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METHAMPHETAMINE REGULATES β APP PROCESSING IN HUMAN NEUROBLASTOMA CELLS

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Running title: β APP processing regulation by methamphetamine

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Abstract

Methamphetamine is a potent and highly addictive psychostimulant whose abuse has turned out to be a global health hazard. The multitudinous effects it exerts at the cellular level induces neurotoxic responses in the human brain, ultimately leading to neurocognitive disorders. Strikingly, brain changes, tissue damage and neuropsychological symptoms due to Meth exposure compels and necessitates to link the probability of risk of developing premature Alzheimer's disease, a progressive neurodegenerative disorder characterized by amyloid plaques composed of amyloid- β peptides and clinical dementia. These peptides are derived from sequential cleavages of the β -amyloid precursor protein by β - and γ -secretases. Previous studies reveals evidence for both positive and negative effects of Meth pertaining to cognitive functioning based on the dosage paradigm and duration of exposure revealing a beneficial psychotropic profile under some conditions and deleterious cognitive deficits under some others. In this context, we proposed to examine the effect of Meth on β APP metabolism and β APP-cleaving secretases in the human neuroblastoma SH-SY5Y cell line. Our results showed that Meth dose-dependently increases BACE1 expression and catalytic activity, while its effect on the α -cleavage of β APP and on the expression and catalytic activity of the main α -secretase ADAM10 display a bell-curve shape. To our knowledge, the present study is the first to demonstrate that Meth can control β APP-cleaving secretases. Moreover, we propose from these findings that the deleterious effect of Meth on cognitive decline might be an outcome of high dosage paradigm whereas acute and short-term drug use which stimulated sAPP α might produce improvements in cognition in disorders such as AD.

1. Introduction

It has long been accepted that long term methamphetamine (Meth) abuse (high repetitive doses) produces neurotoxicity and is associated with cognitive impairments [1], the

neuropsychological deficits being associated with the neurodegenerative effects of this drug as observed in experimental models [2, 3]. Several mechanisms of Meth-induced neurotoxicity have been proposed, including oxidative stress, excitotoxicity, mitochondrial dysfunction and neuroinflammation marked by microgliosis, astrogliosis and cytokine induction [4], concurring to mediate apoptosis and neurotoxicity in the CNS [5]. Beside perturbations in calcium and lipid homeostasis, post Meth exposure is characterized by degeneration of neurons, similar to what is observed in Alzheimer's disease (AD) pathology. Indeed, a neuroproteomic study carried out in rats has shown that the expression of 18 proteins (11 in the hippocampus and 7 in the olfactory bulb) underwent a significant alteration as a result of Meth exposure with the altered proteins being involved in cell death, inflammation, oxidation, and apoptosis [6]. Interestingly, all these pathogenic mechanisms are part of the neurodegenerative cascade of Alzheimer's disease (AD) [7, 8] and involvement of the limbic system and hippocampus in particular exemplifies the connection between Meth abuse and AD pathology.

It has also been demonstrated that the GSK3 β kinase as well as the pleiotropic transcription factor NF κ B, which respectively play key roles in Tau phosphorylation and in inflammatory responses, both participate to the control of A β ₄₂-induced inhibition of ADAM10 expression and augmentation of BACE1 and presenilin 1 transcription [9-11]. Interestingly, Meth is capable to induce pro-inflammatory cytokines and their mediators through the NF κ B pathway [12] and to promote TNF α expression as well as NF κ B nuclear translocation [13]. Given the additional fact that Tau phosphorylation is induced by 3,4-Methylenedioxymethamphetamine ("Ecstasy") *in vivo* in the mouse hippocampus [14], these mechanisms altogether portray that Meth-induced alterations correlate with AD-like pathology.

Nevertheless, although substantial number of evidences validates the pernicious effects of Meth on the CNS, other studies have contrastingly ascertained some neuroprotective effects

of this psychostimulant [15, 16] and the genuine effect of Meth abuse on cognition is still matter to debate [17]. Therefore, Meth presents a challenging ambiguity of neurotoxicity and neuroprotection where the potential underlying mechanisms are precisely regulated by the controlled exposure of Meth dosages. Given the neurodegenerative effects and its sodality with the cognitive functioning, which majorly depends on dosage variation and time of exposure, we reasoned that Meth administration might differentially affect the proteolytic processing of β APP, a field of investigation that has been relatively unexplored so far. Herein, we investigated the effect of various doses of methamphetamine on β APP metabolism and β APP-cleaving secretases *in vitro* in cultured human SH-SY5Y neuroblastoma cells. With emphasis on the benefits and hazards of Meth on cognitive, functional and behavioral manifestations, the idea is to develop a better understanding of the drug action and disease process and the possibilities of modification of the AD pathogenesis and treatment.

2. Materials and methods

2.1 Materials

DMEM complete medium, Opti-MEM, trypsin and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA, USA). Penicillin–streptomycin mix was from PAA. Poly-D-lysine was from Sigma (St. Louis, MO, USA). Tris buffer and glycine were from Vivantis. Skim milk powder was from criterion. ECL and ammonium persulphate were from GE Health care (Piscataway, NJ, USA). SDS was from Amresco. O-Phenanthroline was from Calbiochem (San Diego, CA, USA). (D)-METH hydrochloride was purchased from Alltech-Applied Science (State College, PA, USA).

2.2. Cell culture and treatments

Human neuroblastoma SH-SY5Y cells were cultured at 37°C, 5% CO₂ in DMEM supplemented with 10% inactivated FBS containing penicillin (100 U/ml) and streptomycin (50 mg/ml). For experiments, the cells were seeded in six-35mm well plates and were grown until reaching 80% confluence. Cells were then treated without (control) or with various concentrations of Meth (1, 10, 100 and 1000 µM) for 16 h.

2.3. Western blot analysis in cell lysate

Cells were collected with phosphate-buffered saline (PBS)-EDTA and resuspended in 80-150 µl of lysis buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.5% triton X-100, 0.5% deoxycholate, 5 mM EDTA). Protein concentrations were determined by the Bradford method [18] and 5-50 µg of proteins were separated by SDS-polyacrylamide gel electrophoresis on 8% (βAPP), 10% (ADAM10 and BACE1) or 12% (β-actin) Tris/glycine gels. Proteins were then transferred onto nitrocellulose membranes (45-120 min according to protein size, 100 V), blocked for 1 h in 5% nonfat milk and incubated overnight at 4°C with primary antibodies (in 5% nonfat milk) directed against βAPP (dilution 1/4000, polyclonal A8717, Sigma), ADAM10 (dilution 1/500, polyclonal AB19026, Millipore), BACE1 (dilution 1/1000, monoclonal Ab108394, Abcam) or β-actin (dilution 1/5000, monoclonal 13E5; Cell Signaling) antibodies. Bound antibodies were detected using goat anti-mouse (dilution 1/3000, polyclonal 7076, Cell Signaling) or goat anti-rabbit peroxidase-conjugated (dilution 1/3000, polyclonal 7074, Cell Signaling) antibodies. Immunological complexes were revealed using Immobilon Western Chemiluminescence HRP substrate (Millipore) and detected using an automatic medical X-ray processor (Kodak, Rochester, NY, USA). Films were scanned, band densities were measured with the Image J software (<http://imagej.nih.gov/ij/>), and data were normalized using β-actin as an internal standard.

2.4. Measurement of sAPP α secretion

SHSY5Y cells were cultured in 35-mm dishes until they reached 80% confluence. Then, the cells were treated without (control) or with various concentration of Meth in DMEM containing 1% FBS. Media were then removed and replaced with 1 ml of serum-free DMEM and cells were allowed to secrete for 3 h. Then, TCA precipitation of the 1 ml serum-free secretion media was performed and samples were subjected to Western blot analysis onto 8% SDS-PAGE, transferred onto nitrocellulose membrane (120 min, 100 volts), incubated in 5% non-fat milk blocking solution for 30 min and incubated overnight at 4°C with the human-specific anti-sAPP α antibody DE2B4 (dilution 1/500, monoclonal DE2B4, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Membranes were then incubated with HRP-conjugated anti mouse antibody and processed for quantification as described above.

2.5. α -secretase fluorimetric assay on intact cells

SH-SY5Y cells cultured in 35-mm dishes coated with polylysine (10 μ g/ml) were treated without (control) or with Meth for 16 hours and the α -secretase catalytic activity was measured using the JMV2770 substrate and the ADAM10-specific GI254023X inhibitor as previously described [19].

2.6. β -secretase fluorimetric assay of cell homogenates

SH-SY5Y cell lysate (30 μ g) were treated without (control) or with Meth for 16 hours and assayed for their β -secretase activity using the JMV2236 substrate and the JMV1197 BACE1-specific inhibitor as previously described [20].

2.7. Real-time quantitative polymerase chain reaction (q-PCR)

SH-SY5Y cells were treated in the absence (control) or in the presence of various Meth concentrations as described above. Post treatment, total RNA was extracted and purified with the PureLink RNA mini kit (Ambion, Life Technologies, Austin, TX, USA). Real-time PCR was performed with 100 ng of total RNA using the QuantiFast SYBR Green RT-PCR kit (Qiagen, Singapore) detector system (Eppendorf Mastercycler ep RealPlex) and the SYBR Green detection protocol. The 2x QuantiFast SYBR Green RT-PCR master mix, QuantiFast RT mix, QuantiTectPrimer Assay and template RNA were mixed and the reaction volume was adjusted to 25 μ l using RNase-free water. The specific primers were designed and purchased from Qiagen. Each primer is a 10x QuantiTect Primer Assay containing a mix of forward and reverse primers for specific targets: Hs_ADAM10_1_SG (QT00032641) (human ADAM10), Hs_BACE1_1_SG (QT00084777) (human BACE1) and Hs_GAPDH_1_SG (QT00079247, human GAPDH, housekeeping gene for normalization).

2.8. Statistical analysis

Statistical analyses were performed with the GraphPad Prism software (San Diego, CA, USA) using the unpaired *t*-test for pair wise comparisons.

3. Results

3.1. Effect of methamphetamine on the α -secretase processing of β APP

We first investigated the effect of Meth on the non amyloidogenic α -secretase processing of endogenously expressed β APP in cultured human SH-SY5Y neuroblastoma cells by measuring the production of the neurotrophic, neuroprotective, memory-enhancing and

neurogenesis-stimulating β APP-derived sAPP α metabolite. Our results interestingly showed that Meth dose-dependently increases sAPP α secretion when applied at 1 and 10 μ M, while sAPP α production was not changed when further increasing Meth concentrations up to 100 μ M and 1 mM (Fig. 1). Moreover, none of the Meth doses applied altered β APP immunoreactivity (Fig. 1), thereby indicating that Meth is genuinely controlling the α -secretase processing of β APP rather than altering its expression or maturation.

3.2. Effect of methamphetamine on α -secretase catalytic activity

We then studied the effect of different concentrations of Meth on the catalytic activity of the principal α -secretase ADAM10 in wild-type SH-SY5Y by means of an α -secretase-specific fluorimetric assay. The results show that, as was observed with sAPP α production, 1 and 10 μ M of Meth significantly and dose-dependently increase the GI254023X-sensitive hydrolysis of the JMV2770 substrate when compared to untreated cells (Fig. 2A) whereas higher doses of the psychoactive drug (up to 1 mM) failed to modify ADAM10 activity (Fig. 2A).

3.3. Effect of methamphetamine on ADAM10 transcription and protein expression

To determine whether the observed up regulation of ADAM10 catalytic activity is a consequence of a Meth-dependent transcriptional activation of the protease, we measured the effect of Meth on ADAM10 mRNA by quantitative real-time PCR experiments. The results depict a similar pattern of action of Meth on ADAM10 mRNA expression when compared to the one previously observed for sAPP α production and ADAM10 catalytic activity, with a dose-dependent stimulation at 1 and 10 μ M and no effect at 100 μ M and 1 mM (Fig. 2B) and a similar pattern was observed when assessing the impact of the drug on ADAM10 immunoreactivity/protein levels by western blot (Fig. 2C).

3.4. Effect of methamphetamine on BACE1 catalytic activity and expression

We have then investigated the effects of Meth on the β -secretase BACE1, which is the rate-limiting initiator of A β production. Indeed, following incubation of SH-SY5Y cells with various concentrations of Meth for 16 h, we have evidenced a dose-dependent increase of BACE1 catalytic activity with a significant difference observed only at 1 mM when compared to untreated control cells (Fig. 3A). In a second set of experiments, we have established by quantitative real-time PCR that the drug indeed increases BACE1 mRNA levels when applied at the high 100 μ M and 1 mM concentrations but not at the low 1 and 10 μ M doses, thereby indicating that high Meth can control BACE1 at a transcriptional level (Fig. 3B). Finally, western blot analysis performed under the same conditions have established that Meth treatments positively control BACE1 protein levels (Fig. 3C). The a priori paradoxical observation that Meth at 100 μ M did not show any effect on β -secretase catalytic activity while significantly increasing BACE1 mRNA levels could be due to the presence of Meth-insensitive non-BACE1 β -secretase activities able to cleave the fluorimetric substrate, whereas the quantitative PCR is fully specific for BACE1 and certainly displays a higher sensitivity when compared to the fluorimetric assay.

4. Discussion

The present demonstration that Meth could convey beneficial effect regarding Alzheimer's disease is in line with previous reports having established that psychostimulants can be used medically to fight brain disorders [21]. Indeed, amphetamine and Meth are the first line treatment for attention deficit hyperactivity disorder (ADHD) [22] as well as for the

management of narcolepsy [23]. Furthermore, clinical trials have provided evidence that Meth may be effective in the treatment of otherwise treatment-resistant anxiety disorders [24] and that its administration is involved in supportive and integrative psychotherapy protocol [25].

More specifically considering brain functions, a significant amount of data has evidenced beneficial effects of this psychoactive drug under correct dosage and right timings/duration of exposure. Regarding physiological status, juvenile rats administered with Meth showed improved performance in a spatial navigation task when tested at adulthood [26]. In addition, Meth controls the release of the catecholamine dopamine that are capable of activating multiple neuroprotective pathways in the brain [27]. Now considering pathological conditions, it has been demonstrated that low to moderate doses of Meth induces short-term improvements in learning and memory in preclinical models of stroke and traumatic brain injury [16].

In this context, the present study strongly suggests that the above mentioned beneficial effects of low Meth doses can, at least partly, be due to the enhancement of α -secretase expression and activity leading to an increased production of sAPP α , which is a well-established neuroprotective and neurotrophic factor, but is also a key contributor to synaptic plasticity and spatial memory and has been more recently shown to enhance neurogenesis [28].

When briefly considering the possible mechanisms through which Meth could promote sAPP α production, it is important to firstly underline here that Meth, after its binding to neuronal dopamine receptors, can activate the ERK pathway [29] and the phosphorylation of the CREB transcription factor [30]. Because CREB, a downstream target of ERK, is involved in ADAM10 promoter transactivation [31, 32], low Meth could positively control ADAM10 expression via the CREB pathway, the incapacity of higher concentrations of the drug to

operate in the same manner being due to an desensitization of the receptors. Secondly, because Meth increases BDNF and TrkB in multiple brain regions and since BDNF promotes the non-amyloidogenic processing of β APP and reduces the production of A β peptides in a transgenic mouse model of AD [33], BDNF might play an intermediate role in the Meth-dependent sAPP α augmentation. Thirdly, because Meth induces glutamate release causing over activation of NMDAR and AMPAR receptors in the brain [34] and since synaptic NMDAR receptor activation stimulates α -secretase processing of β APP and inhibits A β production [35], it could be speculated that adjusting the dosage paradigm of Meth would be another channel to regulate and modulate NMDAR receptor activation. Finally, the key regulatory role of calcium in cognition, together with the Ca²⁺-dependent nature of α -secretase [36], might provide an additional route through which Meth could possibly influence β APP processing by α -secretase.

Although disruption of BACE1 has been recently shown to affect amphetamine-dependent dopaminergic signalling in the midbrain, an area implicated in schizophreniform behaviours [37], the present study is the first one to report an effect of Meth on BACE1 and the here described Meth-induced up-regulation of BACE1 expression could possibly be under the control of several molecular mechanisms. Firstly, it has been shown that chronic Meth administration increases α -synuclein protein levels in the hippocampus [38]. Since α -synuclein expression induces BACE1 protein levels [39], it could therefore be inferred that the high Meth-induced rise in BACE1 levels seen in our present study might use α -synuclein as an intermediate factor. Secondly, Meth administration can cause neurotoxicity and neurodegeneration via the up regulation and nuclear translocation of NF κ B [13] and by increasing GSK3 β and tau phosphorylation in a dose- and time-dependent manner [40], thereby triggering apoptosis in a GSK3 β -dependent manner [41]. Because NF κ B and GSK3 β have an impact on β APP metabolism via the modulation of BACE1 expression [11], it can therefore be

speculated that our observed effect of high Meth treatment on BACE1 might involve both NF κ B and GSK3 β . Thirdly, initially reported as the most instantaneous effect of moderate to high dose of Meth is the production of ROS and oxidative stress [42]. Considering this parameter, Meth-induced oxidative stress has been shown to lead to a dysfunctional processing of β APP in rat and human platelets in AD patients [43] with enhanced secretion of sAPP β [44] that further increases BACE1 processing of β APP via BACE1 gene activation [45]. Finally, it is well established that mitochondrial dysfunction affects β APP expression and processing as well as A β accumulation [46]. Moreover, some metabolic disturbances seen in AD likely arise from increased ER-mitochondrial communication that is driven by an increase in the levels of C99, the C-terminal processing product of β APP derived from its cleavage by β -secretase that is present in mitochondria-associated endoplasmic reticulum membranes [47]. In this context, the fact that Meth mediates ER stress leading to apoptosis [48] could provide another mechanism through which Meth could control the BACE1 cleavage of β APP.

Altogether, our data shed light on possible mechanisms through which Meth could convey both beneficial and deleterious effects regarding AD through differential effects on β APP processing, depending on the concentrations applied. Thus, one can first delineate a so-called “therapeutic” window (1-10 μ M) in which sAPP α production is at the pic of the bell-shape curve and BACE1 is not yet activated (Fig. 4, green area), thereby directing the metabolism of β APP towards its amyloidogenic pathway. Secondly, one enter an AD-promoting window in which higher Meth concentrations (100 μ M-1 mM) do not have an effect on sAPP α secretion anymore but dose-dependently increase BACE1 expression and catalytic activity, thereby shifting β APP processing toward the amyloidogenic pathway (Fig. 4, red area). It will now be of particular interest to determine whether Meth could also have an impact on γ -secretase.

Considering the possible therapeutic use of Meth in AD, it is worth noting that this compound has a relatively long half-life and crosses the blood brain barrier rapidly and that low doses of Meth produce very few side effects and the fact that Meth is metabolized to amphetamine, further prolongs its activity in the brain [49, 50]. For these reasons, Meth has significant potential as a neuroprotective agent and could therefore be possibly used as a substitute to manipulate the cognitive outcomes in AD patients under strict supervision. In order to further characterize the pharmacology of Meth as a potential anti-AD agent and define possible parameters of clinical application, the precise therapeutic window in which it would be possible to intervene should now be thoroughly examined *in vivo* in animal models of the disease.

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Legends to figures

Figure 1: Effect of methamphetamine on the α -secretase processing of β APP in cultured human SH-SY5Y neuroblastoma cells. Cultured wild-type human SH-SY5Y neuroblastoma cells were incubated without (control, white bars) or with the indicated concentrations of methamphetamine (in DMEM containing 1% FBS) for 16 hours at 37°C. Cells were then allowed to secrete for 3 h (in 1 ml serum-free DMEM). Media and cell lysates were then collected and sAPP α (media) as well as β APP and β -actin (lysates) were analyzed by Western blot as described in Materials and Methods. Representative gels as well as the statistical analysis for all experiments are shown. Bars correspond to the densitometric analyses (total β APP (mature + immature) being normalized with β -actin), are expressed in arbitrary units (white bars, non-treated cells) taken as 100, and are the means \pm SE of 4 independent determinations. * $p < 0.005$; ** $p < 0.0005$; ns, non-statistically different.

Figure 2: Effect of methamphetamine on α -secretase catalytic activity and ADAM10 expression. Cultured wild-type human SH-SY5Y neuroblastoma cells were treated without

(control) or with the indicated concentrations of methamphetamine for 16 h at 37°C. (A) Cells were then incubated in PBS with or without GI254023X (10 μ M) for 30 min. The enzymatic reaction was initiated by adding the JMV2770 fluorimetric substrate (10 μ M) to all wells. Media (100 μ l) were collected at the indicated times and fluorescence was recorded. (B) Total RNA was extracted and ADAM10 as well as GAPDH mRNA levels were measured. (C) ADAM10 protein amounts were assessed by Western blot. Bars in graphs correspond to the fluorescence expressed as the percentage of control (untreated cells, white bar) for the time period indicated (grey area on the curve graph) (A), ADAM10 mRNA levels normalized with GAPDH (B) or ADAM10 immunoreactivities normalized with β -actin (C), are expressed as a percentage of control (untreated cells, white bar) and represent the means \pm SE of 6 (A), 4 (B) or 3 (C) independent determinations. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; # $p < 0.0001$; ns, non-statistically different.

Figure 3: Effect of methamphetamine on β -secretase catalytic activity and BACE1 expression. Cultured wild-type human SH-SY5Y neuroblastoma cells were treated without (control) or with the indicated concentrations of methamphetamine for 16 h at 37°C. (A) Cells were collected, homogenized and 30 μ g of proteins were assayed for their JMV2236-sensitive JMV2236-hydrolyzing β -secretase activity. (B) Total RNA was extracted and BACE1 as well as GAPDH mRNA levels were measured. (C) BACE1 protein amounts were assessed by Western blot. Bars in graphs correspond to the fluorescence expressed as the percentage of control (untreated cells, white bar) for the time period indicated (grey area on the curve graph) (A), to BACE1 mRNA levels normalized with GAPDH (B) or to BACE1 immunoreactivities normalized with β -actin (C), are expressed as a percentage of control (untreated cells, white bar) (B) and represent the means \pm SE of 6 (A), 4 (B) or 7 (C) independent determinations.

* p<0.05; ** p<0.02; *** p<0.01; & p<0.001; # p<0.0001; ns, non-statistically different.

Figure 4: Schematic representation of the concentration-dependent beneficial and deleterious effects of methamphetamine on β APP processing. This diagram illustrates the two antagonistic windows of action of methamphetamine regarding β APP processing by the nonamyloidogenic α -secretase and the amyloidogenic β -secretase activities. On one hand is the low-dose (1-10 μ M)-associated putative therapeutic window of Meth due to stimulation of ADAM10 expression leading to an increased production of the neurotrophic, neuroprotective, and memory-enhancing and neurogenesis-stimulating sAPP α fragment (green). On the other hand is the AD-promoting window (100 μ M-1 mM), which might correspond to drug abusing condition and that no longer augments sAPP α production but instead increases BACE1 expression and activity (red).

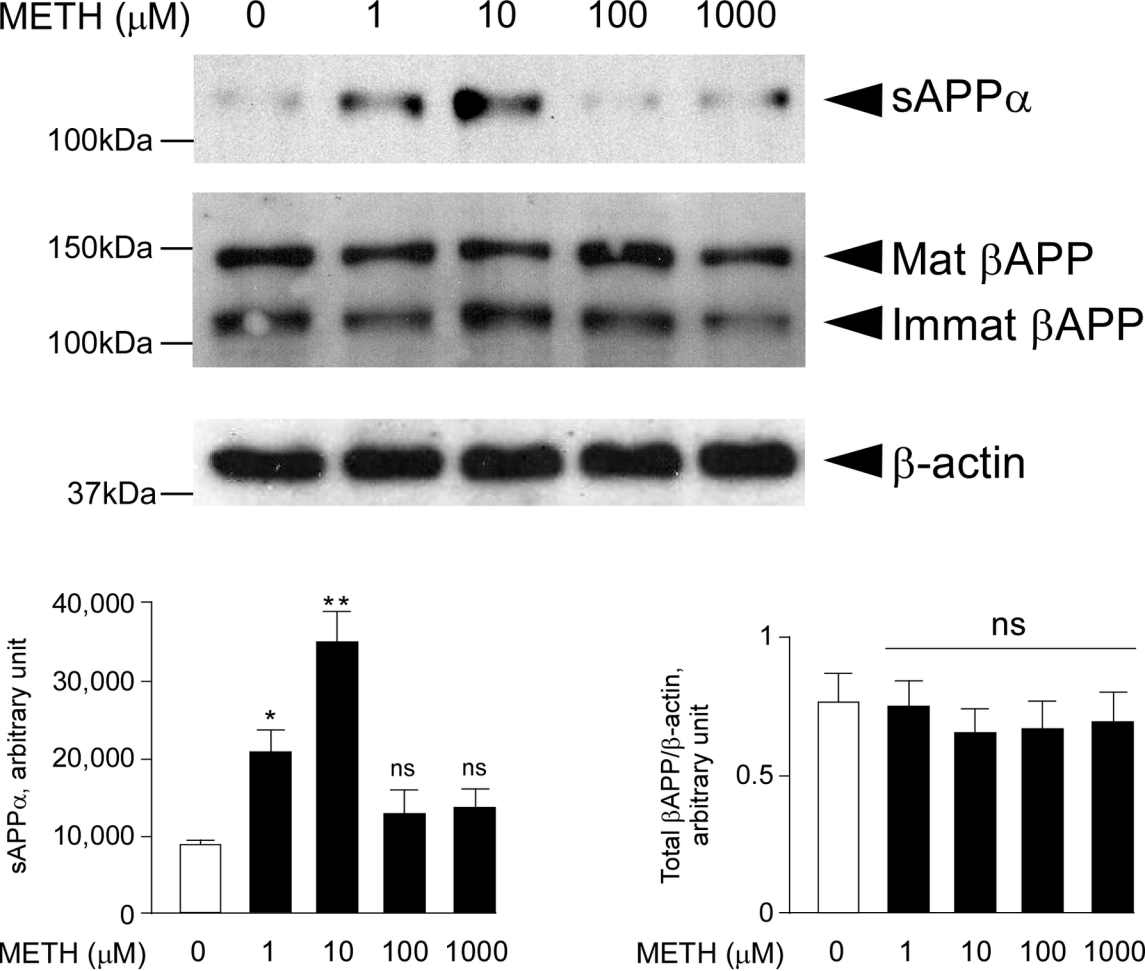
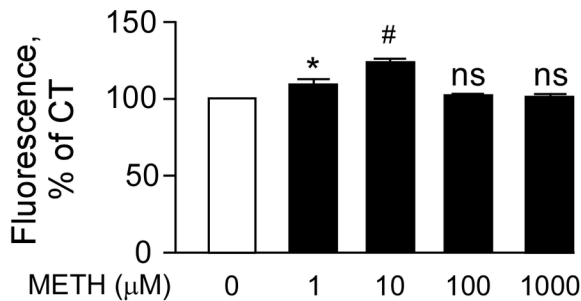
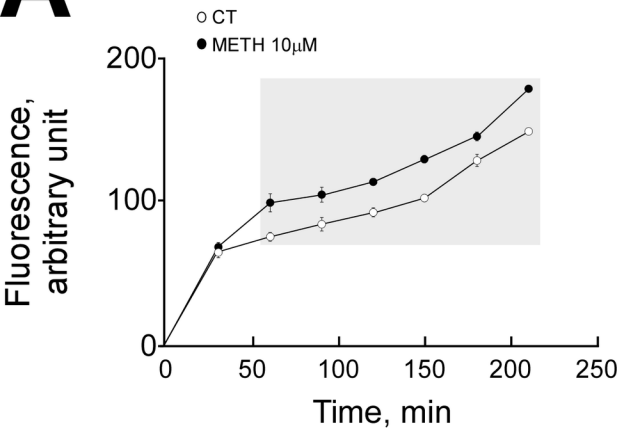
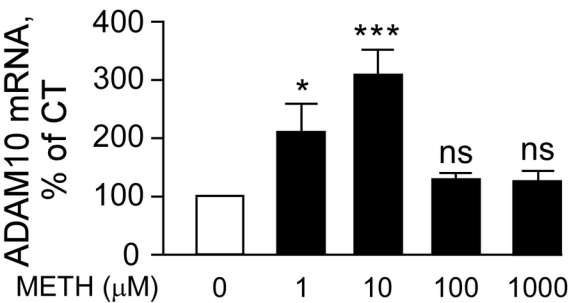
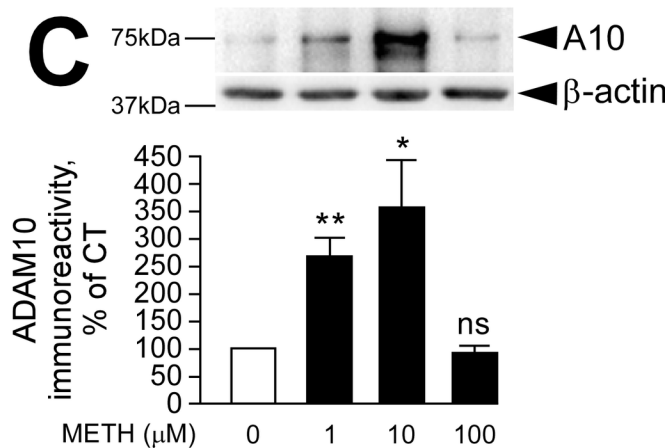


Figure 1

A**B****C****Figure 2**

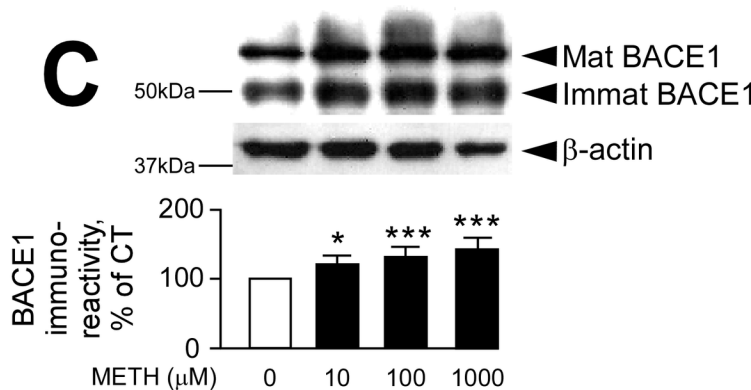
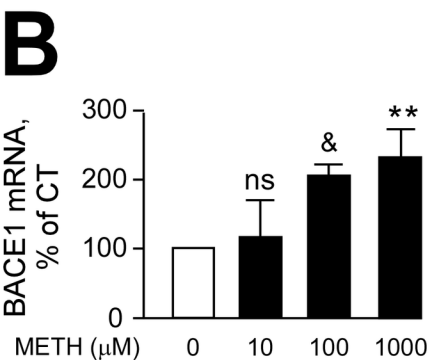
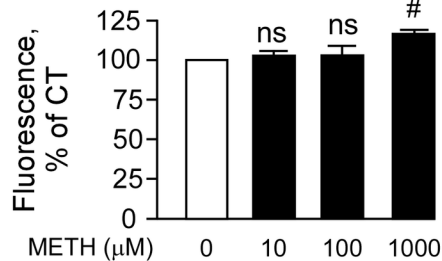
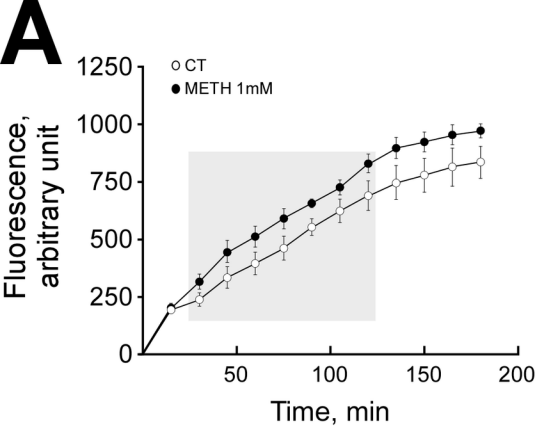


Figure 3

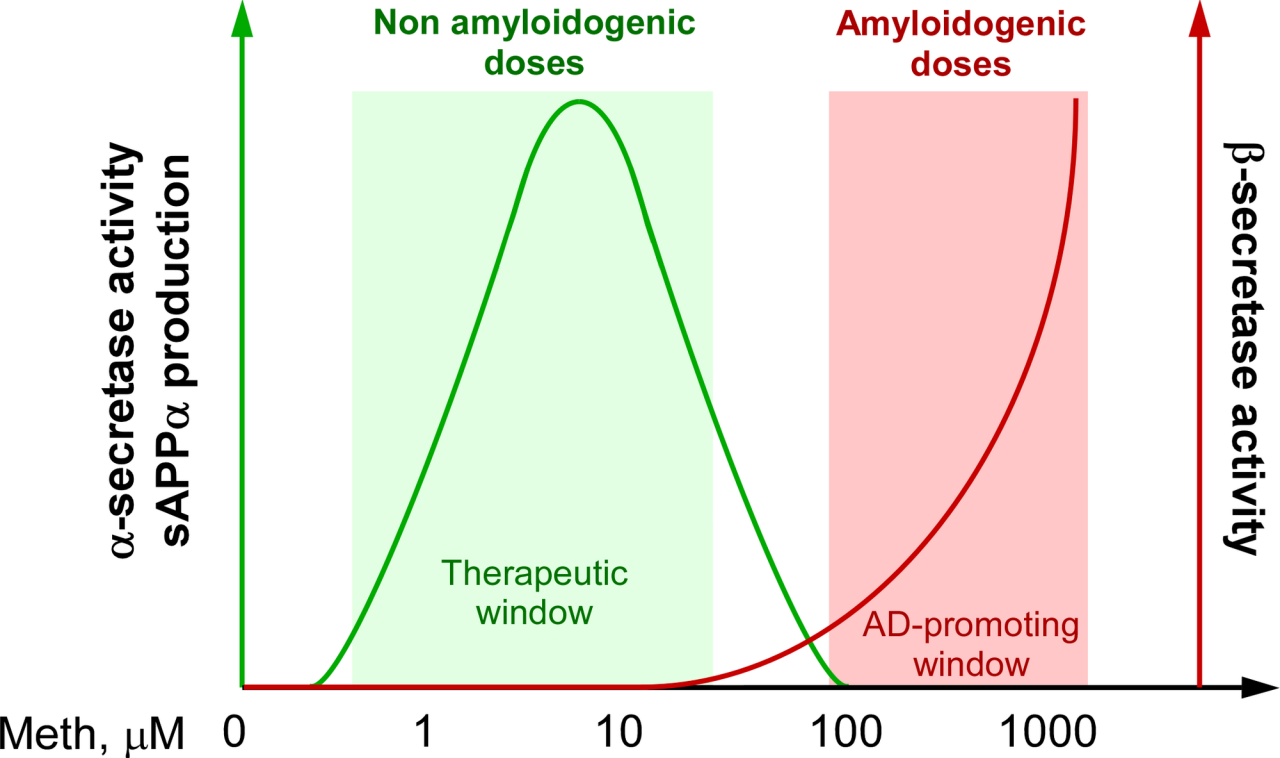


Figure 4