become increasingly important.

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Cell types historically used in AD drug discovery, such as immortalized or genetically transformed neuronal lines, lack complete phenotypic properties of primary neurons. Dissociated primary neuronal cultures, though a better physiological model compared to cell lines, have major limitations due to survival time in culture, cellular heterogeneity and inability for large scale production (McNeish, 2004; Pouton and Haynes, 2007). Stem cell technology furnishes a novel opportunity for the generation of a physiologically relevant disease model using directed differentiation of stem cells into the optimal model cell type suitable for functional study and drug discovery (McNeish, 2004; Pouton and Haynes, 2007; Rubin and Haston, 2011).

Creation of a human neuronal model derived from human embryonic stem (ES) cells or patient-derived induced pluripotent stem (iPS) cells would ultimately serve as the ideal disease model, eventually able to predict drug responsiveness in cells originating from the patient's own tissue (Yamanaka and Blau, 2010; Jang et al., 2012) However, despite great promise, productive and reliable use of human ES or iPS cells in drug discovery remains challenging (Ebert and Svendsen, 2010; Han et al., 2011). For drug screening applications such as high throughput screening (HTS), human ES and iPS protocols require laborious maintenance which make automation and differentiation into functional neurons challenging (Ebert and Svendsen, 2010). Furthermore, genetic and epigenetic variability among patients may influence pharmacological responsiveness, hindering the practical application of stem cells from human patient origin for drug discovery (Han et al, 2011). In contrast to human stem cells, mouse ES (mES) cells harbor several notable advantages for bioassays and drug discovery applications, including the relatively homogenous nature of differentiated cells, potential derivation from disease models and convenient expandability (Pouton and Haynes, 2007). It has been shown that mES cells can be differentiated into specific neuronal subtype(s), including pyramidal neurons, a significantly vulnerable neuronal population in AD (Mann, 1996; Morrison and Hof, 2002).

To establish an AD-centric primary neuronal model, we developed phenotypic cell-based assays for the biogenesis and synaptic action of A $\beta$  using mouse ES cell-derived neuronal cultures enriched in functional pyramidal neurons. We isolated ES cells from a mouse model of the disease, Tg2576 which harbors human APP with the Swedish mutation (APP<sub>sw</sub>). To validate application of mouse embryonic stem cell-derived neurons (ESNs) we screened a library of clinical compounds and identified four compounds capable of reducing A $\beta$  levels. Our neuronal model is therefore highly suitable for phenotypic screening in AD drug discovery and has the potential to enhance the predictive value of cell-based screening assays for lead compounds with a higher probability of surviving advanced rounds of vetting for efficacy and diminished toxicity.

## RESULTS

### Characterization of Pyramidal Cell Enriched ESN Cultures

A neuronal culture highly enriched in pyramidal neurons was produced by directed differentiation of mES cells by modifying published protocols (Figure 1A) (Bibel et al., 2004; Bibel et al., 2007). Neuronal morphology was apparent after 1 day *in vitro* (DIV) and the majority of cells displayed neuronal morphology by DIV 5 (Figure 1A). By DIV 7 neuronal proteins including neuronal  $\beta$ -tubulin III (TUJ-1) and pyramidal neuron marker EMX1 were expressed indicating a highly homogenous neuronal population enriched in pyramidal neurons (Figures 1B and 1D) (Chan et al., 2001). Glial cells, identified by glial fibrillary acidic protein (GFAP) were only rarely observed (Figures 1C and 1D). Neuron specific proteins, neuronal  $\beta$ -tubulin (TUJ-1) and synaptophysin, as well as glutamatergic neuronal proteins, TrkB and CaMKII  $\alpha$ , increased from DIV 1-7 and there was a gradual decline in the levels of the p75 neurotrophin receptor (p75<sup>NTR</sup>) consistent with maturing

pyramidal neurons (Figure 1E) (Buck et al., 1988). The subcellular distribution of endogenous pre-synaptic synaptophysin and post-synaptic PSD-95 as adjacent puncta along dendrites recapitulated mature synapses in primary neurons (Figure 1F). Electron microscopy (EM) analysis revealed that both presynaptic vesicles as well as postsynaptic densities were apparent in this culture (Figure 1G). Expression of PSD-95 and synaptophysin was evident by Western analysis by DIV 14 (Figure 1H).

### Modeling A $\beta$ -induced synaptic alterations in ESNs

Oligomeric A $\beta$  induces both structural and functional alterations at synapses in cultured neurons (Pozueta et al., 2012). To model this using mouse ESNs, we examined the effects of A $\beta$  treatment on synaptic morphology and changes in cell signaling events indicative of synaptic plasticity. Synapse loss is documented in AD mouse models and after treatment of neurons with oligomeric A $\beta$ , correlating with the loss of immunoreactivity of PSD-95, a postsynaptic protein (Gyls et al., 2004; Roselli et al., 2005; Cerpa et al., 2010). In ESNs, oligomeric A $\beta$  treatment decreased PSD-95 puncta count, fractional area and total area but synaptophysin puncta, a presynaptic protein, remained unchanged (Figures 2A-2F) (Hu, et al., 2003; King ad Arendash, 2002; Jacobsen et al., 2006). A $\beta$  treatment also triggers morphological defects in synaptic spines including reduction in dendritic spine density (Hsieh et al., 2006; Shankar et al., 2007; Lacor et al., 2007). Diolistic labeling of neurons (Moolman et al., 2004; McIntire et al., 2012) revealed that treatment of ESNs with A $\beta$  oligomers reduced spine density (Figure 2G, H). After A $\beta$  oligomer treatment, spine length increased modestly and slightly decreased dendritic diameter (Figures 2I and 2J) (Calabrese et al., 2007; Lacor et al., 2007). In addition to morphological spine changes, at a functional level, A $\beta$  has been shown to induce characteristic molecular changes in neurons, including suppressed phosphorylation of cyclic AMP response element binding protein, CREB, a memory-associated transcription factor (Vitolo et al., 2002; Snyder et al., 2005). In ESNs, A $\beta$  oligomer treatment prevented NMDA stimulation dependent phosphorylation of CREB (pCREB) (Figures 2K and 2L). Treatment of ESNs with A $\beta$  oligomer at high concentrations led to neuronal death (Figure S1).

### Expression of Secretase Components in ESNs

To determine if ESNs would effectively model A $\beta$  biogenesis, we determined expression of enzymatic components of APP processing. ESNs express the  $\beta$ -site APP cleaving enzyme (BACE1) at later stages of differentiation but not in ES cells (Figures 3A and 3C) (O'Brien and Wong, 2011; Zheng and Koo, 2011). In contrast, PS1 was detected in mES cells consistent with its described role in development (Figure 3B) (Wong et al., 1997). Further, APP and BACE1 were enriched in the late endosomal compartment enriched for syn6 and rab11 and de-enriched in early endosomes containing early endosomal protein A1 (EEA1) consistent with published distribution in cultured primary neurons (Figure 3C) (Tang, 2009; Finan et al., 2011; Wen et al., 2011).

### Genetic Modeling of Expression of FAD-associated APP and PS1 in ESNs

To model familial AD (FAD), wild type ESNs were infected with APP<sub>sw</sub> lentivirus and APP processing was analyzed. Treatment of infected ESNs with BACE1 inhibitor (BSI) (Stachel, 2006) reduced secretion of sAPP $\beta$ , A $\beta$  40 and A $\beta$  42 while treatment with  $\beta$ -secretase inhibitor (GSI) (Seiffert, 2000) inhibited A $\beta$  40 and A $\beta$  42 secretion without affecting the levels of secreted APP $\beta$ -cleaved fragment (sAPP $\beta$ ) or secreted APP $\alpha$ -cleaved fragment (sAPP $\alpha$ ) (Figures 3D and 3F). Consistent with previous studies in cell models and mouse brain, C-terminal fragments (CTFs) resulting from APP cleavage, accumulated in response to GSI treatment (Figure 3D) (Hardy and Selkoe, 2002; O'Brien and Wong, 2011; Zheng and Koo, 2011; Landman et al., 2006; Okada et al., 2010).

To model PS1-linked FAD phenotypes in ESNs, three different FAD mutant PS1 transgenes (E9, M146V, and L286V) were electroporated into mES cells to produce ES lines stably expressing the mutants. Resulting stable mES cells were subjected to directed differentiation into pyramidal neurons. A representative Western blot shows the PS1 transgene and normal neuronal protein expression in ESNs derived from these clonal mES cell lines (Figures 3G and 3H). An increase in the A<sub>42</sub>/A<sub>40</sub> ratio, a key pathogenic phenotype associated with PS1 FAD (O'Brien and Wong, 2011; Zheng and Koo, 2011) was observed in ESNs expressing E9, M146V, and L286V mutant forms of PS1 after APP<sub>sw</sub> lentiviral infection (Figure 3I).

### ESNs from Tg2576 Mouse Model of AD

In order to achieve robust and reproducible neuronal expression of human APP, mES cells were isolated from the inner cell mass of blastocysts from a well characterized mouse model of AD, Tg2576 (Hsiao et al., 1996). ESNs from Tg2576 showed normal neuronal differentiation by morphology and neuronal protein expression (Figures 4A and 4B), comparable to neuronal differentiation of wild-type ES cells. Transgene-derived human APP<sub>sw</sub> was expressed at DIV 1-7, but little expression was detected in the undifferentiated mES cells (Figure 4B). APP processing at DIV 7 was sensitive to BSI, GSI and an  $\gamma$ -secretase inhibitor, TAPI-2. APP CTF expression increased in response to GSI treatment, but levels of neuronal proteins synaptophysin, CaMKII and neuronal  $\alpha$ -tubulin did not change with any pharmacological treatment (Figure 4C). Soluble sAPP<sub>40</sub> and sAPP<sub>42</sub> were reduced by TAPI-2 and BSI treatment respectively (Figure 4D). Further, human A<sub>40</sub> and A<sub>42</sub> were reduced by treatment of neurons with BSI and GSI as predicted (Figures 4E and 4F) (Stachel, 2006; Seiffert, 2000).

### High Throughput Screening Assay for A $\beta$ in Tg2576 ESNs

High-throughput screening (HTS) using a miniaturized assay allows rapid and parallel examination of the bioactivities of large numbers of compounds. To accommodate HTS in ESNs, we miniaturized an A $\beta$  detection assay using Tg2576 ESNs cultured in a 96 multi-well plate. We determined the optimal cell density and assay parameters to optimize suitability and robustness for HTS application. The optimal cell density of  $1.5 \times 10^5$  cells/cm<sup>2</sup> maximized the cell viability signal (Figures 5A and 5C). Two parameters commonly used to evaluate the robustness of a HTS assay are the coefficient of variance (%CV) and Z' factor (Inglese et al., 2007). Typically, an assay with %CV value <15% and Z' factor >0.5 is considered a robust assay suitable for HTS (Inglese et al., 2007). At the plating density of  $1.5 \times 10^5$  cells/cm<sup>2</sup>, with 5 replicate wells, both the %CV (Figure 5B) and Z' factor (Figure 5C) were optimized. Using the optimized 96 well platform, ESNs derived from the Tg2576 mouse model displayed characteristic inhibitor response kinetic profiles for both BSI and GSI (Figures 4E and 4F) (Stachel, 2006; Seiffert, 2000). Values of %CV for A<sub>40</sub> and A<sub>42</sub> indicated acceptable variance for signal detection (Inglese et al., 2007). Additional metrics including signal to noise (S:N), signal to background (S:B) and signal window also indicated detection of A<sub>40</sub> and A<sub>42</sub> was sufficiently higher than variance of the assay (data not shown).

### Identification of Clinical Compounds that Reduce A $\beta$ Secretion in ESNs

To fully validate the ESNs-based assay, we screened for inhibitors of A $\beta$  secretion using the NIH clinical collection (NCC) which consists of 446 small molecules that have a history of use in human (Noorbakhsh et al., 2009). Compounds were identified as hits if they reduced A<sub>40</sub> by 40% and deviated 4 standard deviations from the average value of negative controls (Figure 6A) but maintained cell viability within 2 standard deviations of control (Figure 6B). The most potent hits identified were amiridine, icariin, phenelzine and progesterone (Figure 6C). The compounds were obtained from an independent source,

activity was confirmed and they were subjected to IC50 determination (Figure 6D). To investigate the possible mechanisms of the A $\beta$ -reducing activity of these compounds, alterations in APP processing were determined in response to compound treatment. Icaritin, phenelzine and progesterone reduced sAPP $\beta$  levels, suggesting that these compounds may modulate the activity of BACE1 (Figures 6E and 6F). None of the compounds significantly affected levels of sAPP $\beta$  or full length APP (Figures 6D, 6G and 6H). A $\beta$ 40 was confirmed to be reduced by the hit compounds (Figure 6I).

### Pharmacological Response of Hit Compounds in ESNs and Cell Lines

An advantage of phenotypic screening in ESNs is the preservation of the cellular context of physiological neurons compared to commonly used cell-based screening assays typically based on transformed cell lines. Therefore, we determined the ability of hit compounds to exert A $\beta$ -lowering activity in APP, PS1 and BACE1 expressing cell lines. Interestingly, icaritin, phenelzine and progesterone did not significantly reduce A $\beta$  secretion in any of the three alternate cell lines we tested, including Neuro2a expressing APP<sub>sw</sub>, CHO expressing APP and mutant PS1 (E9), and SH-SY5Y cells expressing BACE1 and APP (Figure 7). Amiridine reduced A $\beta$  secretion in the SH-SY5Y cell line however, A $\beta$ -reducing activity was not observed in Neuro2a or CHO cell lines (Figure 7).

## DISCUSSION

Our current study presents practical applications of mouse ESNs for AD and CNS drug discovery research. ESNs serve as an infinite source of neurons which functionally and morphologically recapitulate pyramidal neurons as well as harbor the appropriate machinery for APP processing and synaptic function. We show that ESNs can be cultured and assayed in a platform suitable for drug screening. Using ESNs from the Tg2576 mouse model of AD, we established and validated a high throughput screening (HTS) platform for an A $\beta$  detection assay and also provided examples of how this platform can be used to probe various key biological and pathophysiological pathways in intact primary neurons, such as analyses of alterations in synaptic function and structure, and APP processing. It is plausible that our ESN platform is also applicable to parallel detection of multiple A $\beta$ -amyloid phenotypes described in this paper, in addition to other AD-associated phenotypes such as tau-associated abnormalities. Thus, to address the complex biological mechanisms underlying AD, our model uniquely permits the investigation of druggable pathways or networks in intact neurons (Noorbakhsh et al., 2009).

The mouse ESN model has appreciably enhanced suitability for HTS compared to human ES and iPS cell-derived neurons. In general, much longer expansion and differentiation periods are required for ESNs and iPS-derived neurons, which typically require 21+ days for differentiation and up to 5 weeks for maturation in culture to display desired phenotypes (Barker, 2012; Dhara and Stice, 2008; Schwartz et al., 2008). Thus, mouse ES cells and ESNs harbor an advantage over human systems for ease of expandability in a practical amount of time for automation (Erceg et al., 2009; Kim et al., 2011; Nistor et al., 2011). Furthermore, it is difficult to obtain relatively homogenous neuronal populations from human pluripotent cells in contrast to mouse ESNs implying that our mouse ESN-based approach is more suitable for quantitative detection and assays owing to relatively homogenous and reproducible neuronal cultures (Engle and Puppala, 2013; Ebert and Svendsen, 2010; Han, 2011). Human iPS cells from AD patients have recently been successfully differentiated into neurons displaying several expected phenotypes including elevated A $\beta$  (Yagi et al., 2011; Israel et al., 2011). However, iPS cell-derived neurons from one sporadic AD patient lacked the A $\beta$  increase altogether, highlighting the inherent variability among patient derived iPS cells due to differences in genetic background compounded with inherent variability among clones from the same patient (Israel et al.,

2011; Choi and Tanzi, 2012; Ooi et al., 2013). Further, since AD has a mixed etiology, selecting appropriate control lines for iPS is challenging considering the variability in genetic background of each patient (Barker, 2012; Choi and Tanzi, 2012). In contrast, mouse ES cell lines can be harvested from the numerous existing mouse models, giving rise to disease models and near-clonal appropriate controls. Genetic and epigenetic integrity in iPS cells may be compromised by insertional defects owing to the integration of ectopic transcription factors (Han et al., 2011; Papp and Plath, 2011). Synapse loss, a critical feature in AD correlating with disease severity, was not observed in recent publications on iPS from AD patients, perhaps due to limited culture duration (Yagi et al., 2011; Choi and Tanzi, 2012), however, mouse ESN display robust synapse formation as well as A $\beta$ -triggered synapse loss (Figure 2). Thus, mouse ESNs uniquely harbor both phenotypic relevance and HTS adaptability. Though mouse ESN are favorable for HTS, human derived ESN and iPS derived neurons currently hold high value for secondary confirmatory studies for compound efficacy and toxicity in the human genetic background.

The four most potent hits identified in our screen are known CNS drugs. Amiridine belongs to the class of aminopyridine acetylcholinesterase inhibitors including tacrine, which have been used in the clinic for the symptomatic treatment for AD (Braginskaya et al., 2001; Yoshida and Suzuki, 1993). An interesting future investigation would be to determine whether the A $\beta$  lowering activity is a common property of all aminopyridine compounds in this drug class and if this activity potentially influences clinical variables. Phenzelzine belongs to a class of drugs, the monoamine oxidase inhibitors (MAOI), which have been studied for potential clinical use in AD, based on their ability to promote neurotransmission and improve cognition (Freedman et al., 1998; Mangoni et al., 1991; Sano et al., 1997; Volz and Gleiter, 1998). We found that phenelzine reduced A $\beta$  and sAPP secretion suggesting that this compound may reduce A $\beta$  through the inhibition of BACE1 in neurons. In the previous studies, derivatives of this class of drug have been reported to inhibit  $\beta$ -secretase activity and stimulate sAPP production in cell lines (Lee et al., 2010; Weinreb et al., 2009; Yodanis et al., 2003). Thus, it is conceivable that phenelzine confers A $\beta$ -reducing activities through multiple mechanisms involving both  $\beta$ - and  $\gamma$ -secretase pathways through cellular target(s) have not yet been identified. Icaritin has been reported to reduce A $\beta$ -induced synaptic and cellular deficits (Li et al., 2010; Zeng et al., 2010) and ameliorate memory impairment in A $\beta$  treated rats and a mouse model of AD (Nie et al., 2010; Urano and Tohda, 2010). Icaritin has also been reported to reduce the expression of BACE1 (Nie et al., 2010). Consistent with its reported role on BACE1, we found that icaritin reduces secretion of sAPP which may have contributed to cognitive improvements observed in previous mouse model studies. Lastly, our screen identified progesterone as a A $\beta$ -reducing compound. In previous in vivo studies, progesterone was shown to reduce amyloid load and improve cognitive performance of AD mouse models (Carroll et al., 2010; Frye and Walf, 2008). Since progesterone lowered sAPP, A $\beta$  40 and A $\beta$  42 production in neurons, it is conceivable that A $\beta$ -reducing activity may underlie the observed rescue of pathological and behavioral changes in mouse models of AD. Collectively, our approach therefore could be useful in evaluating potential of known drugs for a new use in AD in the context of phenotypically intact neurons as well as screening for novel small molecules.

## EXPERIMENTAL PROCEDURES

### Directed differentiation of mouse embryonic stem cells to pyramidal neurons

mES were maintained for two passages on mouse embryonic fibroblast (MEF) feeder cells in the presence of Leukemia Inhibitory Factor (LIF) (Millipore). For embryoid body (EB) formation, mES were deprived of MEFs for two passages. EB were grown in suspension for 8 days and in the presence of retinoic acid (RA) for the last 4 days prior to plating as neurons. Optimized neuronal media containing N2 supplement (Invitrogen) was used for the

first 2 days after plating and B27 (Invitrogen) supplemented media was used for the duration of the culture. Detailed media composition and protocols are found in Supplemental Experimental Methods.

### **Analysis of synaptic proteins, morphology and spine density**

After DIV21, ESN were treated with 200nM A oligomer prepared as previously described (Dahlgren et al., 2002; McIntire et al., 2012) for 24 hours then fixed in 4% paraformaldehyde and 4% sucrose in PBS for 20 minutes. PSD-95 (Thermo Scientific) and synaptophysin (S-physin) (Epitomics) antibodies were used and detected with Alexa tagged secondary antibodies (Molecular Probes). For spine analysis, after fixation, neurons were labeled with DiI (Molecular Probes) as previously described (Moolman et al., 2004; McIntire et al., 2012). Images were collected with an inverted Olympus microscope with 100x objective using Nikon C2 confocal laser microscope system. For spine analysis, 0.5 $\mu$ M z-sections were taken at 3x zoom. Images were stacked, used for 2-D projection with ImageJ 1.4g software (NIH), and measured with the segmented line tool. For particle analysis, the RGB colors were split, threshold was set for each image, and the analyze particles tool was used.

### **Phospho-CREB detection**

Neurons were grown to maturity and pre-treated for 1 hour in the presence or absence of A oligomer preparation. Subsequently, cultures were treated 10 minutes under stimulating conditions (150mM, 5mM KCl, 2mM CaCl<sub>2</sub>, 30mM glucose, 10mM HEPES, pH7.4, 10 $\mu$ M NMDA) (Synder et al., 2005) or non-stimulating conditions (120mM NaCl, 3mM KCl, 2mM CaCl<sub>2</sub>, 2mM MgCl<sub>2</sub>, 15mM glucose, 15mM HEPES, pH 7.4) in the presence or absence of A oligomer (Dahlgren et al., 2002). Protein was solubilized immediately in Laemmli sample buffer and loaded (v/v) to 4-20% Tris/Glycine gel (Invitrogen). Protein was separated using SDS-PAGE and quantitative Western blotting was accomplished using infra-red secondary antibody (Rockland) detection with the Licor Odyssey Infrared Imager with solid-state diode laser at 685 nm and 785 nm. Total CREB primary antibody was from Santa Cruz Biotechnology and phospho-CREB (S133) primary antibody was from Invitrogen.

### **Subcellular Fractionation**

Differentiated mouse embryonic stem cells were homogenized using a ball-bearing homogenizer in homogenization buffer (0.25M sucrose, 10mM Tris, pH7.4, 2mM MgAC, and 0.5 mM EDTA, supplemented with protease inhibitors (Roche)). Homogenate was loaded onto a 0.25M – 2M sucrose step gradient and centrifuged for 2.5h at 39,000rpm at 4°C. Ten fractions were collected and analyzed by Western blotting. Primary antibodies used were: anti-sortilin (BD Biosciences), anti-BACE1 (Zhoa et al., 2007), anti-APP-CTmax (Landman et al., 2006; Okada et al., 2010), anti-syn6 (BD Biosciences), anti-EEA1 (Affinity BioReagents) and anti-Rab11 (BD Biosciences).

### **Lentiviral Expression of APP<sub>sw</sub> in ESNs and generation of FAD mutant PS1 stable ES cell lines**

To model human APP processing, neurons were infected with APP<sub>sw</sub> lentivirus for 24 hours and 48 hours after initial infection, treated with BACE inhibitor IV (BSI) (Calbiochem) or -secretase inhibitor (GSI) (Calbiochem) for 24 hours. Uninfected neurons were treated with 0.1% saponin (Sap) as a positive control for loss of cell viability. To generate stable lines of mES cells expressing PS1 wild-type and FAD variants 3 $\times$ 10<sup>6</sup> ES cells were resuspended in Nucleofector solution (Amaxa Neucleofector, Lonza) and mixed with 20  $\mu$ g plasmid carrying human wild-type or FAD mutant PS1 and neomycin resistance in the pcDNA3.1

vector. Cells were electroporated using program A-13 (Amaxa), resuspended, plated on feeder cells and subjected to G418 (250-500  $\mu\text{g}/\text{ml}$ ) selection 24 hours after electroporation.

### Analysis of APP processing

At DIV 12-30, neurons were infected with lentivirus carrying the Swedish variant of human APP (pLenti6/hAPP<sub>sw</sub>). After 24 hrs, cell medium was changed and 48 hours after infection, cells were treated with secretase inhibitors. Conditioned media was collected after 24 hrs and the levels of A<sub>42</sub> and A<sub>40</sub> were determined using sandwich ELISA (Invitrogen) according to the manufacturer's protocol. Total protein levels were determined by BCA protein assay (Pierce). Total A<sub>42</sub> was detected by immunoprecipitating conditioned medium with 6E10 (Covance) or 7N22 (Invitrogen) antibodies, running samples on NuPAGE gels and detected by quantitative Western blotting with 6E10 antibody. Full-length APP was detected by Western blot analysis using 6E10 (Covance) or polyclonal antibody against the C-terminus of APP (APP-CTmax) (Landman et al., 2006; Okada et al., 2010). For detection of sAPP<sub>42</sub> and sAPP<sub>40</sub> conditioned medium was harvested 24 hours after a media change.

Immunoprecipitation of sAPP<sub>42</sub> was performed using s<sub>sw</sub> antibody (Figure S2). Protein G plus/Protein A agarose suspension (Calbiochem) was used to precipitate the antibody-protein complex. The immunoprecipitate was subjected to SDS-PAGE followed by Western blot detection using 22C11 (Chemicon) or LN27 (Covance). sAPP<sub>42</sub> was immunoprecipitated with 6E10 and detected on Western blot with LN27. BACE inhibitor IV, compound E and TAPI-2 were from Calbiochem.

### Isolation of ES cells from a mouse model of AD

Tg2576 mice were outbred strain of Swiss Webster  $\times$  B6D2F1 which is a C57BL/6  $\times$  DBA/2 F1 cross harboring the APP transgene with the Swedish mutation under the control of the prion promoter (Hsiao et al., 1996) C57BL/6 females 23 days old were induced for superovulation with a standard protocol using intraperitoneal injection with pregnant mares serum gonadotropin (PMSG) on day 1 and human chorionic gonadotropin (hCG) on day 3. Immediately following the final injection, the female was mated with a male positive for the APP<sub>sw</sub> transgene. After 3.5 days blastocysts were flushed from the uterus and cultured in 96-well feeder plate wells containing mitomycin C-treated MEF feeders in ES medium for six days without medium change. Inner cell mass (ICM) outgrowth was disaggregated with 0.05% trypsin into small clumps and plated in one well of 24-well feeder plates. ES colonies were apparent on day three after re-plating and ES cells were passaged upon confluence and maintained as above. DNA was isolated using the DNeasy and Tissue DNA isolation kit (Qiagen). ES lines harboring the transgene were identified using PCR based genotyping protocol with primers against the human APP<sub>sw</sub> gene sequence (sense primer 1: CCGAGATCTCTGAAGTGAAGATGGATG and antisense primer 2: GTGGATACCCCTCCCCAGCCTAGACC).

### Small molecule screen of clinical compound library for reduction of A $\beta$

ESNs were plated in 96 well black plates at a density of  $1.5 \times 10^5$  cells/cm<sup>2</sup> (45,000 cells/well). After DIV 8, ESNs were treated with 10 $\mu\text{M}$  of each compound from a library of 446 clinically relevant compounds which have a history of use in human, the NIH clinical collection (NCC) (NIH) for 24 hours in B27 media. Each plate of ESNs was treated additionally with DMSO (negative control), and positive controls for A<sub>42</sub> reduction, BSI and GSI using 6 replicates. After 24 hours, conditioned media was harvested and frozen at -80°C until A<sub>40</sub> analysis using human specific ELISA (Invitrogen). The remaining cells were subjected to cell viability assay CellQuanti Blue (BioAssay Systems). Compounds were considered hits if they reduced A<sub>40</sub> by 40% compared to the negative control (DMSO) and reduced A<sub>40</sub> by 4 standard deviations from the average value of the negative control but

maintained cell viability. Cell viability was defined by maintenance of CellQuanti Blue (BioAssay Systems) fluorescent signal within two standard deviations of the control treated (DMSO) neurons. For confirmation studies, amiridine, phenelzine and icariin were obtained from Sigma and progesterone was obtained from Tocris.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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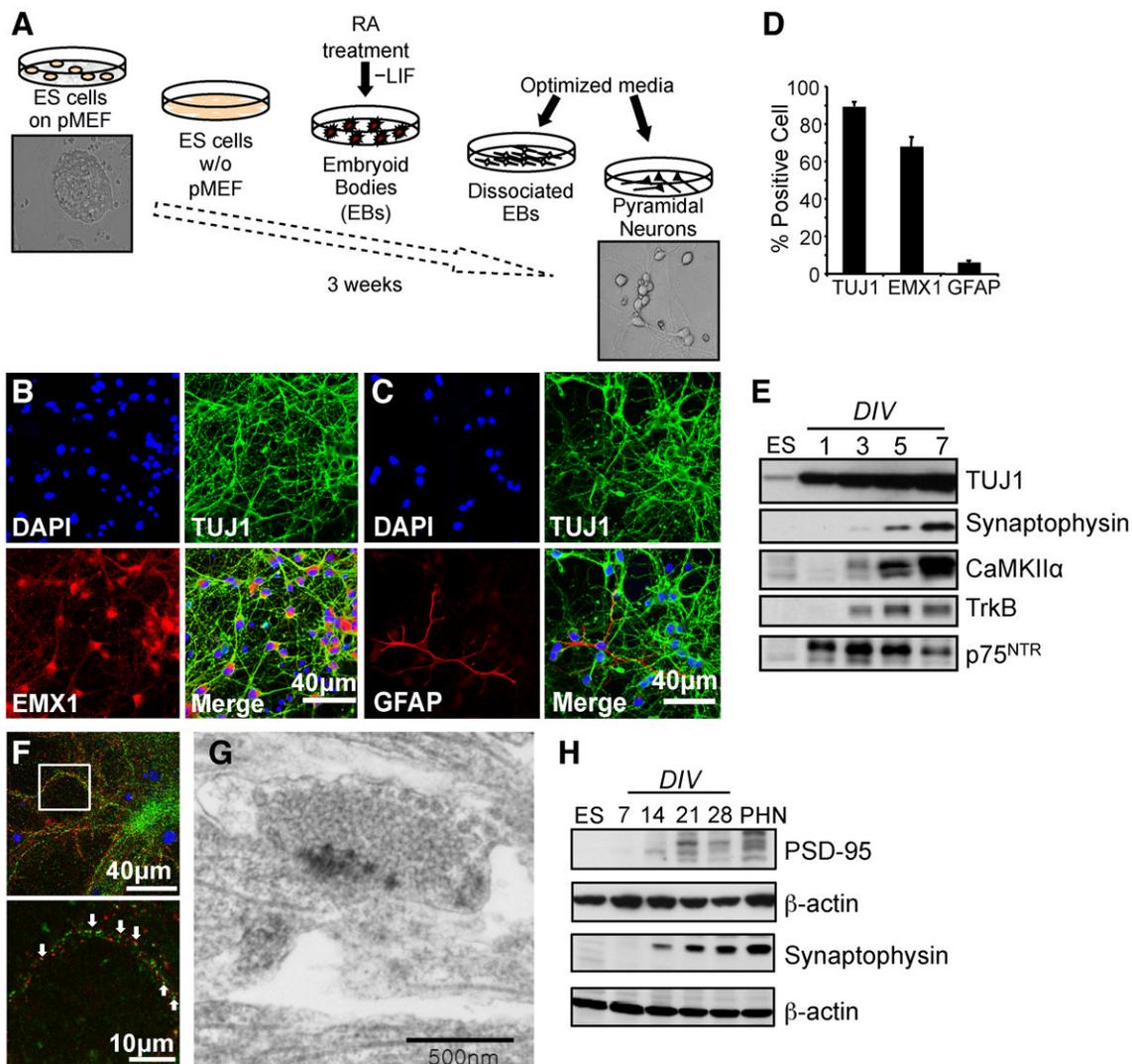
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**HIGHLIGHTS**

- Scaling and optimizing primary neurons derived from mouse ES cells
- Modeling phenotypic assays using mouse ESNs for biogenesis and synaptic action of A
- Isolation and use of ES cells originating from a mouse model of AD
- Screen of clinical compounds and identification of A<sup>-</sup>-lowering small molecules

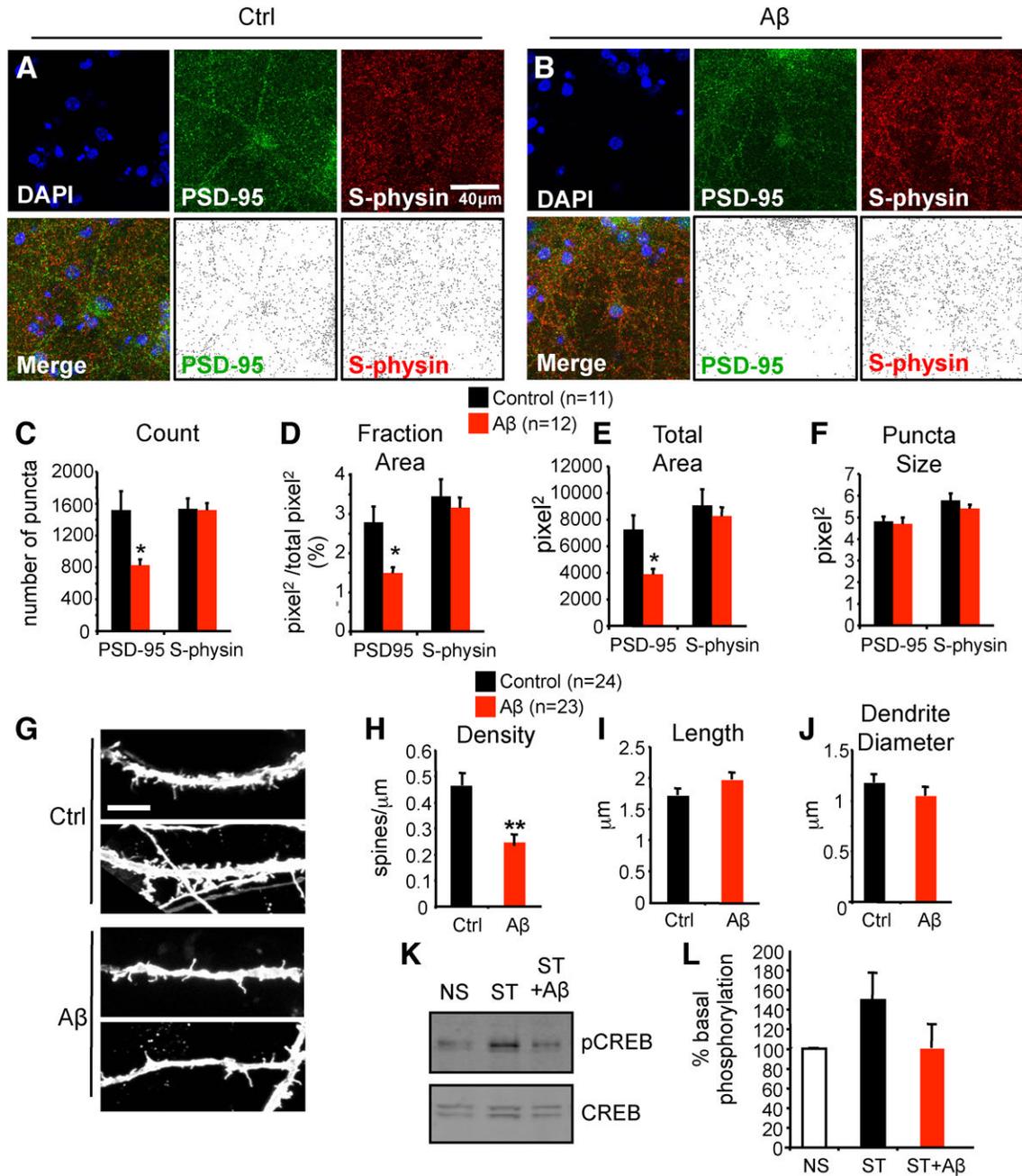
### SIGNIFICANCE

Our current study describes practical applications of mouse embryonic stem (ES) cell-derived neurons (ESNs) for AD and CNS drug discovery research. Our studies indicate that ESNs could potentially replace dissociated primary neuron cultures as a cell-based model for investigation of neurobiology and pathobiology relevant to AD. We also demonstrated that ESNs from the Tg2576 mouse model of AD can be cultured and assayed in a platform suitable for drug screening. Using this ESN-based assay, we performed phenotypic screening assays and identified four interesting clinical compounds for which A $\beta$ -modulating effects have not been previously reported in primary neurons. There is a growing demand for preclinical models that reliably predict efficacy and safety prior to clinical trials. This demand can be met by specialized disease-relevant cell types. For drug discovery applications, our system offers notable advantages over human patient derived stem cells, which have limited expandability and phenotypic variability. Thus, our model overcomes current challenges in phenotypic HTS assays in neurons, bridging the gap between in vitro screening paradigms and translational drug discovery and has great potential to lead to innovation in AD drug discovery by furnishing pyramidal neurons amenable to screening and functional evaluation of compound efficacy and toxicity in stem cell-derived primary neurons.



### Figure 1. Directed Differentiation of mES Cells Into Pyramidal Cell-enriched Neurons

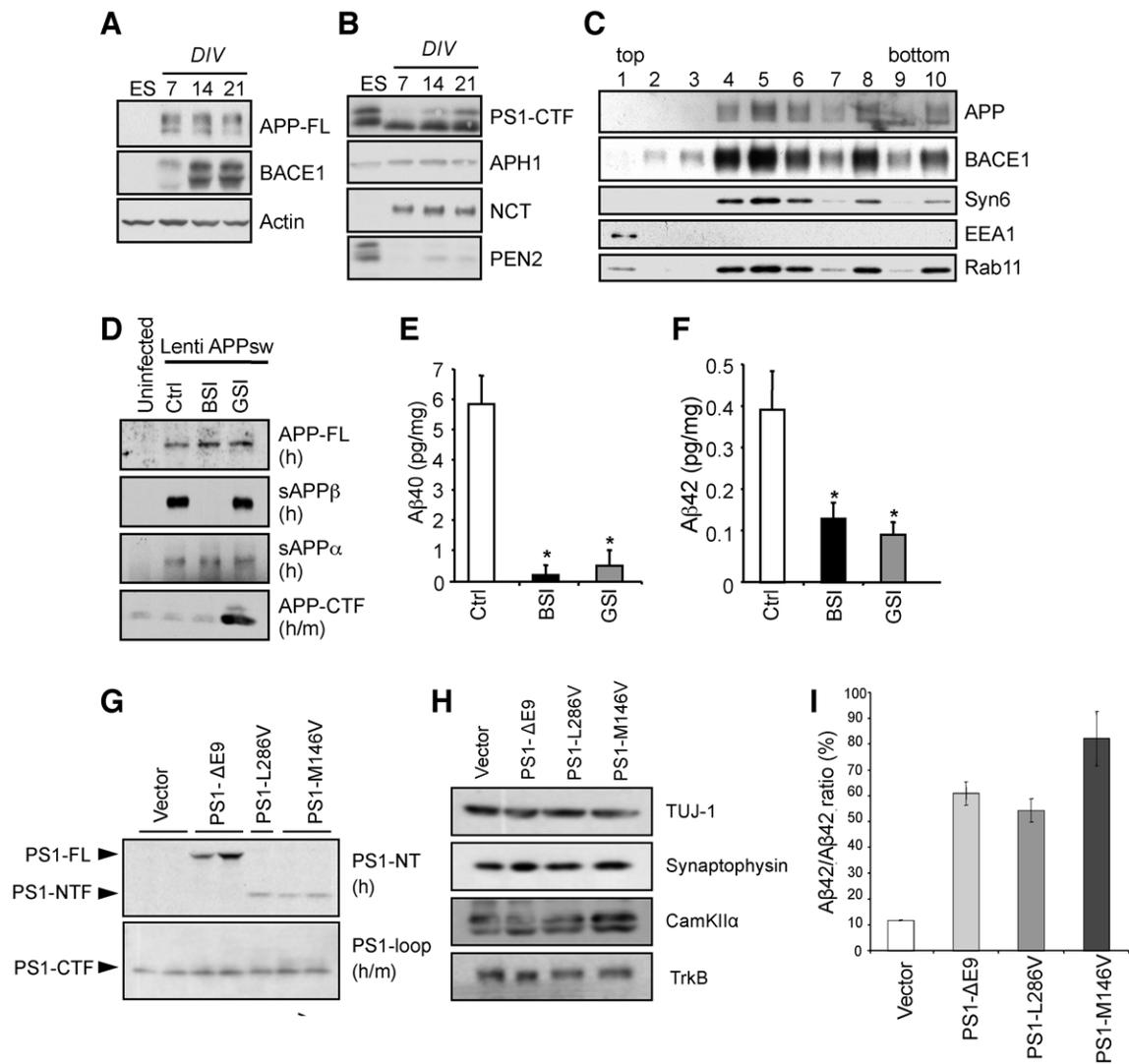
(A) Schematic of the differentiation procedure for mES cell derived neurons using primary mouse embryonic fibroblasts (pMEF) and retinoic acid (RA) showing ES cells and ESNs at DIV 5. (B) ES cell derived neuronal culture at DIV 7 co-labeled with neuronal  $\alpha$ -tubulin III (TUJ1) and EMX1 antibodies and DAPI to identify nuclei. (C) ES cell derived neuronal culture at DIV 7 co-stained with GFAP antibody and TUJ1 antibodies and DAPI to identify nuclei. (D) Quantification of cultures show only 6(±1.5) % of cells stained positive for GFAP, while 68(±10)% cells were positive for EMX1 and 89.5 (±4)% cells were positive for TUJ1. Scale bar represents 40µm. (E) Cultures increasingly express neuronal proteins assessed by Western analysis using indicated antibodies. (F) Confocal images of *DIV21* neurons derived from mES cells detect post-synaptic density protein PSD-95 and pre-synaptic density protein synaptophysin which localize to adjacent puncta at higher magnification. (G) Electron micrograph showing intact synaptic structures including post-synaptic density and pre-synaptic vesicles in *DIV21* cultures. (H) PSD-95 and synaptophysin (S-physin) detection by Western analysis.



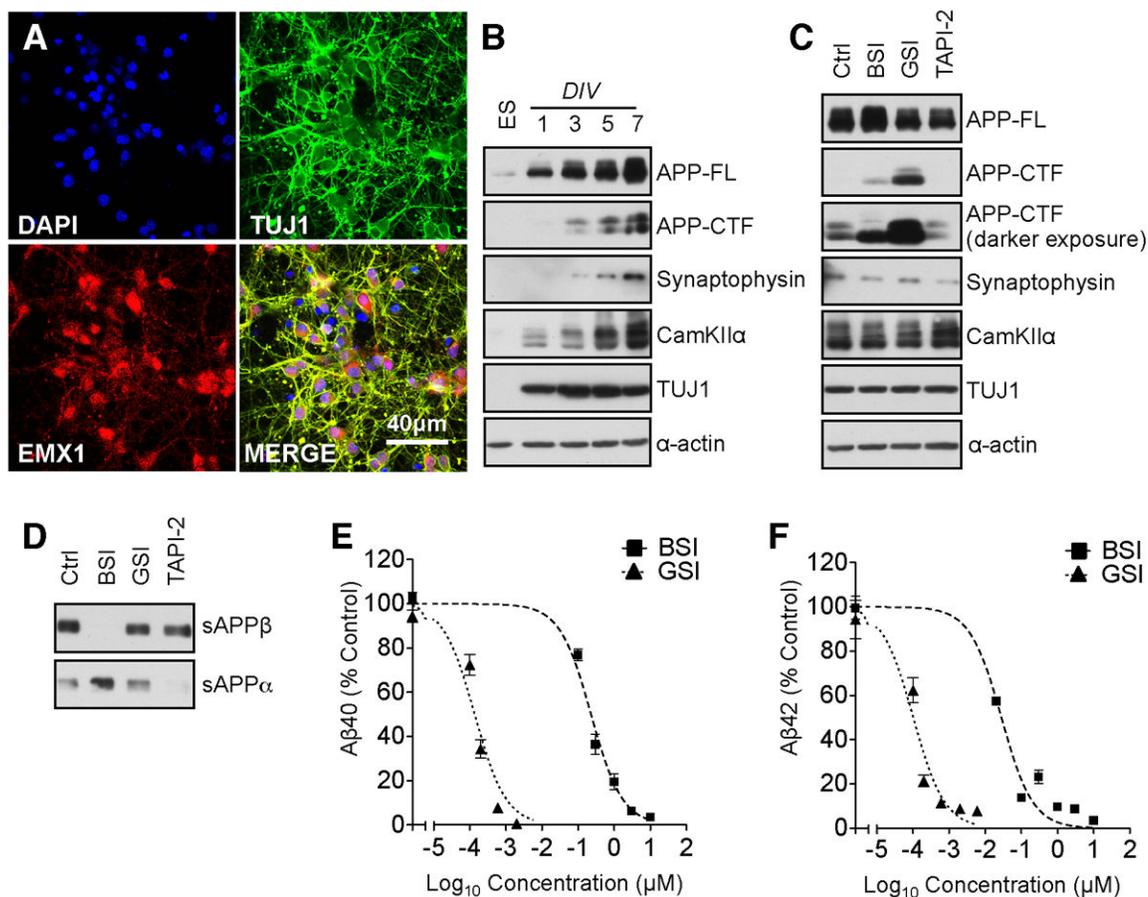
**Figure 2. Modeling A $\beta$ -associated Synaptic Abnormalities in ESNs**

(A and B) At DIV 21 mES derived neurons were treated for 24 hours with DMSO control (Ctrl) or A $\beta$  42 oligomer, subsequently fixed and co-stained for synaptic proteins PSD-95 (green) and synaptophysin (S-physin) (red). Nuclei are labeled with DAPI (blue). PSD-95 and S-physin particles were analyzed using Image J software. (C-F) Quantification of PSD-95 positive particles and S-physin positive particles using Image J software particle analysis. (G) DIV 21 ESN were treated with control or A $\beta$  42 oligomer, fixed, diolistically labeled and imaged with confocal microscopy. (H-J) Spine density, length and dendrite diameter were quantified using Image J software. (K) Phosphorylation of CREB was detected with a pCREB specific antibody after no stimulation (NS) or NMDA stimulation

(ST) of cultures with or without A<sub>42</sub> oligomer (A<sub>42</sub>) pretreatment. (L) Quantification of CREB and pCREB using infrared quantitative Western blot imaging system with SEM shown by error bars.

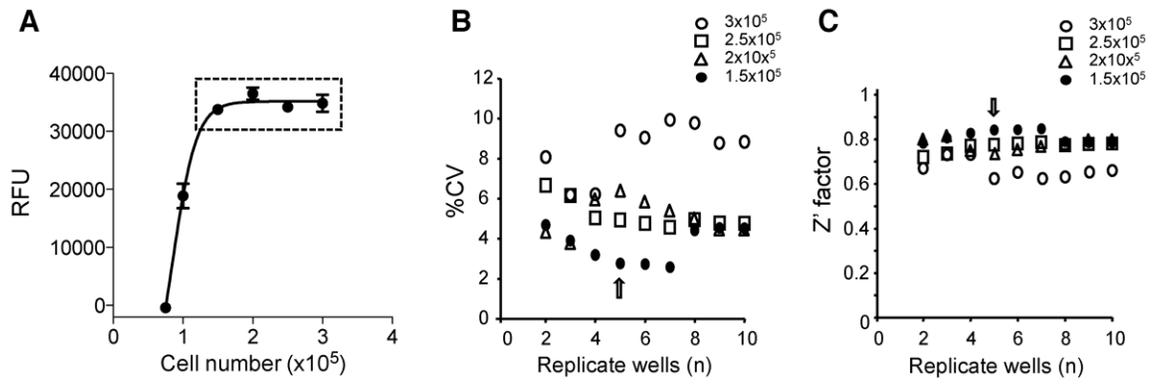


stably expressing PS-FAD mutants infected at DIV 7 or 14 with APP<sub>sw</sub> Lentiviral particles resulted in enhanced ratio of A<sub>42</sub>/A<sub>40</sub>. Lentiviral gene transfer did not affect the viability of ESNs (data not shown).



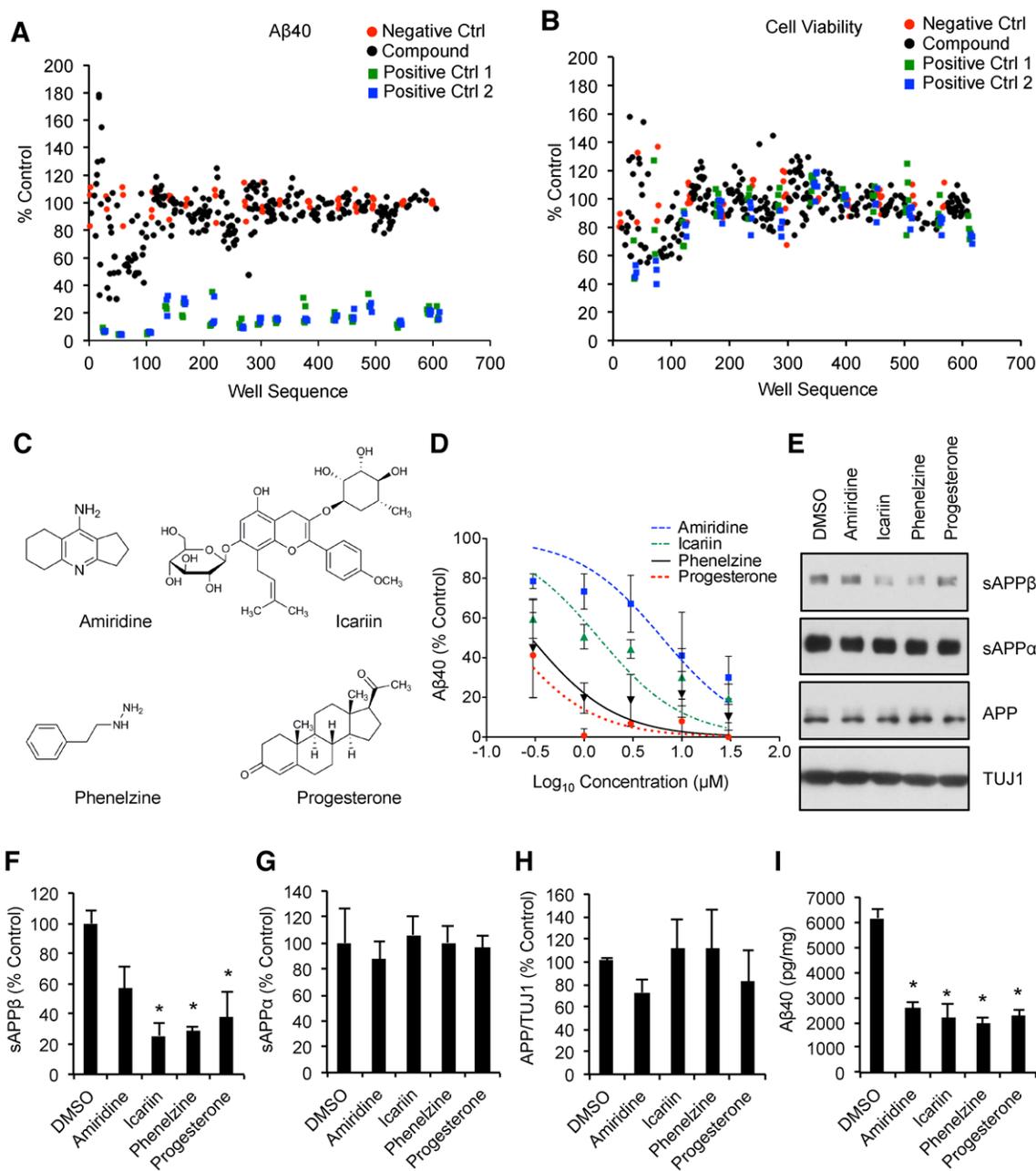
**Figure 4. Analysis of mES Cells and ESNs Isolated From Tg2576 AD Mouse Model**

(A) Tg2576 mES-derived neurons identified by nuclear DAPI stain, express neuronal  $\beta$ -tubulinIII (TUJ-1) (green) and pyramidal cell protein EMX1 (red). Scale bar represents 40 $\mu$ m. (B) Western blot analysis of protein lysates from Tg2576 mES-derived neuronal culture harvested at indicated *DIV* express APP, cleaved APP fragments (APP-CTF) and neuronal proteins synaptophysin, CamKII and neuronal  $\beta$ -tubulinIII (TUJ-1).  $\alpha$ -Actin is a loading control. (C) Cell lysates from *DIV*7 Tg2576 ES-derived neurons treated with indicated compounds were subjected to Western blot detection with 6E10 (APP-FL), APP-CTmax (APP-CTF) and antibodies to indicated proteins. (D) Secreted sAPP $\beta$  and APP were immunoprecipitated from conditioned medium after treatment of Tg2576 ES-derived neurons with the indicated compounds. (E, F) Secreted A $\beta$  levels detected using human specific A $\beta$ 40 or 42 ELISA kit. A $\beta$ 40 and A $\beta$ 42 show characteristic sensitivity to BSI and GSI inhibition. IC $_{50}$  values for the BACE1 inhibitor were 229.7nM and 28.1nM for A $\beta$ 40 and A $\beta$ 42 respectively. IC $_{50}$  values for Compound E were 136.9pM and 102.2pM A $\beta$ 40 and A $\beta$ 42 respectively. The Z' factors were 0.85 for A $\beta$ 40 and 0.5 for A $\beta$ 42 indicating an excellent assay for HTS.



**Figure 5. Miniaturization and Optimization of 96 Well Platform for Mouse ESNs**

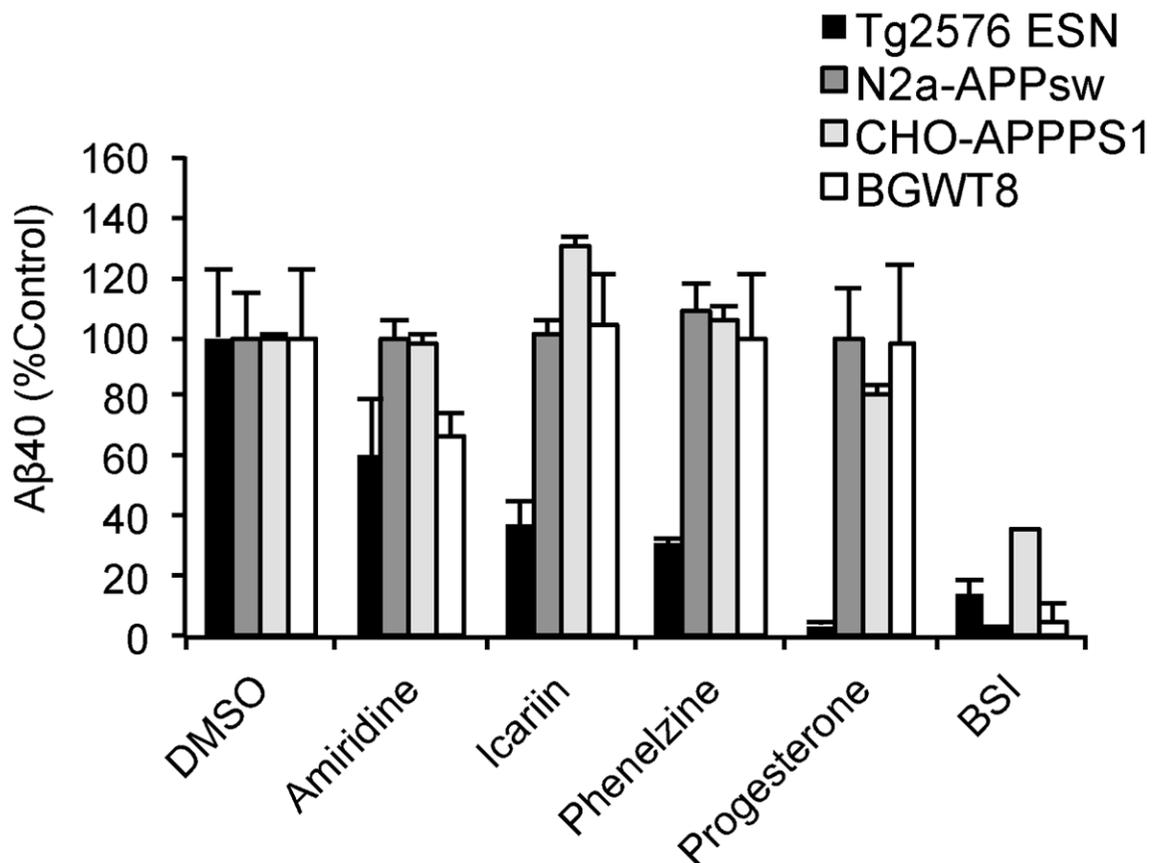
(A) DIV 8 Tg2576 ES-derived neurons were plated at increasing density in a 96 well plate and subjected to a cell viability assay quantified in relative fluorescent units (RFU). (B) %CV and (C)  $Z'$  were calculated with increasing number of wells. The minimum %CV and maximum  $Z'$  are optimized at  $n=5$  wells at a plating density of  $1.5 \times 10^5$  cells/cm<sup>2</sup> (arrows in B and C).



### Figure 6. Screening NIH Clinical Collection (NCC) for A $\beta$ Biogenesis

(A) DIV 7 Tg2576 ESNs plated in 96 well plate platform were treated with compounds from the NCC compound collection for 24 hours and A $\beta_{40}$  was detected in conditioned media and is shown as % Control, DMSO treated wells. Negative control (Ctrl) was DMSO, while Positive Ctrl 1 was BACE inhibitor IV and Positive Ctrl 2 was Compound E. (B) The remaining cells were subjected to cell viability assay CellQuanti Blue. (C) Potent hits were obtained from an independent source and subjected to IC<sub>50</sub> determination in the 96-well platform for A $\beta_{40}$  biogenesis (Amiridine, 6.29  $\mu\text{M}$ ; Icaritin, 1.412  $\mu\text{M}$ ; Phenelzine, 0.2834  $\mu\text{M}$ ; Progesterone, 0.1746  $\mu\text{M}$ ) determined by Prism analysis log(inhibitor) vs. normalized response curve fit with baseline level of A $\beta_{40}$  subtracted. (D) Chemical structures of hit compounds (E) Representative Western blot analysis of APP processing determined using

immunoprecipitation of secreted fragments and from condition media. Full length APP was determined in protein lysate using Western blot analysis with 6E10 antibody and CTFs were detected using CTMaxi antibody. Neuronal  $\alpha$ -tubulin was detected using TUJ1. (F,G) Quantification of sAPP<sup>1-40</sup> and sAPP<sup>1-42</sup> (H) Full length APP was quantified by Image J analysis and normalized to tubulin (TUJ1). (I) Quantification of A $\beta$  40 after treatment with indicated compounds normalized to protein level. Shown are means and SEM (n=3).



**Figure 7. Disparity In Pharmacological Response of Hit Compounds Between Tg2576 ESNs and Cell Lines**

(A) Tg2576 ES cell derived neurons; Neuro2a cells stably overexpressing APP<sub>sw</sub> (N2a-APP<sub>sw</sub>); Chinese hamster ovary cells stably overexpressing wild type APP and PS1 with the E9 mutation (CHO-APPPS1); and SH-SY5Y cells stably overexpressing GFP-BACE1 and HA-tagged wild type APP (BGWT8) were plated in 96 well plates and treated with hit compounds (10μM) for 6 hours after which media was harvested and tested for A<sub>40</sub> content which was normalized to DMSO treated cells for each type (% control). Absolute values for control (DMSO) treated cells are Tg2576 ESNs: 129±29.2 pg/ml; N2a-APP<sub>sw</sub>: 346±50.7 pg/ml; CHO-APPPS1: 62.5±7 pg/ml; and BGWT8: 28.8±6.4 pg/ml.