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Impaired β -Amyloid Secretion in Alzheimer's Disease Pathogenesis

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A central question in Alzheimer's disease (AD) research is what role β -amyloid peptide ($A\beta$) plays in synaptic dysfunction. Synaptic activity increases $A\beta$ secretion, potentially inhibiting synapses, but also decreases intraneuronal $A\beta$, protecting synapses. We now show that levels of secreted $A\beta$ fall with time in culture in neurons of AD-transgenic mice, but not wild-type mice. Moreover, the ability of synaptic activity to elevate secreted $A\beta$ and reduce intraneuronal $A\beta$ becomes impaired in AD-transgenic but not wild-type neurons with time in culture. We demonstrate that synaptic activity promotes an increase in the $A\beta$ -degrading protease neprilysin at the cell surface and a concomitant increase in colocalization with $A\beta$ 42. Remarkably, AD-transgenic but not wild-type neurons show reduced levels of neprilysin with time in culture. This impaired ability to secrete $A\beta$ and reduce intraneuronal $A\beta$ has important implications for the pathogenesis and treatment of AD.

Introduction

A defining neuropathological feature of Alzheimer's disease (AD) is the aberrant accumulation of β -amyloid peptide ($A\beta$). $A\beta$ accumulation can lead to alterations in synapses and memory (Selkoe, 2002; Almeida et al., 2005; Hsieh et al., 2006). The site(s) and mechanism(s) whereby $A\beta$ initiates dysfunction of synapses in AD are of major interest. Secreted, extracellular $A\beta$ has traditionally been viewed as the source of $A\beta$ -induced toxicity to synapses in AD, since addition of $A\beta$ 1–42 impairs synaptic function (Cleary et al., 2005; Shankar et al., 2008). In contrast, picomolar levels of extracellular $A\beta$ were recently shown to enhance synaptic plasticity (Puzzo et al., 2008). Remarkably, synaptic activity increases levels of secreted, extracellular $A\beta$ (Kamenetz et al., 2003; Cirrito et al., 2005). Since the default network of the brain is particularly prone to the development of AD, it has been hypothesized that brain regions with the highest baseline metabolic activity are prone to AD because of high amounts of secreted $A\beta$ (Cirrito et al., 2008; Palop and Mucke, 2010). However, it is unclear why such elevated levels of secreted $A\beta$ from default network activity cause problems only with aging. Moreover, at-risk individuals for AD show reduced brain activity decades before clinical symptoms (Reiman et al., 2004), which might predict

reduced $A\beta$ secretion. There is increasing support for an alternative scenario focusing on aberrant intracellular accumulation of $A\beta$ within vulnerable neurons (Gouras et al., 2010). In fact, we recently demonstrated that $A\beta$ -related synapse damage and memory impairment in AD-transgenic mice correlated with this intracellular pool of $A\beta$ but not with plaques (Tampellini et al., 2010).

We now provide evidence for reduced $A\beta$ secretion with time in culture in AD-transgenic but not wild-type neurons. Furthermore, we show that synaptic activity is able to reduce levels of intracellular $A\beta$ in AD-transgenic neurons at 12 but not at 19 days *in vitro* (DIV). We demonstrate that levels of neprilysin are reduced in AD-transgenic but not wild-type neurons with time in culture. Finally, we provide mechanistic evidence consistent with neprilysin leading to degradation of $A\beta$ 42 at the cell surface with synaptic activity.

Materials and Methods

Neuronal cultures. Primary neuronal cultures were prepared from cortices and hippocampi of embryonic day 15 Tg2576 (AD-transgenic) (Hsiao et al., 1996) and wild-type littermate mouse embryos, as described previously (Tampellini et al., 2009). Tg2576 male and wild-type female mice (Jackson Laboratories) were bred to generate the embryos. Mice were used in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Antibodies. For immunofluorescence, the following antibodies were used: neprilysin (H-321; Santa Cruz Biotechnology), early endosomal antigen-1 (BD Transduction Laboratories), tumor susceptibility gene 101 (GeneTex), amyloid precursor protein (APP) intracellular domain (AICD; Covance), postsynaptic density-95 (PSD-95; Millipore), $A\beta$ 42 (C terminus; Covance). For Western blot, the following antibodies were used: human-specific $A\beta$ /APP 6E10 (Covance), murine and human $A\beta$ /APP 4G8 (Covance), rabbit polyclonal APP (C terminus) 369, phospho-CaMKII (Millipore), total CaMKII (Millipore). Secondary antibodies were conjugated to Alexa Fluor-488 or -546 (Invitrogen) or horseradish peroxidase (GE Healthcare).

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The authors declare no competing financial interests.

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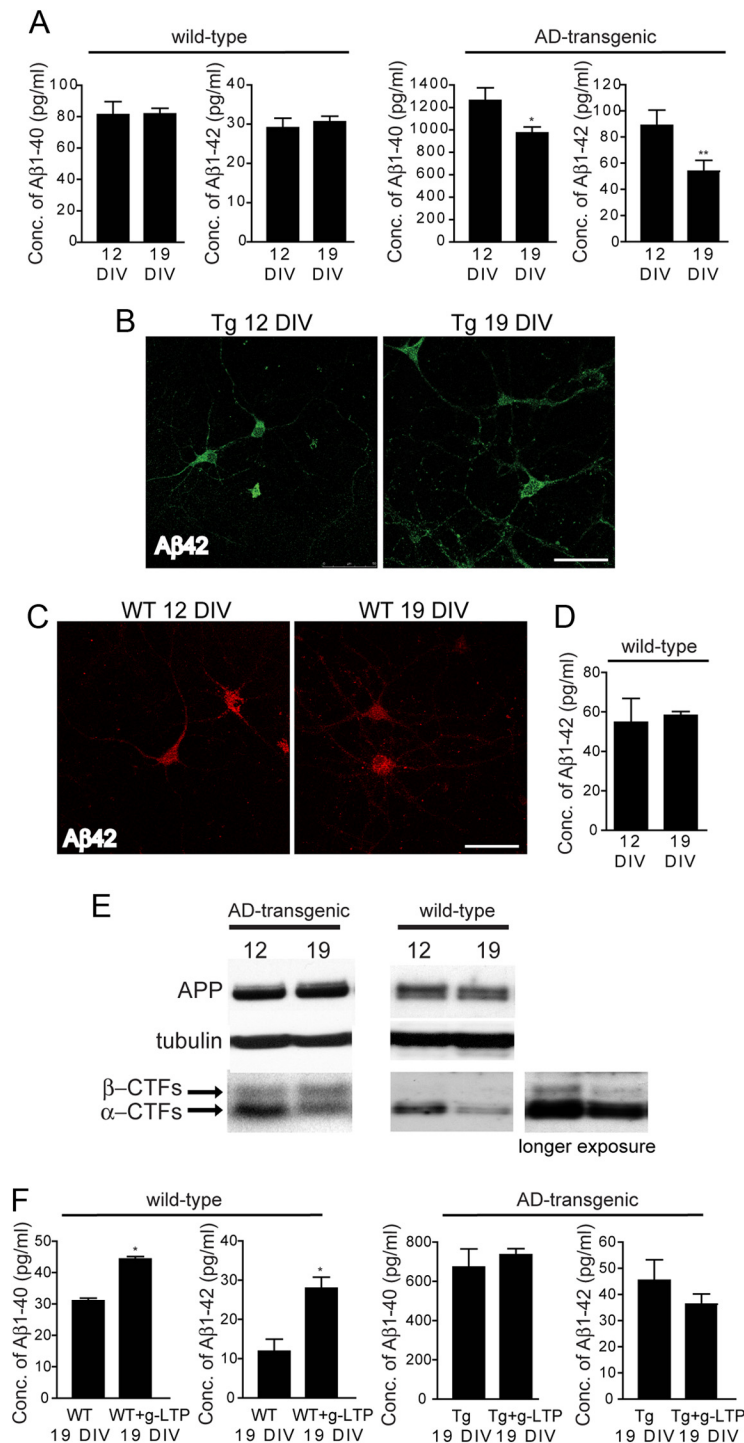


Figure 1. Decreased A β secretion in AD-transgenic (Tg) but not wild-type (WT) neurons with time in culture. **A**, Levels of A β 1–40 and A β 1–42 were assayed by ELISA in media of AD-transgenic or wild-type neurons. While wild-type neurons secrete comparable amounts of A β peptides at 12 and 19 DIV ($n = 6$; left), AD-transgenic neurons showed decreased levels of A β 1–40 and A β 1–42 in media at 19 compared with 12 DIV ($n = 10$; * $p < 0.05$, ** $p < 0.01$; right). **B**, Levels of intraneuronal A β 42 were increased by $46 \pm 17\%$ in AD-transgenic neurites at 19 compared with 12 DIV ($n = 4$; $p < 0.05$). **C**, **D**, Levels of intraneuronal A β 42 were unchanged in wild-type neurons at 19 compared with 12 DIV, as quantified by confocal immunofluorescence (**C**) and ELISA (**D**) ($n = 4$). **E**, Left, Levels of α -CTFs were decreased by $52 \pm 2\%$, while levels of β -CTFs and full-length APP were unchanged in AD-transgenic neurons at 19 compared with 12 DIV ($n = 4$; $p < 0.01$). Right, Levels of α -CTFs were decreased by $32 \pm 9\%$ ($n = 5$; $p < 0.05$), while levels of β -CTFs (longer exposure) showed a trend for a $49 \pm 16\%$ decrease ($p = 0.074$) in wild-type neurons at 19 compared with 12 DIV. **F**, At 19 DIV, AD-transgenic neurons failed to enhance secretion of both A β 1–40 and A β 1–42 during g-LTP ($n = 6$, right). In contrast, wild-type neurons were still able to increase A β 1–40 and A β 1–42 secretion during g-LTP ($n = 4$; * $p < 0.05$, left). Conc., Concentration. Scale bars, 50 μ m.

Treatments. For glycine-induced long term potentiation (g-LTP), neurons were treated as described previously (Tampellini et al., 2009). In experiments on neprilysin and A β 42 surface colocalization during g-LTP stimulation, 50 μ M thiorphan was added to neurons during the 15 min incubation with or without glycine; 50 μ M thiorphan was then added during the following 1 h chase to prevent A β degradation.

ELISA analysis. To measure A β secretion from primary neurons (10 cm dish) at steady state, media were replaced with 1 ml of fresh neurobasal medium and collected after 5 h. To measure A β secretion with or without g-LTP, LTP buffer was collected after the 1 h chase. Concentrations of A β 1–40 and A β 1–42 were measured using the respective ELISA kits (Bio-source) for mouse (wild-type neurons) or human (AD-transgenic neurons) A β .

Western blot. Neuron lysates were prepared as described previously (Tampellini et al., 2009). Membranes were immunoblotted with antibodies 6E10 or 369, and intensities were quantified using Scion Image software (NIH).

Immunofluorescence. Immunofluorescence and its quantification were performed as described previously (Tampellini et al., 2009). Localization of surface neprilysin with A β 42 and subcellular markers was determined using a colocalization algorithm (Leica Application Suite 1.8.2 software). To count AICD-positive nuclei, all nuclei were marked with Hoechst stain. Counts of AICD-positive and total nuclei were performed with MetaMorph on 6–10 fields per coverslip at 20 \times magnification.

Statistical analysis. Statistical comparisons were made using two-tailed unpaired t tests with significance placed at $p < 0.05$.

Results

We previously reported progressive intraneuronal A β 42 accumulation, alterations in endosomal trafficking, and AD-like synapse alterations in AD-transgenic neurons with time in culture, analogous to changes seen *in vivo* in AD-transgenic mouse brains with aging (Takahashi et al., 2004; Almeida et al., 2005, 2006). In this study, we examined whether time in culture affects A β secretion in AD-transgenic neurons. In wild-type neurons, levels of secreted A β 1–40 and A β 1–42 remained unchanged between 12 and 19 DIV (Fig. 1A, left). In contrast, in AD-transgenic neurons, levels of secreted A β 1–40 and A β 1–42 fell by $33 \pm 4\%$ and $39 \pm 9\%$, respectively, between 12 and 19 DIV (Fig. 1A, right). We next examined levels of intraneuronal A β with time in culture. There was a $46 \pm 17\%$ increase in levels of intraneuronal A β 42 in AD-transgenic neurites at 19 compared with 12 DIV, as quantified by confocal immunofluorescence microscopy (Fig. 1B). In contrast, levels of intraneuronal A β 42 were unchanged in wild-type neurons at 19 compared with 12 DIV (Fig. 1C,D).

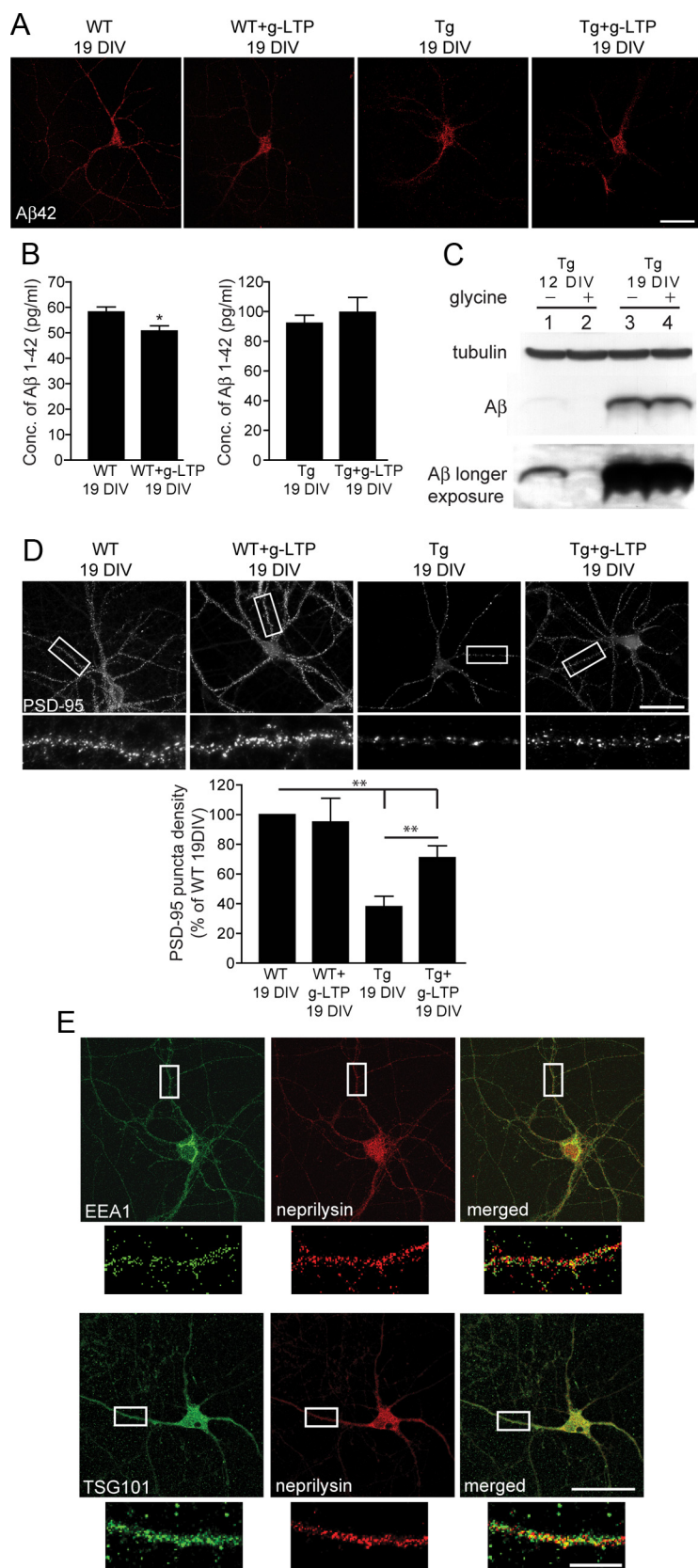


Figure 2. Impaired ability for synaptic activity to reduce intraneuronal $\text{A}\beta_{42}$ and protect synapses in AD-transgenic neurons with time in culture. **A**, g-LTP reduced levels of intraneuronal $\text{A}\beta_{42}$ by $23 \pm 4\%$ in wild-type (WT; left) but not AD-transgenic (Tg; right) neurons at 19 DIV, as determined by confocal immunofluorescence ($n = 4$; $p < 0.01$). **B**, $\text{A}\beta_{1-42}$ ELISA of lysates from g-LTP compared with untreated neurons at 19 DIV revealed reduced levels of $\text{A}\beta_{1-42}$ in wild-type but not in AD-transgenic neurons ($n = 4$; $*p < 0.05$). **C**, Western blot of cell lysates demonstrated reduced levels of intraneuronal $\text{A}\beta$ in g-LTP-treated compared with -untreated AD-transgenic neurons at 12 (lanes 1 and 2, longer exposure) but not 19 (lanes 3 and 4) DIV ($n = 3$). **D**,

Interestingly, the levels of APP α C-terminal fragments (α CTFs) fell by $52 \pm 2\%$ between 12 and 19 DIV in AD-transgenic neurons, whereas levels of APP β C-terminal fragments (β CTFs) and full-length APP remained unchanged (Fig. 1E, left). Thus, amyloidogenic processing of APP, as reflected by the β CTF/ α CTF ratio, increased approximately twofold from 12 to 19 DIV in AD-transgenic neurons. Levels of α CTFs and β CTFs were both comparably decreased in wild-type neurons at 19 compared with 12 DIV (Fig. 1E, right), and therefore the β CTF/ α CTF ratio did not change.

It has been shown that $\text{A}\beta$ secretion is enhanced by synaptic activation (Kamenetz et al., 2003; Cirrito et al., 2005; Tampellini et al., 2009). Given the fall in steady-state secretion of $\text{A}\beta$ in AD-transgenic neurons with time in culture, we investigated whether $\text{A}\beta$ secretion was impaired during synaptic activity in AD-transgenic neurons at 12 DIV (Tampellini et al., 2009). We now show that, although g-LTP increases $\text{A}\beta$ secretion in wild-type neurons at 19 DIV, it failed to increase $\text{A}\beta$ secretion in AD-transgenic neurons at 19 DIV (Fig. 1F).

We reported that synaptic activation reduced intracellular $\text{A}\beta_{42}$ and restored levels of PSD-95 back to wild-type levels in AD-transgenic neurons at 12 DIV (Tampellini et al., 2009). To investigate whether these effects are modulated by aging *in vitro*, we induced g-LTP in 19 DIV AD-transgenic neurons and quantified levels of intraneuronal $\text{A}\beta$ and PSD-95. At 19 DIV, g-LTP reduced levels of intraneuronal $\text{A}\beta_{42}$ by $23 \pm 4\%$ in wild-type neurons, but there was no decrease in AD-transgenic neurons as determined by confocal immunofluorescence (Fig. 2A). These data were confirmed by ELISA (Fig. 2B) and Western blot (Fig. 2C) of neuronal lysates. We confirmed that 12 DIV lysates showed decreased levels of intraneuronal $\text{A}\beta$ in g-LTP-treated compared with -untreated AD-transgenic neurons (Fig. 2C; lanes 1 and 2, longer ex-

PSD-95 puncta increased in g-LTP-activated AD-transgenic neurons at 19 DIV, although they failed to reach wild-type levels ($n = 5$; $**p < 0.01$). **E**, Wild-type neurons (12 DIV) showed a $74 \pm 21\%$ greater relative colocalization of neprilysin with the MVB/late endosomal marker tumor susceptibility gene 101 (TSG101) than the early endosomal marker early endosomal antigen-1 (EEA1) ($n = 3$; $p < 0.01$). Conc., Concentration. Scale bars: **A**, **D**, **E**, 50 μm ; **E**, inset, 10 μm .

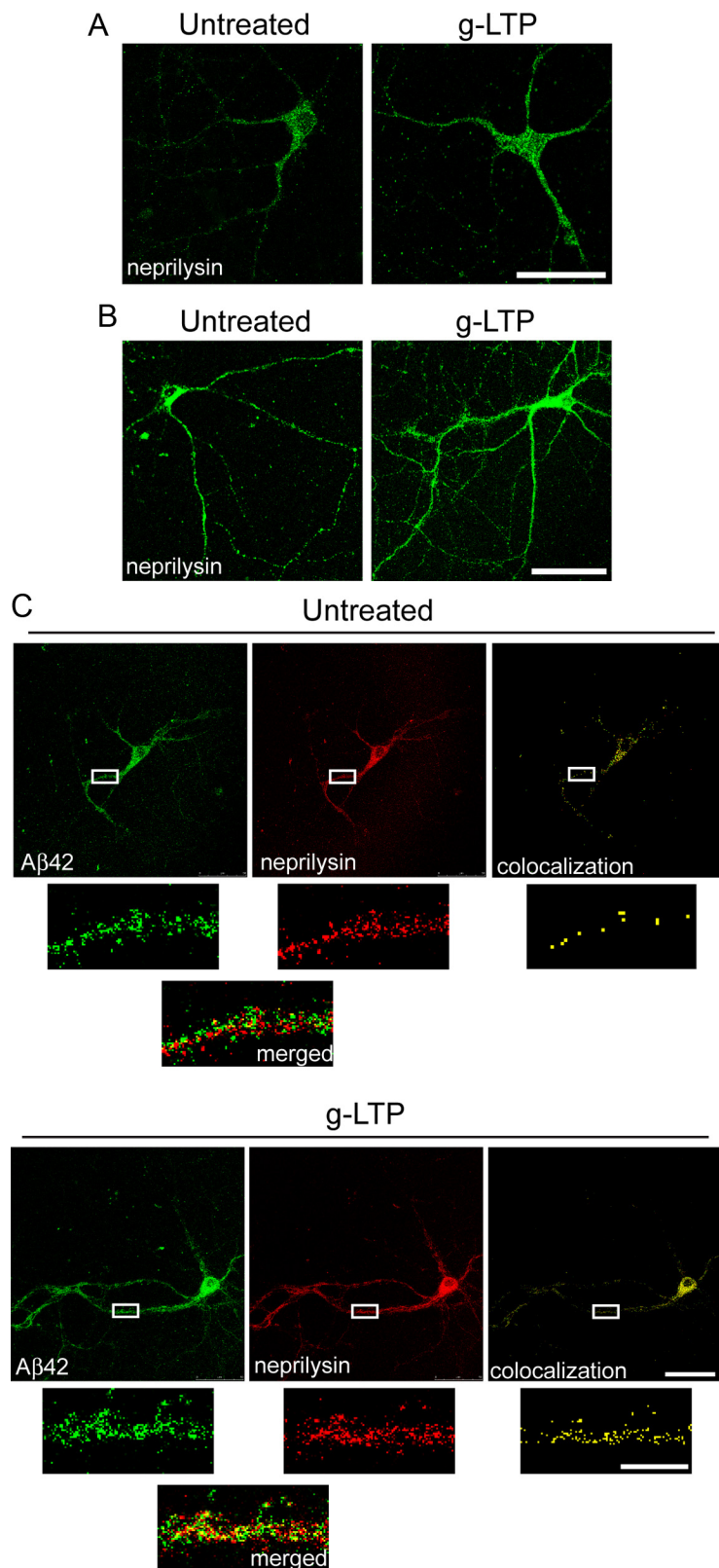


Figure 3. Synaptic activation increases neprilysin surface levels and colocalization with A β 42. **A**, g-LTP increased levels of surface neprilysin by $37 \pm 7\%$ in g-LTP-treated compared with -untreated wild-type neurons at 12 DIV ($n = 6$; $p < 0.01$). **B**, g-LTP also increased levels of surface neprilysin in g-LTP-treated compared with untreated AD-transgenic neurons at 12 DIV ($n = 3$; $p < 0.01$). **C**, g-LTP increased the relative colocalization of neprilysin with A β 42 by $54 \pm 21\%$ at the cell surface in AD-transgenic neurons at 12 DIV ($n = 3$; $p < 0.01$). Scale bars: **A–C**, 50 μm ; **C**, insets, 10 μm .

posure). Activity-induced reduction in levels of intraneuronal A β promoted recovery of PSD-95 puncta to wild-type levels in AD-transgenic neurons at 12 DIV (Tampellini et al., 2009). In contrast, levels of PSD-95 did not recover back to wild-type levels in g-LTP-treated compared with -untreated 19 DIV AD-transgenic neurons (Fig. 2D), although there was still an increase compared with untreated AD-transgenic neurons.

We showed that activity-dependent reduction in intraneuronal A β is dependent on the A β -degrading protease neprilysin (Tampellini et al., 2009). Data on the subcellular localization of neprilysin showed marked relative colocalization of neprilysin predominantly with late endosomes (Fig. 2E). To investigate the mechanism of neprilysin-dependent A β 42 clearance with synaptic activity, levels of neprilysin were quantified as a function of synaptic activation. g-LTP did not change total levels of neprilysin (data not shown); however, it did increase levels of surface neprilysin by $37 \pm 7\%$ in stimulated compared with unstimulated wild-type neurons (Fig. 3A). Surface neprilysin also increased in stimulated compared with nonstimulated AD-transgenic neurons (Fig. 3B). We next explored whether the increased localization of neprilysin to the cell surface could be responsible for the augmented neprilysin-dependent A β 42 degradation. To test this hypothesis, g-LTP was induced in AD-transgenic neurons in the presence of the neprilysin inhibitor thiorphan to prevent loss of A β 42 labeling from degradation. g-LTP increased the relative colocalization of neprilysin with A β 42 at the cell surface by $54 \pm 21\%$ (Fig. 3C).

Since synaptic activation failed to reduce levels of intraneuronal A β in AD-transgenic neurons at 19 DIV, we next investigated whether levels of neprilysin were altered in AD-transgenic neurons with time in culture. Remarkably, levels of neprilysin were decreased by $18 \pm 8\%$ in 19 compared with 12 DIV AD-transgenic but not wild-type neurons, as quantified by confocal immunofluorescence (Fig. 4A). Previous work reported that neprilysin is regulated by the AICD (Pardossi-Piquard et al., 2005). To investigate whether reduction of neprilysin might be related to reduced levels of AICD in the nucleus with time in culture, we double-labeled nuclei of 12 and 19 DIV AD-transgenic neurons with Hoechst stain and an AICD-specific antibody. Remarkably, the number of AICD-positive nuclei was decreased by $27 \pm 6\%$ in 19 compared with 12 DIV AD-transgenic neurons (Fig. 4B), suggesting reduced synthesis of neprilysin with time in culture.

Discussion

The data presented here provide evidence for an impaired ability of familial AD-transgenic neurons to efficiently modulate levels of secreted and intraneuronal A β with time in culture. We demonstrate that the ability to secrete A β falls with time in culture in AD-transgenic but not wild-type neurons. Activity-induced A β secretion also falls with time in culture in AD-transgenic but not wild-type neurons. Moreover, with time in culture, AD-transgenic neurons become impaired in their ability to reduce intracellular A β and protect synapses in response to synaptic activity. We provide evidence that the mechanism by which synaptic activity reduces intracellular A β is dependent on the A β -degrading protease neprilysin. Synaptic activity leads to recruitment of neprilysin to the cell surface and increased colocalization with A β 42. Finally, we show that neprilysin levels fall with time in culture in AD-transgenic but not wild-type neurons, providing a mechanism for the failure of synaptic activation to reduce intracellular A β in aged AD-transgenic neurons (Fig. 4C,D).

These data have important implications for AD. Decreased neuronal secretion of A β with aging in AD could explain previous data that baseline interstitial A β 42 levels appear to be reduced in older compared with younger AD-transgenic mice (Cirrito et al., 2005). Reduced A β 42 levels are also seen in CSF in human AD, even in subjects who are cognitively normal but later develop AD (Fagan et al., 2009). The traditional explanation for reduced CSF A β 42 with AD is that secreted A β is decreased by sequestration to extracellular plaques. Decreased secretion would also contribute to reduced A β 42 levels in CSF or interstitial fluid. Of note, other pathological conditions linked with AD, such as brain injury, also show reduced levels of extracellular A β (Brody et al., 2008) and increased intraneuronal A β (Gouras et al., 2010).

Reduced A β secretion fits well with increasing evidence for early intraneuronal A β accumulation with AD pathogenesis, as reported in human AD, Down syndrome, and in AD-transgenic rodents (Gouras et al., 2000; D'Andrea et al., 2001; Wirths et al., 2001; Takahashi et al., 2002; Oddo et al., 2003; Cataldo et al., 2004; Echeverria et al., 2004; Lord et al., 2006; Oakley et al., 2006; Knobloch et al., 2007; Gandy et al., 2010). Intraneuronal A β accumulates and oligomerizes preferentially in distal processes even before plaques (Takahashi et al., 2004), and is associated with memory impairment, decreased synaptic plasticity, and subcellular pathology (Mori et al., 2002; Takahashi et al.,

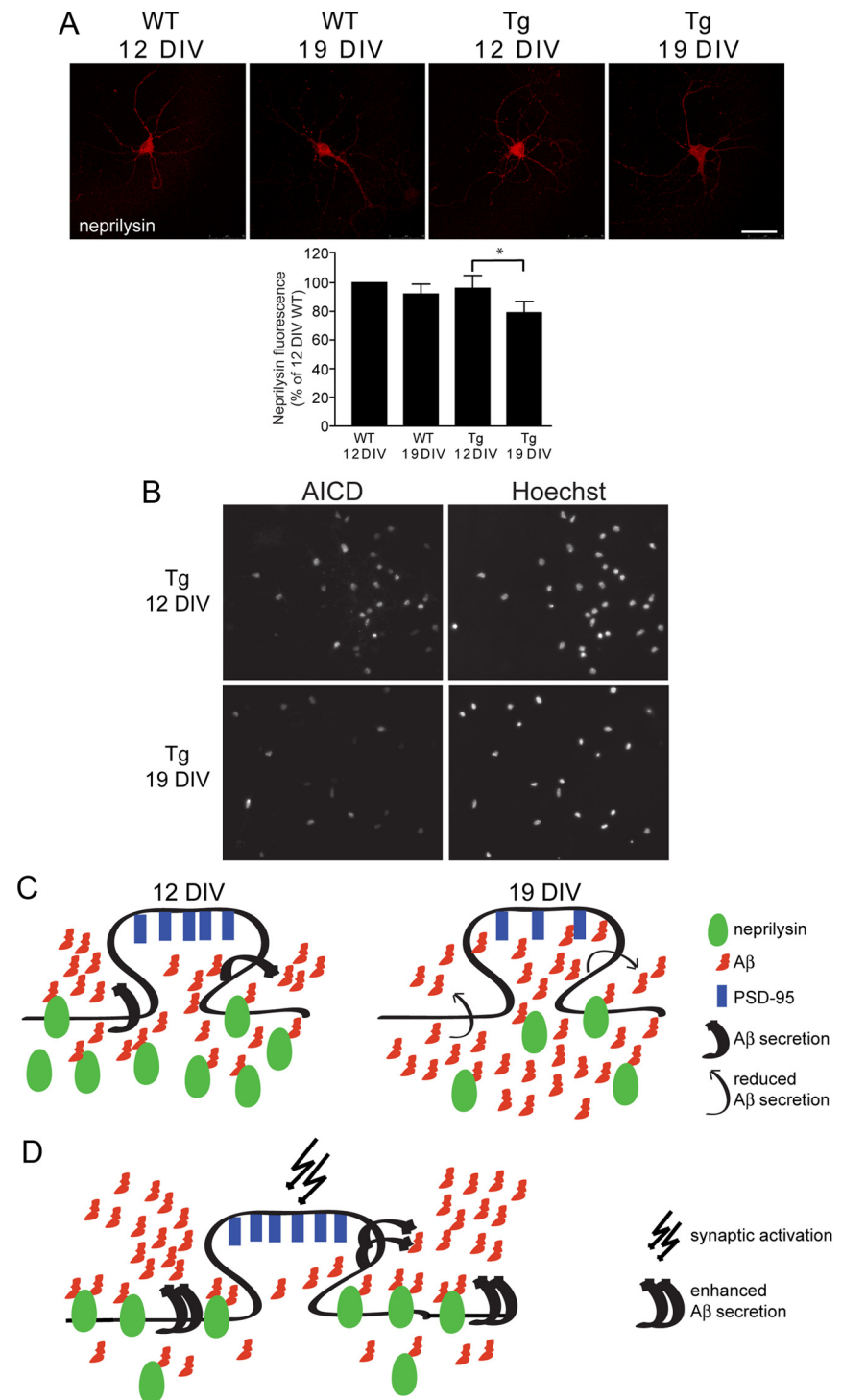


Figure 4. Decreased levels of neprilysin in AD-transgenic neurons with time in culture. **A**, Levels of neprilysin were decreased by $18 \pm 8\%$ in 19 compared with 12 DIV AD-transgenic neurons ($n = 4$; $*p < 0.05$; scale bar, $50 \mu\text{m}$). In contrast, 19 compared with 12 DIV wild-type neurons did not show a decrease of neprilysin. **B**, The number of AICD-positive nuclei was decreased by $27 \pm 6\%$ in 19 compared with 12 DIV AD-transgenic neurons ($n = 3$; $p < 0.05$). **C**, The ability to secrete A β falls with time in culture in AD-transgenic neurons. Neprilysin levels fall with time in culture, suggesting a mechanism for progressive intracellular A β accumulation and loss of synaptic proteins (PSD-95) in aged AD-transgenic neurons. **D**, Synaptic activity enhances A β secretion, leads to neprilysin recruitment to the cell surface, and increases colocalization of neprilysin with A β 42.

2002; Oddo et al., 2003; Almeida et al., 2005; Billings et al., 2005; Tomiyama et al., 2010). Intraneuronal A β 42 localizes and accumulates preferentially in late endosomes, including multivesicular bodies (MVBs) (Takahashi et al., 2002; Langui et al., 2004; Almeida et al., 2006). Interestingly, neprilysin also localizes to late endosomes/

MVBs (Fig. 2E). We show that neprilysin significantly relocates to the cell surface during synaptic activation. Neprilysin is a transmembrane neutral endopeptidase with the catalytic site at the C terminal side, which can be either extracellular or luminal. Since neprilysin works best at neutral pH, the cell surface would be the optimal location for it to degrade A β 42. In fact, we observed that colocalization of neprilysin and A β 42 was increased at the cell surface with synaptic activation. This pool of A β 42 could derive from amyloidogenic APP processing occurring within synaptic endosomes with synaptic activity (Cirrito et al., 2008; Tampellini et al., 2009), followed by trafficking of A β to the cell surface (Rajendran et al., 2006). The finding that neprilysin normally declines with aging in synaptic layers of wild-type mouse brain (Iwata et al., 2002) supports the theory that it plays a critical role in synaptic accumulation of A β with aging and AD. We show that neprilysin is reduced in AD-transgenic but not wild-type neurons with time in culture, although it is possible that a decline would eventually occur later in wild-type neurons. Reduced neprilysin in AD-transgenic neurons with time in culture can explain the impaired ability to reduce intraneuronal A β , in particular A β 42, with synaptic activity, since we previously reported the inability of synaptic activity to reduce A β 42 in neprilysin knock-out neurons or when neprilysin activity was blocked by treatment with thiorphan (Tampellini et al., 2009). In addition, it has been shown that extracellular A β can upregulate intracellular A β (Yang et al., 1999; Tampellini et al., 2009), which could lead to a vicious cycle of A β -induced elevation and synapse damage.

Overall, this study provides novel evidence for reduced A β secretion and impaired ability to reduce intraneuronal A β with time in culture in AD-transgenic neurons, and further underscores the important role of neprilysin in regulating neuronal A β .

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