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Cathepsins B and L differentially regulate  
amyloid precursor protein processing

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Running Title Page

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List of abbreviations:

A $\beta$	amyloid beta
AD	Alzheimer's disease
APP	amyloid precursor protein
APP <sup>swe</sup>	Swedish mutation of APP
BTM	batimastat
catB	cathepsin B
catL	cathepsin L
catL inh IV	cathepsin L inhibitor IV
CHO	Chinese hamster ovary cells
CTF	C-terminal fragments of APP
DMEM	Dulbecco's modified eagle's medium
FBS	fetal bovine serum
N2A	neuroblastoma 2A
TTX	tetrodotoxin

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## Abstract

Previous studies have shown that cathepsins control amyloid beta (A $\beta$ ) levels in chromaffin cells via a regulated secretory pathway. In the present study, this concept was extended to investigations in primary hippocampal neurons to test if A $\beta$  release was co-regulated by cathepsins and electrical activity, proposed components of a regulated secretory pathway. Inhibition of cathepsin B (catB) activity with CA074Me or attenuation of catB expression through siRNA produced decreases in A $\beta$  release, similar to levels produced with suppression of BACE1 expression. To test if the catB-dependent release of A $\beta$  was linked to ongoing electrical activity, neurons were treated with tetrodotoxin (TTX) and CA074Me. These comparisons demonstrated no additivity between decreases in A $\beta$  release produced by TTX and CA074Me. In contrast, pharmacological inhibition of cathepsin L (catL) selectively elevated A $\beta$ 42 levels, but not A $\beta$ 40 or total A $\beta$ . Mechanistic studies measuring C-terminal fragments of amyloid precursor protein (APP) suggested that catL elevated  $\alpha$ -secretase activity, thereby suppressing A $\beta$ 42 levels. The mechanism of catB-mediated regulation of A $\beta$  release remains unclear but may involve elevation of  $\beta$ -secretase. In summary, these studies provide evidence for a significant alternative pathway for APP processing that involves catB and activity-dependent release of A $\beta$  in a regulated secretory pathway for primary neurons.

## Introduction

Accumulation of the amyloidogenic peptide A $\beta$ 42 is the hallmark pathology of Alzheimer's disease (AD). Although it is evident that N- and C-terminal processing of APP can regulate A $\beta$  peptides by BACE1 and gamma secretase in many systems, we have sought to examine other potential APP processing enzymes, particularly as these alternative pathways may be linked to synaptic activity in primary neurons. The impetus to explore alternative processing enzymes is the uncertainty of the inherent dependency of previous studies on cell-based systems that involve both the Swedish mutation of APP (APP<sup>swe</sup>) and the use of cell systems that have a predominance of a constitutive pathway for APP processing. Only 2% of all AD cases are caused by APP<sup>swe</sup> though most models of AD involve cells overexpressing APP<sup>swe</sup>. Additionally, much of the APP processing research has been performed on cell lines transfected with APP, which exhibit constitutive secretion of A $\beta$ , as opposed to cells expressing endogenous A $\beta$  that is primarily secreted through the regulated pathway. It is therefore of interest to investigate whether alternative proteases are involved in processing wild-type APP in the regulated secretory pathway as it related to spontaneous synaptic activity. Cathepsins are of particular interest because they associate with A $\beta$  (Cataldo and Nixon, 1990), are localized to endosomes where APP is processed (Golde et al., 1992; Haass et al., 1992), and their expression is altered in aged brains (Nakanishi et al., 1994).

Cathepsins reside in the endosomal-lysosomal system and are generally known for bulk proteolysis; however, new evidence suggests they also exhibit specific proteolytic functions

(Kobayashi et al., 1991; Murphy et al., 1992; Yasothornsrikul et al., 2003). Cathepsin D, an aspartyl protease, was initially implicated as a  $\beta$ -secretase candidate (Brown et al., 1996; Chevallier et al., 1997), though reduction in cathepsin D activity never effectively lowered  $A\beta$  levels (Saftig et al., 1996). Similarly, overexpression of cathepsin S resulted in enhanced  $A\beta$  secretion, (Munger et al., 1995). Lysosomal cysteine proteases have also received attention in their ability to modulate  $A\beta$  levels. CatB exhibits  $\beta$ -secretase activity in chromaffin vesicles (Hook and Reisine, 2003) and inhibitors to cysteine proteases lower  $A\beta$  levels in guinea pigs by reducing  $\beta$ -secretase and elevating  $\alpha$ -secretase activity (Hook et al., 2007). In contrast, general cysteine protease inhibitors did not significantly alter  $A\beta$  levels in a variety of systems (Siman et al., 1993; LeBlanc and Goodyer, 1999) and a recent paper showed that catB actually protected neurons from  $A\beta_{42}$  toxicity by degrading  $A\beta_{42}$  into less toxic peptide species (Mueller-Steiner et al., 2006). For the present studies, novel mechanisms for cathepsins in modulating  $A\beta$  are explored. Our cellular model of Alzheimer's disease utilizes primary hippocampal neurons, which secrete detectable levels of  $A\beta$  that can be manipulated with the pharmacological control of spontaneous synaptic activity. We show that two cysteine proteases, typically thought to act on similar substrates, exhibit dramatically different roles in modulating APP processing. While catB elevates  $\beta$ -secretase activity to drive APP processing into amyloidogenic peptides, catL elevates  $\alpha$ -secretase activity and thereby suppresses  $A\beta$  levels. Importantly, the effect of catB on modulating  $\beta$ -secretase activity is specific to neurons expressing endogenous  $A\beta$  secreted through the regulated pathway. These data illustrate why the roles of cathepsins in APP processing may aid in broadening the perspective of therapeutic targets in Alzheimer's disease as well as relating these concepts to changes in synaptic activity.

## Methods

*Cell culture:* Primary hippocampal neurons were isolated from E18 embryonic hippocampi (Brainbits LLC, Springfield, IL) using the Worthington papain dissociation system (Worthington Biochemical Corporation, Lakewood, NJ) according to manufacturers directions. Neurons were plated on poly-L-lysine-coated multiwell plates (BD Biosciences, San Jose, CA) in media containing Neurobasal with B27 supplement and glutamax (Invitrogen Corporation, Carlsbad, CA). Neurons were cultured for 2-3weeks and fed twice per week. Mouse neuroblastoma 2A (N2A) cells were passaged in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). Chinese hamster ovary (CHO) cells stably transfected with APP-751 (a gift from R.Tanzi, Massachusetts General Hospital, Boston, MA) were passaged in DMEM containing 10% FBS and G418 (100 $\mu$ g/mL).

*Drug treatments:* CA074Me, tetrodotoxin (TTX), chloroquine, and E64D were purchased from Sigma (St. Louis, MO). CatL inhibitor (catL inh) I, II, III, and IV and Ro32-0432 were from EMD Chemicals, Inc. (San Diego, CA). DAPT, batimastat (BTM) and BACE1 inhibitor were synthesized at J&JPRD. Since treatment with CA074Me caused some toxicity in primary neurons when applied for more than 12h, CA074Me was only added for 2-4h and then rinsed thoroughly. CA074Me was left on N2A and CHO cells for the duration of the experiment. Chloroquine and BTM were used at 50  $\mu$ M, Ro32-0432 was used at 3  $\mu$ M and BACE1 inhibitor was used at 100 nM. All remaining compounds were added at 10  $\mu$ M for the duration of the experiment.

*Gene knockdown:* Primary hippocampal neurons were transfected with ON-TARGET<sup>plus</sup> SMARTpool siRNA targeting catB or BACE1 (Dharmacon Inc., Lafayette, CO) using Lipofectamine RNAiMAX (Invitrogen), according to manufacturers instructions.

*Sandwich ELISA for A $\beta$  detection:* Primary neurons were fed with fresh media and incubated for 24-72h before media collection. For N2A and CHO cells, media was replaced with serum-free Ultraculture media (BioWhittaker Inc., Walkersville, MD). JRF/rAB/2, JRF/cAB40/10, or JRF/cAB42/26 was used to capture A $\beta$ 1-x, A $\beta$ x-40 or A $\beta$ x-42, respectively (Pype et al., 2003), A $\beta$  was detected with biotinylated 4G8 (Covance, Princeton, NJ), incubated with streptavidin-HRP, and exposed with QuantaBlu Fluorogenic Peroxidase Substrate (Pierce, Rockford, IL) was used as the substrate. For measurement of A $\beta$ 1-42 and A $\beta$ 1-40, A $\beta$  was detected with biotinylated JRF/rAB/2. Synthetic A $\beta$  peptides (California Peptides, Napa, CA) were used to calibrate fluorescence levels to A $\beta$  concentrations. For all experimental groups, six individual wells were assayed for A $\beta$  detection. Most data were normalized to untreated controls and t-tests were used to determine statistically significant differences between pre-determined groups.

*Western blots:* Proteins were lysed in RIPA buffer containing 2% SDS, HALT protease inhibitors with EDTA (Pierce) and 1  $\mu$ M pepstatin A on ice. Lysate was sonicated briefly and centrifuged at 14,600g for 10minutes. Supernatant was collected and stored at -80°C. Proteins were separated electrophoretically on pre-cast 10-20% tricine gels or 4-12% bis-tris gels (Invitrogen) and transferred to nitrocellulose membranes. Full length and C-terminal fragments of APP were stained with A8717 (Sigma). sAPP $\alpha$  was detected with SIG-39139 (Covance) and normalized to full length APP (07-667, Millipore, Billerica, MA). Bands were illuminated with



SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). Band intensity was quantified using ImageJ image analysis software and t-tests were used to assess statistical significance.

Sample sizes are indicated on figures.

*Protease activity assays:* Proteins were lysed in Cytobuster lysis buffer (EMD Chemicals) for 30min on ice. CatB activity was detected with the InnoZyme™ Cathepsin B activity fluorogenic assay kit from EMD Chemicals according the manufacturers directions. For  $\alpha$ -secretase assays, identical lysates were treated with or without inhibitors and incubated at 37°C with a fluorogenic peptide containing the  $\alpha$ -secretase cleavage site of wild-type APP (EMD Chemicals).  $\alpha$ -secretase activity in the presence of inhibitor was measured by resultant fluorescence and normalized to that of untreated samples. Six replicates were performed for each experimental group.

*A $\beta$  degradation assay:* Synthetic human A $\beta$ 42 (300pM) was added to N2A cells and media was collected at 1, 4, 8, or 24h after application. Exogenous hA $\beta$ 1-42 and hA $\beta$ 1-x was measured by ELISA. JRF/cAB42/26 or JRF/ABN/25 was used to capture and biotinylated 6E10 or 4G8 was used to detect hA $\beta$ 1-42 and hA $\beta$ 1-x, respectively. For all groups, six replicates were performed.

## Results

After 72h, media was collected from hippocampal neurons (plated at 30,000 cells/well of a 96well plate with 160 $\mu$ L media/well) and assayed for A $\beta$ 1-x, A $\beta$ x-40, A $\beta$ x-42 and A $\beta$ 1-42. The most abundantly detected A $\beta$  peptide was that cleaved at the 40<sup>th</sup> amino acid (419 $\pm$ 28pM), whereas only 105 $\pm$ 3pM of A $\beta$ x-42 and 319 $\pm$ 10pM A $\beta$ 1-x were detected in the media (Fig. 1).

Given the larger amount of A $\beta$ <sub>x</sub>-40 than A $\beta$ <sub>1-x</sub>, it is likely that not all detected A $\beta$ <sub>x</sub>-40 was cleaved at the first amino acid. A $\beta$  is also cleaved by  $\beta$ -secretase at the 11<sup>th</sup> amino acid (Wang et al., 1996; Cai et al., 2001). Indeed, only 9 $\pm$ 1pM of the 105 $\pm$ 3pM A $\beta$ <sub>x</sub>-42 was cleaved at the 1<sup>st</sup> amino acid. Therefore, A $\beta$ <sub>1-x</sub> represents total A $\beta$  cleaved at the 1<sup>st</sup> amino acid while A $\beta$ <sub>x</sub>-40/A $\beta$ <sub>x</sub>-42 represents total A $\beta$  cleaved at either the 1<sup>st</sup> or 11<sup>th</sup> amino acid and also at the 40<sup>th</sup> or 42<sup>nd</sup> amino acid. (While the detection region of the A $\beta$ <sub>x</sub>-40/42 assay included A $\beta$  cleaved at position 17 or lower, the assay was relatively insensitive to the non-amyloidogenic peptide, p3; data not shown).

The role of catB in APP processing was examined in multiple cell types that exhibited regulated secretory pathways. Inhibition of catB with a commercially available inhibitor, CA074Me, significantly decreased A $\beta$ <sub>x</sub>-40, A $\beta$ <sub>x</sub>-42 and A $\beta$ <sub>1-x</sub> in primary hippocampal neurons (Fig. 2A). A similar, though slightly less effective, reduction in A $\beta$  was observed in N2A cells treated with CA074Me (Fig. 2B). In order to ensure that the reduction in A $\beta$  with CA074Me was not due to off-target effects of the inhibitor, catB expression was knocked down in primary hippocampal neurons with siRNA. Six days after transfection with siRNA, catB activity was significantly reduced by up to 50% and this corresponded to a significant reduction in both A $\beta$ <sub>x</sub>-42 and A $\beta$ <sub>1-x</sub> (p<0.001 vs. untreated controls; Fig. 2C, 2D). Note that a similar reduction in A $\beta$  with BACE1 siRNA was also evident, indicating that BACE1 is also involved in APP processing in primary neurons as previously reported (Kao et al., 2004; Nishitomi et al., 2006).

We next investigated whether catB-dependent APP processing was specific to A $\beta$  secreted through either the regulated or constitutive pathway. Synaptic activity was suppressed with TTX, a voltage-dependent sodium channel blocker that inhibits action potential propagation and subsequent neurotransmitter release. After three days of treatment, TTX caused a significant

reduction in A $\beta$ <sub>x-42</sub> and A $\beta$ <sub>1-x</sub>. Importantly, in the absence of activity-dependent secretion of A $\beta$ , CA074Me-dependent A $\beta$  suppression was eliminated (Fig. 3). The reduction in A $\beta$  with CA074Me was less effective than that reported in Fig 2 because of the three-day incubation time, thus allowing synthesis of new uninhibited catB. (Note that even three days after inhibition of catB with CA074Me, catB levels were still  $37.6 \pm 2.8\%$  of untreated cells.) The necessity of catB in A $\beta$  secreted through the regulated pathway was further verified by testing whether catB inhibition modulated APP processing in CHO cells that were stably transfected with APP751 but only exhibited A $\beta$  secretion via the constitutive pathway. Inhibition of catB with CA074Me did not significantly alter A $\beta$  secreted from CHO-APP751 cells (Fig. 4), indicating that catB does not modulate A $\beta$  secreted through the constitutive pathway.

Since catL cleaves many substrates similar to those cleaved by catB, we investigated whether catL also inhibited APP processing. Following 24hrs of treatment with 3 of 4 catL inhibitors, we found that catL inhibition significantly lowered levels of A $\beta$ <sub>x-40</sub> and A $\beta$ <sub>1-x</sub>. Surprisingly though, inhibition of catL dramatically elevated levels of A $\beta$ <sub>x-42</sub> and A $\beta$ <sub>1-42</sub> (Fig. 5A, 5B), suggesting the two cysteine proteases acted very differently in modulating APP processing. The one inhibitor of catL that did not result in elevated A $\beta$ <sub>42</sub> was impermeable to cells. The specific modulation of A $\beta$ <sub>42</sub> was not only duplicated in N2A cells, but also in CHO APP751 cells, showing that catL-dependent A $\beta$ <sub>42</sub> elevations were not specific to A $\beta$  secreted through the regulated pathway (Fig. 5C, D). The opposing effects of catL and catB on APP processing may explain why previous investigators have found general cysteine protease inhibitors to have no effect on A $\beta$  secretion (Siman et al., 1993; LeBlanc and Goodyer, 1999). Indeed, inhibition of all cysteine proteases with E64D only reduced A $\beta$ <sub>1-x</sub> and A $\beta$ <sub>x-42</sub> levels by about ten percent and A $\beta$ <sub>x-40</sub> by 25% (Fig. 6A), despite a complete elimination of catB activity

(data not shown). Blockade of all proteases within the endosomal-lysosomal pathways with the weak base, chloroquine, further emphasized the opposing actions of catB and catL. Chloroquine significantly reduced both A $\beta$ <sub>x-40</sub> and A $\beta$ <sub>1-x</sub> levels, as observed with inhibitors to both cathepsins. However, specific upregulation of A $\beta$ <sub>42</sub> with catL inhibitors was negated in the presence of chloroquine (Fig. 6B), despite no change in cell viability (data not shown).

In order to elucidate the mechanism of cathepsin-dependent APP processing, we measured C-terminal fragments (CTFs) of APP with western blots. Only full length APP was observed in lysates from untreated primary neurons or those treated with catB or catL inhibitors (data not shown), suggesting that drugs did not block gamma secretase activity. Therefore, we treated cells with a gamma secretase inhibitor, DAPT, in order to increase the levels of APP CTFs above detectable limits. In the presence of DAPT, a single low molecular weight band was observed on western blots. The intensity of the low molecular weight band was significantly reduced with inhibition of either  $\alpha$ -secretase ( $p < 0.01$ ) or  $\beta$ -secretase ( $p < 0.05$ ), indicating it is likely a combination of CTFs generated by  $\alpha$ - and  $\beta$ -secretase. While catL inhibition did not alter CTF expression, inhibition of catB significantly reduced the intensity of APP CTF ( $p < 0.01$ ; Fig. 7). Together with the fact that catB inhibition lowers A $\beta$  secretion, these data imply that catB modulates APP processing by elevating  $\beta$ -secretase activity. It remains unclear whether catB also elevates  $\alpha$ -secretase activity.

Even though catL inhibition did not significantly alter the processing of APP CTFs, we hypothesized that catL elevated A $\beta$ <sub>42</sub> levels by modulating  $\alpha$ -secretase activity. It is likely that a reduction in  $\alpha$ -secretase would be accompanied by an elevation in  $\beta$ -secretase, thereby causing no net change in total APP c-terminal fragments. Moreover, a similar A $\beta$ <sub>42</sub> specific elevation was observed in the presence of BTM, a matrix metalloprotease inhibitor shown to block  $\alpha$ -

activity (Parvathy et al., 1998)). Indeed, catL inhibition had no additional effects on A $\beta$  secretion in the presence of BTM (Fig. 8). The role of catL on  $\alpha$ -secretase activity was further assessed by measuring sAPP $\alpha$  expression. Since catL was shown to be acting within the endosomal-lysosomal pathway, sAPP $\alpha$  was measured in lysates from N2A cells, rather than that cleaved from the cell membrane. Both BTM ( $p < 0.001$ ) and catL inh IV ( $p < 0.005$ ) significantly lowered sAPP $\alpha$  expression, relative to untreated controls (Fig. 9). Additionally, we measured  $\alpha$ -secretase activity using a fluorescent peptide assay, which mimicked the  $\alpha$ -secretase cleavage site of human APP. Lysates from primary neurons were treated with fluorescent substrates in the presence or absence of various inhibitors. Inhibition of catL significantly reduced  $\alpha$ -secretase cleavage of fluorescent substrate ( $p < 0.05$ ) while catB inhibition had no effect on  $\alpha$ -secretase activity, compared to untreated controls (Fig. 10).

Unlike  $\beta$ -secretase,  $\alpha$ -secretase activity is evident in all tested cell lines and can occur either constitutively or after PKC activation. Since PKC-mediated  $\alpha$ -secretase activity takes place intracellularly (Jolly-Tornetta and Wolf, 2000; Skovronsky et al., 2000), where catL was found to be active, we tested whether catL specifically modulated PKC-mediated  $\alpha$ -secretase activity. We blocked PKC activation with Ro32-0432 in primary hippocampal neurons and measured whether catL inh IV still elevated A $\beta$ 42. While we found that PKC inhibition slightly reduced A $\beta$ 42 secretion ( $p < 0.001$ , compared to untreated controls), the dependence of A $\beta$ 42 on catL inh IV disappeared when PKC activation was blocked ( $p = 0.35$ ; Fig. 11)

While it was evident that catL inhibition reduced  $\alpha$ -secretase activity, thereby elevating A $\beta$  levels, it remained unclear why the upregulation was specific to A $\beta$ 42. A recent study showed that catB specifically degraded A $\beta$ 42 into less toxic A $\beta$  peptides (Mueller-Steiner et al.,

2006), so we hypothesized that catL also specifically cleaved A $\beta$ 42 peptides. N2A cells were treated with human A $\beta$ 42 (hA $\beta$ 42; 300pM) and hA $\beta$ 1-42 and hA $\beta$ 1-x degradation were measured subsequently. Neither hA $\beta$ 1-42 nor hA $\beta$ 1-x was detected in media not treated with synthetic hA $\beta$ 42, indicating measurement was specific to the exogenous hA $\beta$ . hA $\beta$  levels subsided gradually over the course of the 24h experiment. Inhibition of either catB or catL significantly slowed the degradation process of hA $\beta$ 42 but did not affect levels of hA $\beta$ tot, compared to untreated controls (Fig. 12). These data indicate that both catB and catL are involved in degrading A $\beta$ 42 into less toxic A $\beta$  species and may help to explain why catL specifically elevated A $\beta$ 42 secretion.

## Discussion

The present study illustrates two novel and opposing mechanisms by which cysteine proteases modulate APP processing as well as a similar mechanism by which cathepsins degrade A $\beta$ . CatB promotes A $\beta$  formation via the regulated, activity-dependent secretory pathway, likely through its role in elevating  $\beta$ -secretase activity. In contrast, catL reduces the formation of A $\beta$ 42 peptides by cleaving APP within the A $\beta$  peptide sequence. Additionally, both cathepsins B and L degrade A $\beta$ 42 into less toxic A $\beta$  peptides. These findings precisely illustrate how cathepsins B and L modulate A $\beta$  levels and aid in understanding the discrepancies between past studies. Moreover, evidence from aged rats, where catB activity is upregulated and catL activity is downregulated (Nakanishi et al., 1994), shows that impaired cathepsin activity may participate in the onset of AD.

Compelling evidence exists in the literature to suggest that catB is involved in processing APP. Not only does catB co-localize with A $\beta$  in secretory vesicles (Hook et al., 2005), but also

cystatin C, an endogenous cysteine protease inhibitor with high affinity for catB, is one of the few downregulated biomarkers evident in human CSF of AD patients (Simonsen et al., 2007). CatB was first identified as the primary  $\beta$ -secretase responsible for A $\beta$  formation in chromaffin vesicles (Hook and Reisine, 2003) and was later shown to be involved in processing APP in chromaffin cells and guinea pig synaptosomes (Hook et al., 2007). Using primary hippocampal neurons, a neuronal phenotype implicated in learning and memory that is specifically targeted in AD, we observed a decrease in A $\beta$  secreted after either treatment with small molecule inhibitors of catB or transfection with catB siRNA. In contrast, Mueller-Steiner et al did not observe any reduction in either  $\beta$ -secretase or  $\alpha$ -secretase activity in hAPP/catB $^{-/-}$  mice (Mueller-Steiner et al., 2006). In fact, knockdown of catB in hAPP-infected primary neurons actually elevated A $\beta$  levels and transfection with catB lowered A $\beta$ . Several distinctions exist between these studies that may aid in understanding the conflicting results, including the use of cells exhibiting endogenous vs. transfected APP, wildtype APP vs. APP<sup>swe</sup>, and regulated vs. constitutive secretion of A $\beta$ . Indeed, both wildtype APP and regulated secretion of A $\beta$  have been shown to be important co-factors in catB-mediated APP processing (Hook and Reisine, 2003). Alternatively, it cannot be ruled out that catB only acts on rodent APP and is not relevant to humans.

Our results showing that catB inhibition only reduced A $\beta$  levels in primary neurons when A $\beta$  was secreted through the regulated pathway were in agreement with a previous study in chromaffin cells (Hook et al., 2005). This distinction illustrates an important reason why cysteine proteases were previously undiscovered as A $\beta$  modulators, and points to the importance of validating potential therapeutic targets in a variety of AD models. As wildtype neurons secrete low levels of A $\beta$ , many investigators utilize cell lines overexpressing APP, to enhance

constitutive release of A $\beta$ . However, detectable levels of A $\beta$  are also evident in untransfected cells when they are either spontaneously active, as in this study, or when synaptic activity is pharmacologically induced (Hook and Reisine, 2003). Moreover, alterations in neuronal excitability are frequently observed in the AD brain, indicating a pathologic rationale for the onset of AD. Whether synaptic activity drives APP endocytosis or A $\beta$  secretion is unknown, though evidence in the literature points towards the former since suppression of neurotransmitter release inhibited  $\beta$ -secretase activity in organotypic hippocampal cultures (Kamenetz et al., 2003). Understanding this distinction will aid in determining how catB elevates APP processed through the regulated pathway.

It is unlikely that catB is the primary  $\beta$ -secretase in primary neurons since BACE1 siRNA and small molecule inhibitors also significantly reduced A $\beta$  secretion. Instead, we believe that catB either acts as a secondary  $\beta$ -secretase or, more likely, indirectly modulates  $\beta$ -secretase activity. For example, catB-dependent APP processing may be specific to the KPI isoform of APP, which is predominately expressed in glia but is upregulated in neurons in both the AD brain (Moir et al., 1998) and after enhanced excitatory activity (Lesne et al., 2005). The KPI domain of APP binds with a serine protease prior to internalization (Knauer et al., 1996). It is feasible that this complex hinders the accessibility of the BACE1 cleavage site of APP and catB cleavage of the complex enhances the affinity of BACE1 for the remaining APP fragment. We attempted to test this hypothesis by using CHO cells transfected with APP751, an APP isoform incorporating the KPI domain, but found no dependence on catB; however, this was at least in part due to the absence of regulated A $\beta$  secretion. Interestingly, neurons were transfected with the non-KPI APP isoform, APP695, in the study that failed to detect catB dependent  $\beta$ -secretase activity (Mueller-Steiner et al., 2006).



In contrast to the  $\beta$ -secretase modulation by catB, we found that catL upregulated  $\alpha$ -secretase activity, presenting an additional source for the contradictory data on cysteine proteases and APP processing. CatL may directly cleave APP within the A $\beta$  sequence or alternatively, cleave the pro-form of another protease and indirectly upregulate  $\alpha$ -secretase activity. While  $\alpha$ -secretase was traditionally thought to cleave membrane bound APP, we observed the effect of catL to be intracellular since the impermeable catL inhibitor had no effect on A $\beta$  levels, intracellular sAPP $\alpha$  decreased with catL inhibition and chloroquine negated the activity of the catL inhibitor. This is in line with newer data that shows PKC-mediated  $\alpha$ -secretase activity typically occurs intracellularly within the transgolgi network (Jolly-Tornetta and Wolf, 2000; Skovronsky et al., 2000). Of the three known  $\alpha$ -secretase candidates that have been identified to date, ADAM9, ADAM10 and ADAM17/TACE (Asai et al., 2003), none are thought to be solely responsible for PKC-mediated  $\alpha$ -secretase activity, suggesting that more candidates are plausible. Moreover, researchers rely on broad spectrum metalloprotease inhibitors to block  $\alpha$ -secretase activity and these inhibitors may indirectly alter catL activity since pro-catL cleavage into its active form has been shown to be mediated by metalloproteases (Hara et al., 1988). Finally, it cannot be ruled out that catL also modulates APP through gamma secretase. A similar specific rise in A $\beta$ 42, accompanied by a reduction in A $\beta$ 40, was observed in CHO-APP751 cells treated with E64D (Figueiredo-Pereira et al., 1999). While we did not observe any accumulation of APP CTFs after catL inhibition, catL may also modulate, rather than inhibit, gamma secretase.

Both catB and catL were also shown to exhibit secondary functions in A $\beta$  modulation by specifically degrading A $\beta$ 42 into less toxic A $\beta$  peptides. While both catB and general cysteine proteases have previously been identified in their roles of A $\beta$  degradation (Mueller-Steiner et al., 2006); (Frautschy et al., 1998), this is the first study to show that catL also specifically degrades

A $\beta$ 42 into less toxic A $\beta$  peptides. Our data suggest that cathepsins degrade A $\beta$  peptides prior to secretion or after internalization by endocytosis, though catB does associate with A $\beta$  plaques and extracellular activity of cathepsins has been confirmed (Cataldo and Nixon, 1990). This secondary role of catB may actually exacerbate its role in elevating  $\beta$ -secretase activity since A $\beta$  application elevates catB activity (Mueller-Steiner et al., 2006). Alternatively, the role of catL in A $\beta$  degradation, in conjunction with its  $\alpha$ -secretase activity, may explain why inhibition of catL specifically elevates A $\beta$ 42, and not A $\beta$ 40.

The opposing roles of catB and catL in APP processing present an ideal opportunity for a therapeutic target to lower A $\beta$  levels in AD patients. CatB and catL share many similar substrates and knockout animals have revealed that the remaining cathepsin compensates for the deficient cathepsin, thereby presenting few significant phenotypic alterations (Deussing et al., 1998; Nakagawa et al., 1998; Guicciardi et al., 2000; Roth et al., 2000). Nonetheless, opposing actions of catB and catL are not unprecedented (Rocken et al., 2005). By developing a drug that lowers catB activity, elevated catL activity would be expected to follow, thereby simultaneously lowering A $\beta$  levels through two distinct pathways.

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## Legends for Figures

Figure 1: A $\beta$  secreted from primary hippocampal neurons over 72hrs were detected by ELISA. Peptides cleaved by  $\beta$ -secretase at position 1, as well as by gamma-secretase at positions 40 and 42 were evident.

Figure 2: Inhibition of catB with CA074Me significantly reduces A $\beta$ 1-x, A $\beta$  x-40 and A $\beta$ x-42 in A) primary hippocampal neurons and B) N2A cells—two cell types exhibiting regulated secretory pathways. Knockdown of catB in primary hippocampal neurons reduces both C) catB activity and D) secreted A $\beta$ . (\* p<0.001 vs. untreated controls)

Figure 3: A $\beta$  secreted through the regulated pathway was blocked in primary hippocampal neurons with TTX. In the absence of regulated secretion of A $\beta$ , CA074Me had no additional efficacy on A $\beta$  suppression. (#p<0.05 compared to untreated.)

Figure 4: Inhibition of catB with CA074Me has no effect on A $\beta$  secreted in CHO cells transfected with APP751, a cell line not exhibiting a regulated secretory pathway.

Figure 5: A) CatL inhibition in primary hippocampal neurons specifically elevates A $\beta$ x-42 while decreasing A $\beta$ 1-x levels with three of the four tested inhibitors. Note that A $\beta$ x-40 was only measured for catL inh IV-treated neurons, and revealed a significant decrease in A $\beta$ x-40 levels. B) A $\beta$ 1-42 is also elevated in primary hippocampal neurons in the presence of catL inh IV, verifying that the effect of catL inhibition is not simply due to an increase in the  $\alpha$ -secretase-

generated p3 fragment of APP. The specific effect on A $\beta$ x-42 is duplicated in C) N2A cells and D) CHO cells, indicating it is not specific to cells with regulated pathways. (\*p<0.001 compared to untreated.)

Figure 6: A) Only slight though significant reductions in A $\beta$  are evident when all cysteine proteases were inhibited in primary hippocampal neurons with E64D. B) APP processing within the endosomal-lysosomal pathways was suppressed with the weak base, chloroquine. Treatment with chloroquine reduced levels of A $\beta$ x-40 and A $\beta$ 1-x, but had no effect on A $\beta$ x-42. The elevation of A $\beta$ 42 in the presence of catL inh IV was negated with chloroquine. (\*p<0.001 compared to untreated; \*\*p<0.01 compared to untreated; #p<0.05 compared to untreated.)

Figure 7: A) Representative western blots show full length APP (APP-FL) and a single CTF of APP between 6 and 14kDa. B) APP processing into CTFs is reduced in the presence of CA074Me,  $\alpha$ -secretase inhibitor (ASI) and  $\beta$ -secretase inhibitor (BSI). (#p<0.05 compared to untreated.)

Figure 8: Both catL inh IV and a non-specific  $\alpha$ -secretase inhibitor, BTM, significantly elevate A $\beta$ 42 in N2A cells. Treatment with both catL inh IV and BTM produces no additional increase in A $\beta$ 42. (\*p<0.001 compared to untreated.)

Figure 9: N2A cells were treated with either BTM or catL inh IV or untreated (ctrl) and sAPP $\alpha$  and full length APP (APP-FL) were detected on western blots. A) Representative blot shows APP-FL and sAPP $\alpha$ . B) The staining intensity of sAPP $\alpha$  was normalized to APP-FL.

Treatment with either BTM or catL inh IV significantly reduced intracellular sAPP $\alpha$ . (\*p<0.001 compared to untreated.)

Figure 10: Cleavage of a fluorescent substrate mimicking the  $\alpha$ -secretase cleavage site of APP was measured in lysates from primary neurons. A) Treatment with 50 $\mu$ M BTM or 10 $\mu$ M catL inh IV significantly reduced  $\alpha$ -secretase activity, compared to matched controls (p<0.05), while inhibition of catB had no effect. Data are presented as percent of untreated controls. B)  $\alpha$ -secretase activity in the presence of the two inhibitors shown to blot  $\alpha$ -secretase activity was detected over a range of concentrations. Data are presented as percent difference from untreated controls. (#p<0.05 compared to untreated.)

Figure 11: Primary neurons were treated with catL inh IV, Ro32-0432 or both and A $\beta$ <sub>x-42</sub> was detected in the media 24hrs later. Inhibition of PKC with Ro32-0432 blocked the elevation in A $\beta$ <sub>42</sub> secreted into media from cells treated with catL inh IV alone. (\*p<0.001 vs. media from untreated cells)

Figure 12: Degradation of exogenously applied hA $\beta$ <sub>42</sub> was detected in N2A cells. Treatment with either catL inh IV or CA074Me did not significantly alter the degradation profile of hA $\beta$ <sub>1-x</sub> (hA $\beta$ <sub>tot</sub>). However, both catL inh IV and CA074Me delayed the degradation process of hA $\beta$ <sub>1-42</sub>. Slight degradation of hA $\beta$ <sub>42</sub> was also observed in the absence of cells. Cells treated with either CA074Me or catL inh IV exhibited significantly more hA $\beta$ <sub>42</sub> than untreated cells after 4h, 8h, and 24h hA $\beta$ <sub>42</sub> treatment. (&p<0.01 both CA074Me and catL inh IV vs. untreated.)

Figure 1:

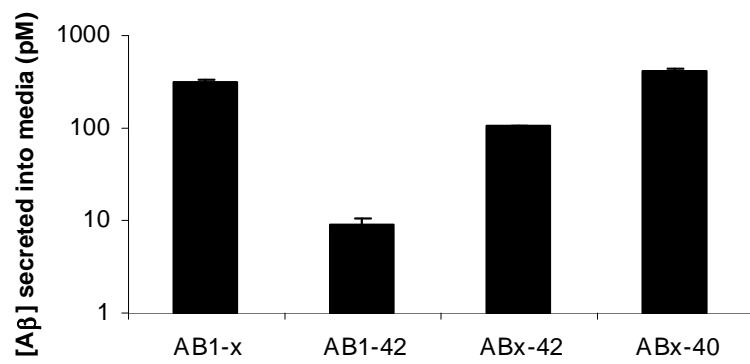
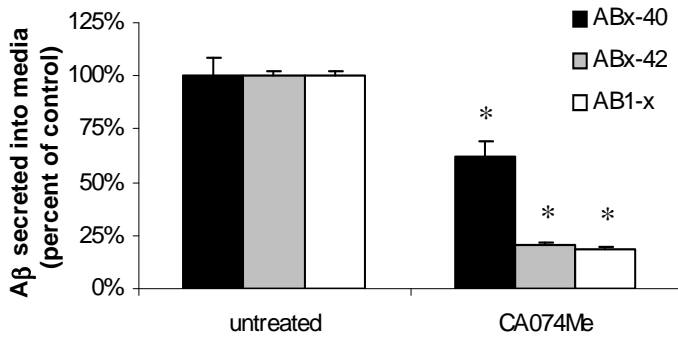
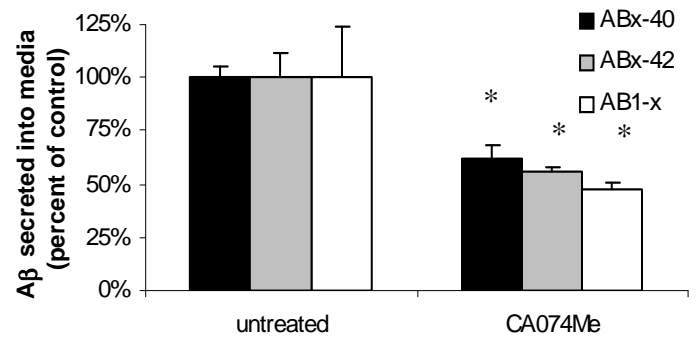


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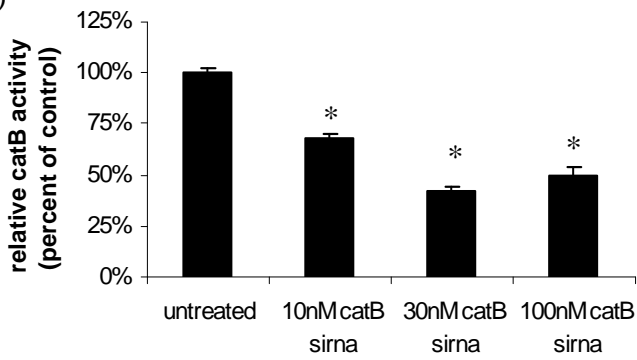
A)



B)



C)



D)

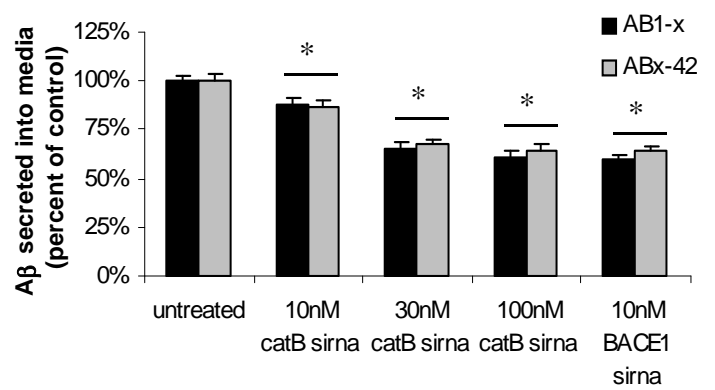


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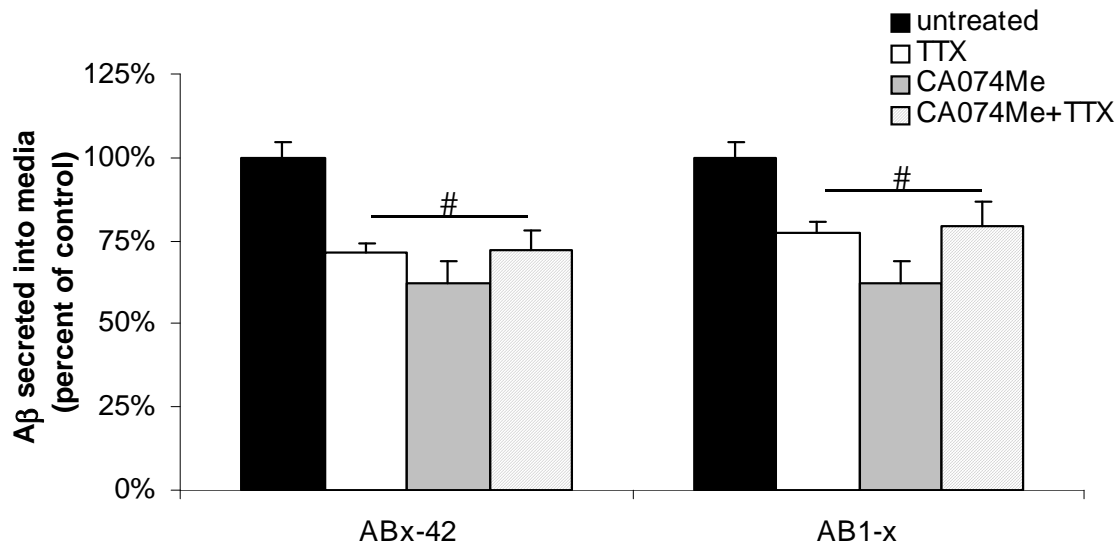


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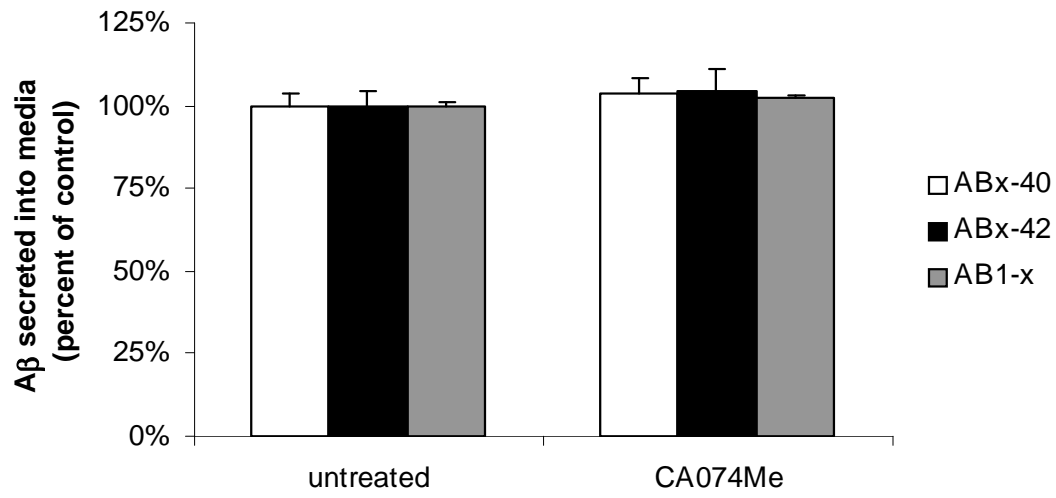




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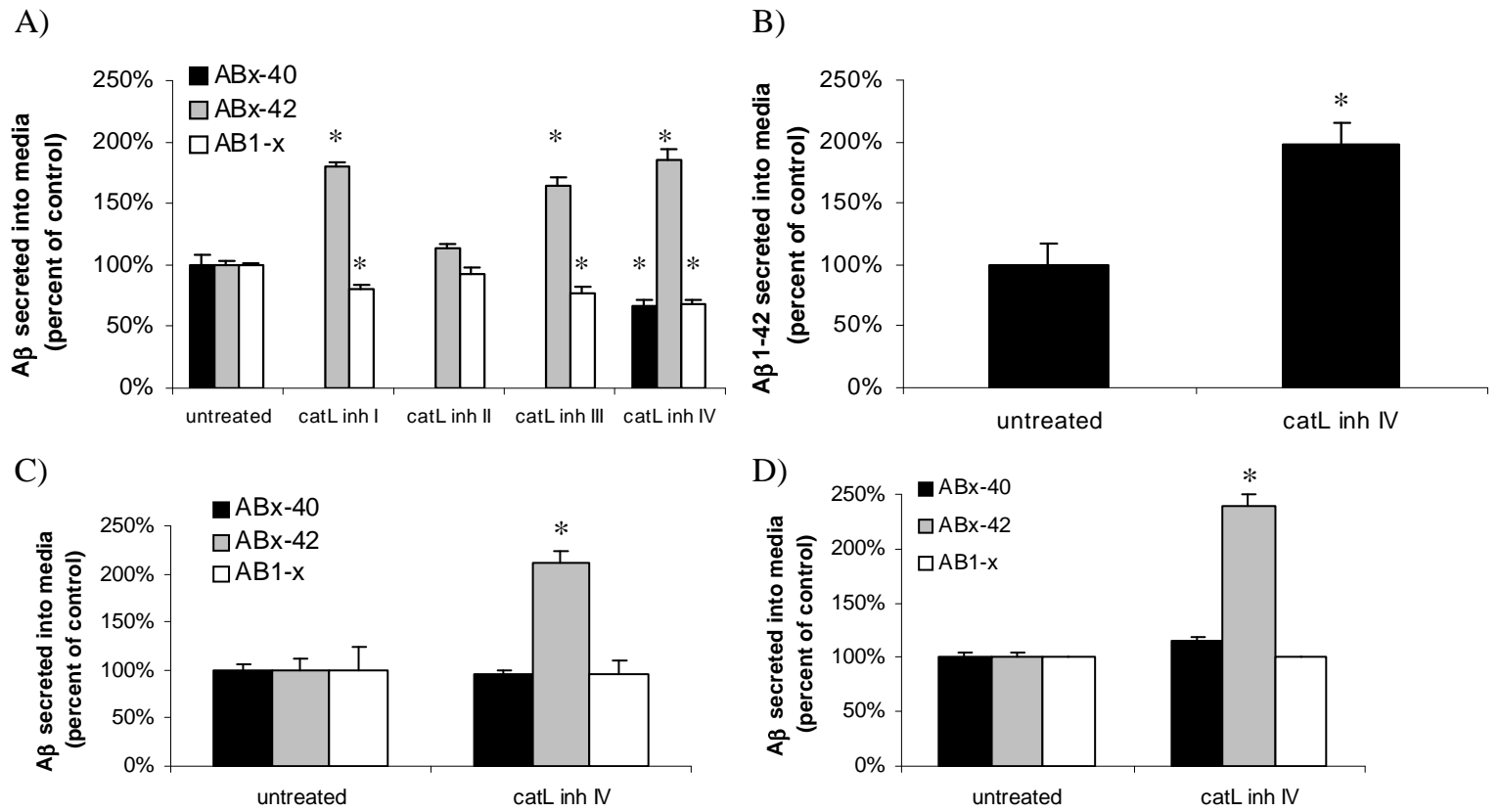


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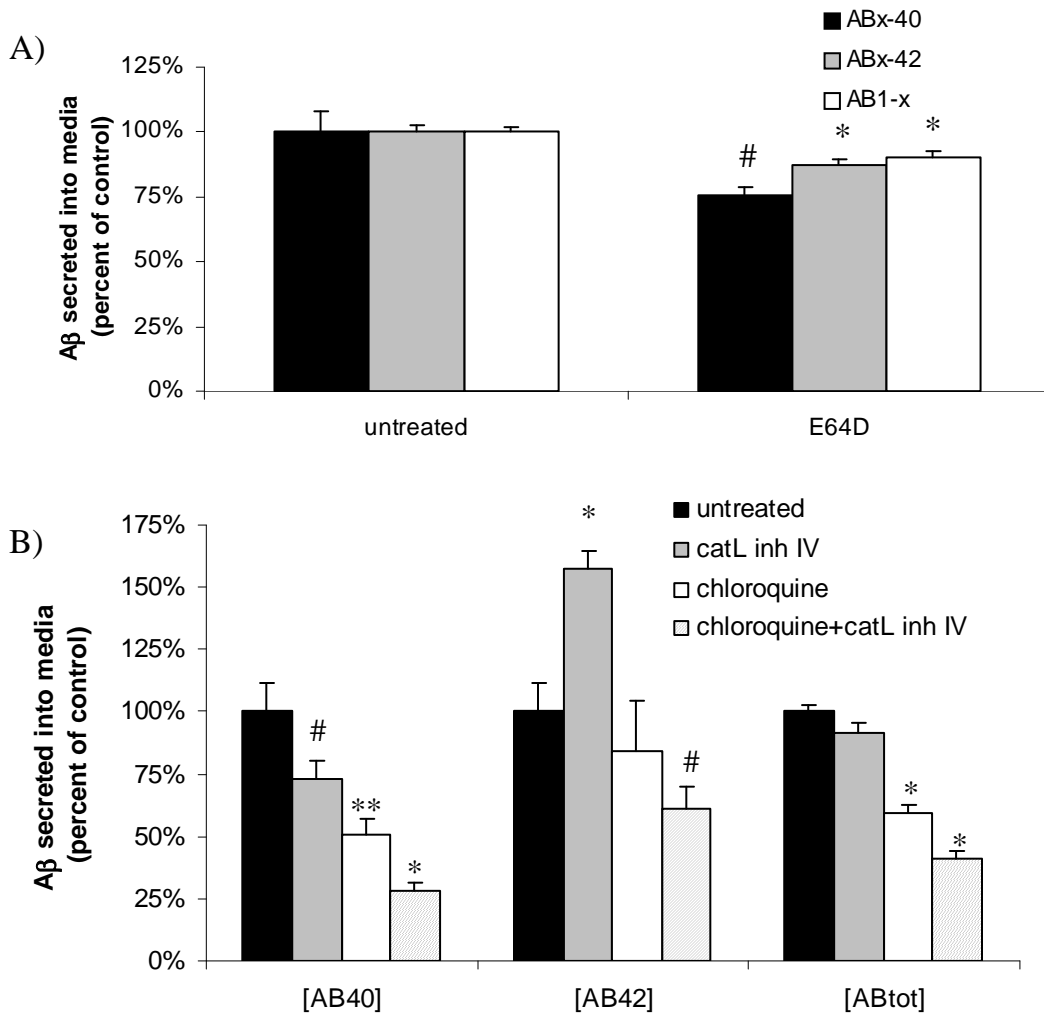


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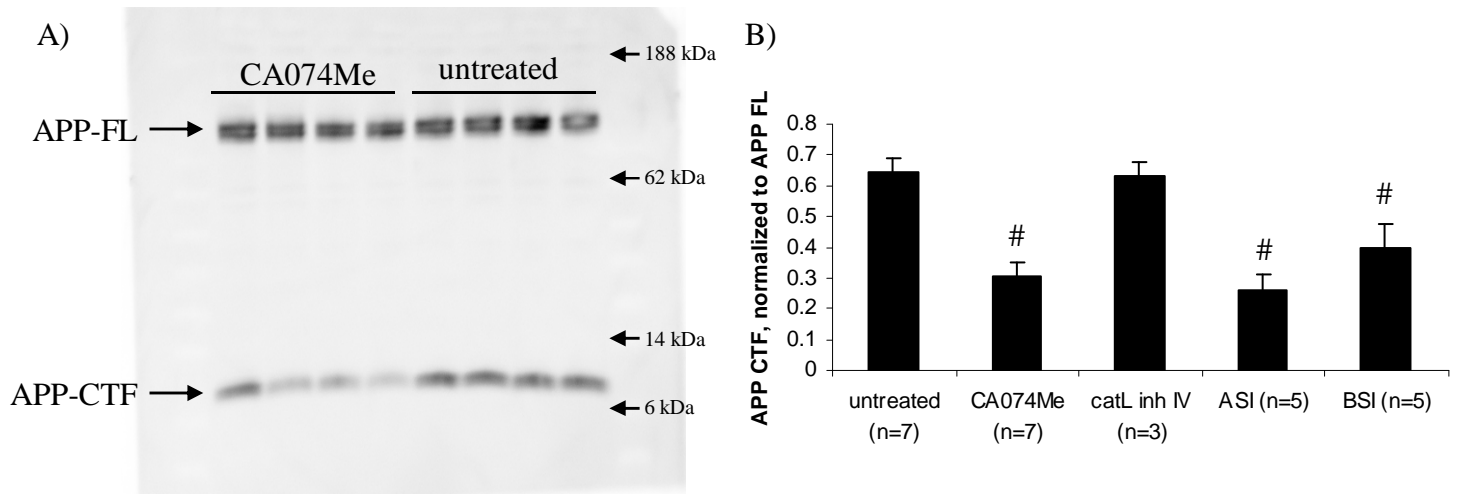


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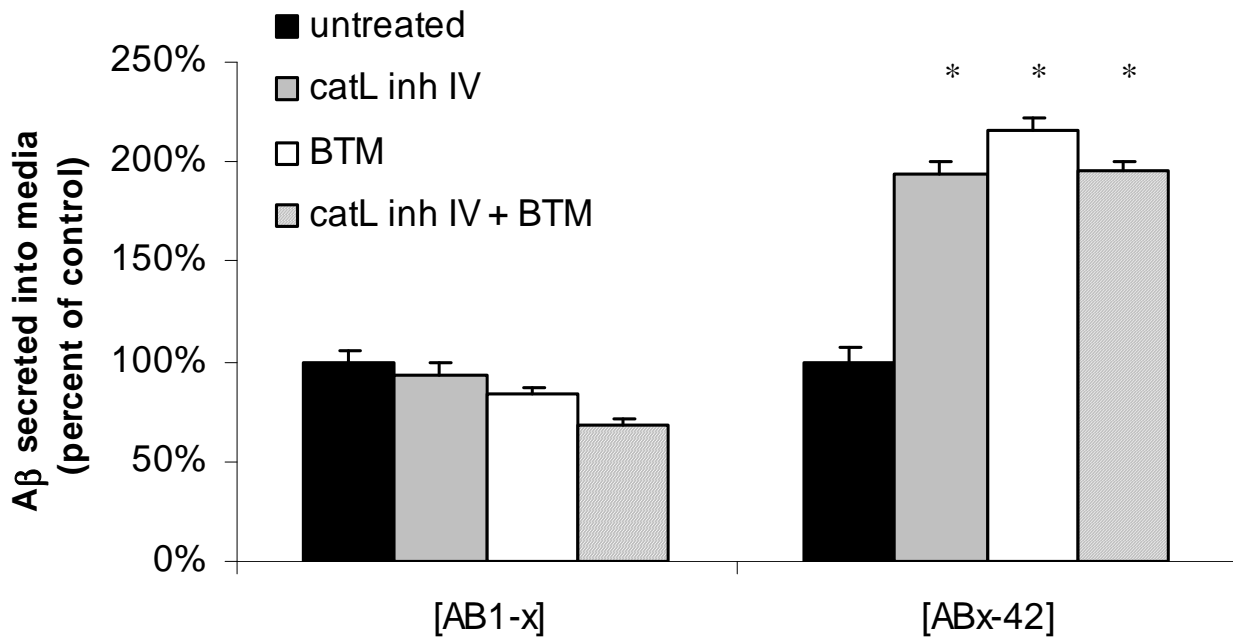


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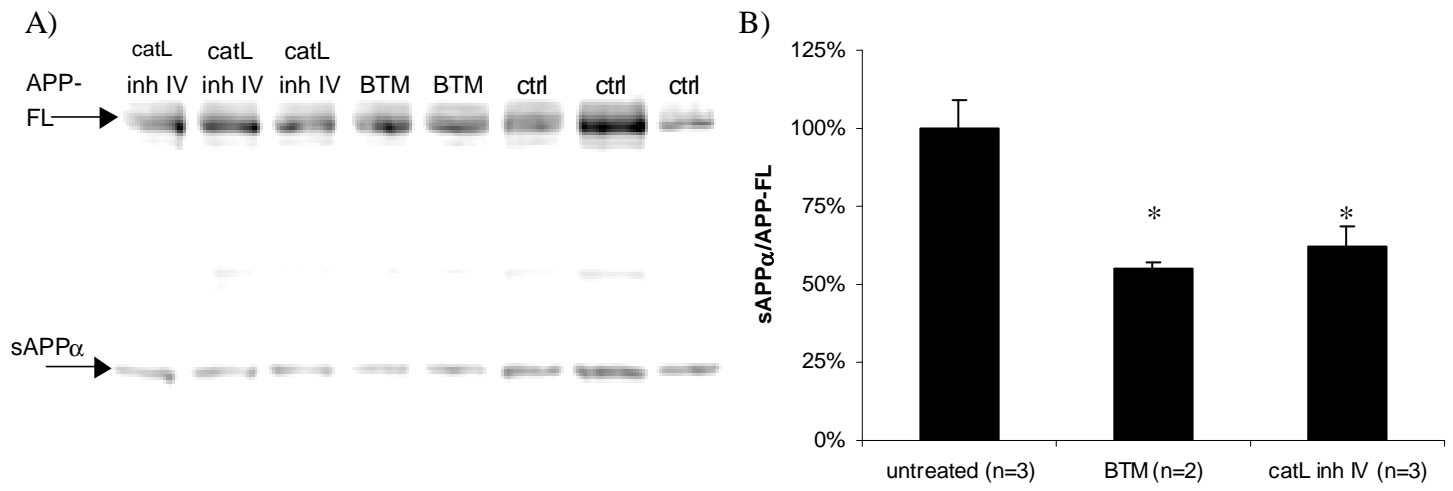


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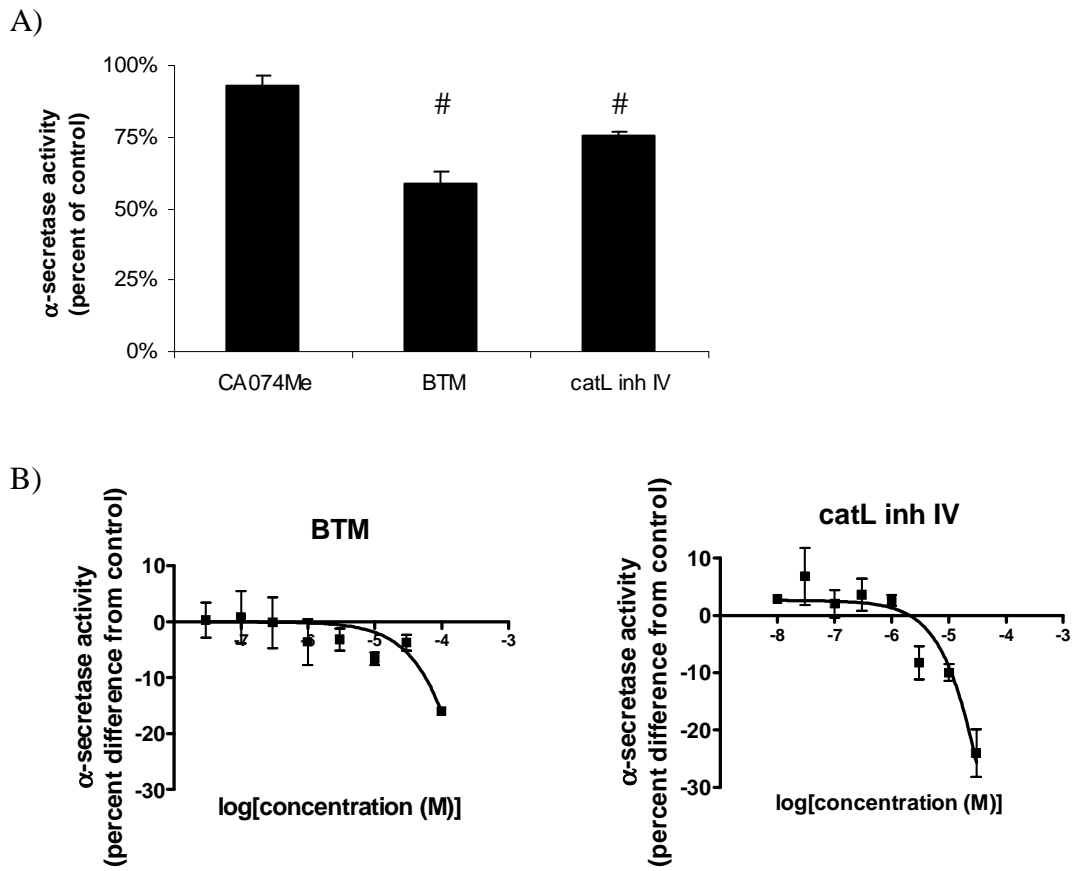


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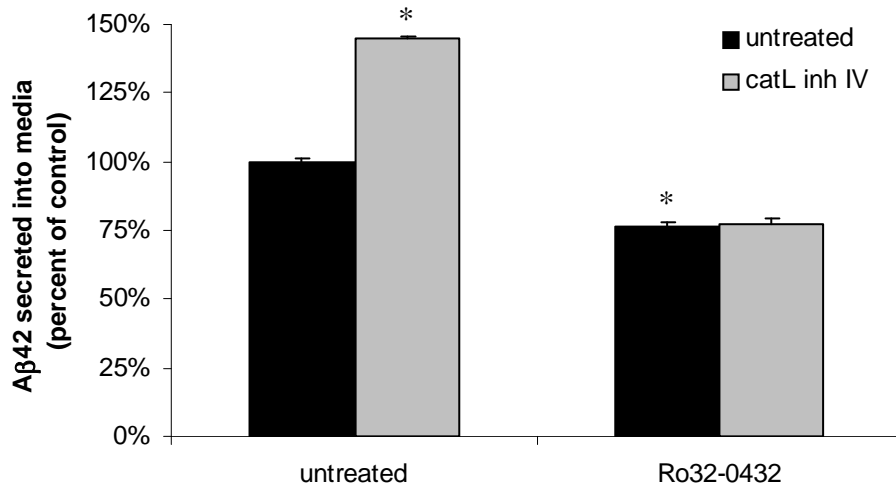


Figure 12:

