

Specific triazine herbicides induce amyloid β 42 production

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Running title: Triazine herbicides induce A β ₄₂ production

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ABSTRACT

Proteolytic cleavage of the amyloid precursor protein (A β PP) by secretases leads to extracellular release of amyloid β (A β) peptides. Increased production of A β ₄₂ over A β ₄₀ and aggregation into oligomers and plaques constitute an Alzheimer's disease (AD) hallmark. Identifying products of the 'human chemical exposome' (HCE) able to induce A β ₄₂ production may be a key to understand some of the initiating causes of AD and to generate non-genetic, chemically-induced AD animal models. A cell model was used to screen HCE libraries for A β ₄₂ inducers. Out of 3500+ compounds, six triazine herbicides were found that induced a β - and γ -secretases -dependent, 2-10 fold increase in the production of extracellular A β ₄₂ in various cell lines, primary neuronal cells and neurons differentiated from human induced pluripotent stem cells (iPSCs). Immunoprecipitation/mass spectrometry analyses show enhanced production of A β peptides cleaved at positions 42/43, and reduced production of peptides cleaved at positions 38 and lower, a characteristic of AD. Neurons derived from iPSCs obtained from a familial AD (FAD) patient (A β PP K724N) produced more A β ₄₂ vs. A β ₄₀ than neurons derived from healthy controls iPSCs (A β PP WT). Triazines enhanced A β ₄₂ production in both control and AD neurons. Triazines also shifted the cleavage pattern of alcadein α , another γ -secretase substrate, suggesting a direct effect of triazines on γ -secretase activity. In conclusion, several widely used triazines enhance the production of toxic, aggregation prone A β ₄₂/A β ₄₃ amyloids, suggesting the possible existence of environmental "Alzheimerogens" which may contribute to the initiation and propagation of the amyloidogenic process in late-onset AD.

INTRODUCTION

Proteolytic processing of A β PP by β - and γ -secretases leads to the production of various A β peptides, including the 42-amino acid form (A β ₄₂) which plays a crucial role in AD [1-3]. The action of β -secretase first leads to a soluble fragment (sA β PP) and a membrane-bound fragment (β CTF, β -carboxyl-terminal fragment). γ -Secretase then cleaves

the β CTF, leading to the generation of A β peptides of various lengths. A β peptides tend to aggregate as extracellular oligomers and ultimately as plaques, one of the clinical hallmarks of AD.

A β ₄₀ is the most abundantly produced A β peptide. Considerable data indicates that generation of the aggregation-prone A β ₄₂ strongly correlates with the onset and development of AD. In early-onset AD (EOAD) (<1% of all cases), mutations in A β PP, or the γ -secretase subunits PSEN1 & PSEN2 [review in 4], all lead to enhanced A β ₄₂ production and/or increased A β ₄₂/A β ₄₀ ratio, a critical factor in AD pathology initiation [5]. Increased A β ₄₂/A β ₄₀ ratio is also found in brain tissue in late-onset AD (LOAD) (>99% of AD cases). A β ₄₂ is more toxic than A β ₄₀, a consequence of its higher stability and strong tendency to oligomerize and to aggregate in plaques [6-8]. A β ₄₃ is also enriched in the brain of AD patients and has been reported as a toxic, aggregation-prone amyloid, inducing strong AD phenotypes in mice [9-12].

We recently reported that some tri-substituted purines, the affins (**A**myloid **β** **F**orty-**T**wo **I**nducers), trigger a robust, secretases-dependent increase in extracellular A β ₄₂ production in cultured cells [13-14]. Under these conditions A β ₃₈ levels dropped while A β ₄₀ remained relatively stable. These results suggest that (i) such molecules might constitute new pharmacological tools to investigate the mechanisms underlying the increased A β ₄₂/A β ₄₀ ratio observed in AD, (ii) these molecules might contribute to generate a chemically induced animal model of AD [15] and (iii) some simple, low molecular weight (LMW) products in our environment might shift the A β ₄₂/A β ₄₀ ratio similarly to what is seen in AD patients and might thus contribute to the development, acceleration or even initiation of LOAD.

We therefore screened for potential A β ₄₂-inducing molecules in libraries of HCE products [16-21]. We here report that a subset of the widely used triazine herbicides is able to shift A β production towards longer, aggregation-prone amyloid peptides (A β ₄₂/A β ₄₃) at the expense of shorter variants (A β ₃₇, A β ₃₈, A β ₄₀). In addition, production of the shorter A β ₁₋₁₆ and A β ₁₋₁₇ peptides, the generation of which is dependent on β - and γ -secretase activities [22, 23], was also enhanced. This effect is observed in various cell lines, primary neuron cultures and neurons differentiated from iPSCs obtained from healthy controls or AD patients. Triazines shift the cleavage pattern of alcadeins α , another γ -secretase substrate [24-28], in a way similar to the A β PP cleavage shift, suggesting a direct effect on γ -secretase rather than on its substrates. Altogether these data support our hypothesis that the HCE contains products able to modulate γ -secretase activity towards the production of high MW, aggregation-prone, AD-associated amyloids. Such products could be qualified as potential “Alzheimerogens”

(name by analogy with “carcinogens”). Their identification and regulation might constitute a key step in AD prevention.

MATERIAL & METHODS

Material and methods are described in full in the SI section. They include:

1. Triazines and other reagents.
2. Cell cultures: cell lines, primary neuron cultures, human iPSCs-derived neurons & HEK293-alcadeina cells.
3. Transient transfections with A β PP truncation mutants.
4. Amyloids assays: ELISA capture assays, absolute quantification of amyloids by tandem mass spectrometry (MS/MS), amyloids profile analysis by immunoprecipitation/mass spectrometry (IP-MS).
5. Cell viability assay.

RESULTS

Screening the HCE reveals triazines as A β ₄₂ inducers

A library of 3500+ LMW (<1000 daltons) products representative of the HCE was assembled. All compounds were tested for their ability to trigger extracellular A β ₄₂ production by N2a cells stably expressing A β PP695 (N2a-APP695), at 1, 10 and 100 μ M (data not shown. A dose-dependent effect was often seen with the active compounds). In parallel, cell viability assays were run to assess cell survival at these concentrations. The vast majority of products were unable to induce A β ₄₂ production. Among the few active products, we identified several triazines, a class of products which are widely used as herbicides, anti-fouling agents or flame retardants [reviews in 29]. We next tested a library of 37 triazines representing the most produced triazines worldwide (**1-37**, SI, Table S1), along with Aftin-5 (**38**) as a positive control, on both N2a-APP695 and CHO-7PA2 cells stably expressing A β PP751 (CHO-7PA2-APP751), for their ability to trigger A β ₄₂ production at 1, 10 and 100 μ M (SI, Table S2) (Fig. 1A).

Six triazines were found to induce more than a 3-fold change in A β ₄₂ levels (Fig. 1A, 1B): ametryn, prometryn, dipropetryn, terbutryn, cybutryne, dimethametryn. As observed with aftins [13, 14], A β ₄₂ production was strongly inhibited by β - (inhibitor IV) and γ -secretases (BMS 299897, DAPT) inhibitors and by a γ -secretase modulator (‘Torrey Pines’ compound) (the latter induces a dose-dependent increase in A β ₃₈ and a decrease in A β ₄₂ and A β ₄₀) [30, 31] (Fig. 1C). Similarly, A β ₃₈ production was strongly reduced, while A β ₄₀ levels

were only modestly affected as measured by ELISA (data not shown) and by mass spectrometry (Fig. 2). Most of the triazines are metabolized in the environment and by organisms. We thus tested some of the cybutryne/terbutryn metabolites (**39-44**) (SI, Fig. S1) for their ability to trigger A β ₄₂ production in N2a-APP695 and CHO-7PA2 cells. None of the tested metabolites was active as an inducer of A β ₄₂ production (data not shown). We next tested a library of 236 triazines that had been synthesized as affinity chromatography reagents, for their ability to induce A β ₄₂ production [32, 33]. Twenty-one of these (**45-65**) showed significant enhancement of A β ₄₂ production (SI, Table S3), showing that A β ₄₂ induction is an intrinsic property of some triazines. Affinity chromatography attempts with immobilized triazines did not allow us to purify specific targets, probably because of unselective hydrophobic interactions and low level expression of the specific targets (data not shown).

Results were confirmed with HEK293 stably expressing A β PPsw (data not shown) and neurons derived from human iPSCs (see below). We also analyzed the effects of triazines on primary neuronal cultures prepared from E18 OFA rat embryo brains. Neurons were exposed to 100 μ M of each triazine for 18 h, and the supernatants were collected for A β determination by ELISA assays. The triazines also induce an increase in A β ₄₂ production by primary neurons and in the A β ₄₂/A β ₄₀ ratios (Fig. 1D). A β ₄₀ production remained relatively stable, while that of A β ₃₈ was reduced by all triazines (Fig. 1D).

Mass spectrometric quantification and profile analysis of induced amyloids

A β ₃₈, A β ₄₀ and A β ₄₂ were quantified in the supernatants of N2a-APP695 (Fig. 2A) and CHO-7PA2-APP751 (Fig. 2B) using LC-MS/MS [34, 35]. Like Aftins [13, 14], triazines induced a reduction in A β ₃₈ levels, a slight increase or modest decrease in A β ₄₀ levels and a strong increase in A β ₄₂ levels (Fig. 2, bottom). A β ₄₂/A β ₄₀ ratios were strongly increased (Fig. 2, top).

We next analyzed, by IP-MS, the range of A β peptides produced by both cell lines exposed to each of the six triazines and aftin-5. Cell supernatants were collected and A β peptides were immunoprecipitated and analyzed using MALDI TOF/TOF [36]. Examples of spectra for cells exposed to terbutryn, aftin-5 and DMSO are provided in Fig. 3 and Fig. S2 (SI). Exposure to triazines increased the production of A β ₁₋₁₇, A β ₁₁₋₄₂, A β ₅₋₄₂ and A β ₁₋₄₂, while the production of A β ₁₋₁₉, A β ₁₋₂₇, A β ₁₋₃₃, A β ₁₋₃₈ and A β ₁₋₃₉ was reduced (Fig. 3). Other amyloid peptides (including A β ₁₋₄₀) showed only modest changes. A β ₁₋₄₃, a highly neurotoxic amyloid

[9-12], was undetectable in supernatants of control cells, but strongly induced in aftin-5 and triazine-treated cells.

Neurons differentiated from human iPSCs of AD patient and healthy control

We next tested the effects of aftin-5 and the active triazines on neurons differentiated from human iPSCs derived from a healthy individual (A β PP WT, wild-type) or from a patient with familial AD (A β PP K724N mutation) [37, 38] (Fig. 4). Neurons from the healthy control were first differentiated for either 4 or 10 weeks before 24 h exposure to 100 μ M aftin-5 or terbutryn (Fig. 4A). Treatment resulted in a 2-3 fold increase in A β ₄₂ levels compared to neurons exposed to DMSO. A β ₄₀ levels remained essentially unchanged. All six triazines were next tested on neurons derived from the control and the AD patient differentiated for 4 weeks [38, 39] (Fig. 4B). A β PP K724N neurons produced more A β ₄₂ versus A β ₄₀ compared to A β PP WT neurons. Addition of aftin-5 or any of the six active triazines resulted in further increase in A β ₄₂ production, in both A β PP WT and A β PP K724N neurons.

A β PP sequence requirements for A β ₄₂ induction by triazines

To investigate the molecular mechanisms and possible ϵ cleavage sites requirement for the induced A β ₄₂ production, we generated six A β PP truncations (T1-T6) and expressed them transiently in N2a cells (Fig. 5A). Cells expressing these truncations were then exposed to 100 μ M aftin-5 and A β ₄₂ production was measured (Fig. 5B). Full-length (FL) A β PP and the first three truncations displayed enhanced A β ₄₂ production (Fig. 5B). In contrast, the three remaining truncations did not allow enhanced A β ₄₂ production when cells were exposed to aftin-5. Cells expressing FL A β PP and truncations T1, T3, T4 were next exposed to 100 μ M of each triazine (Fig. 5C). Although T3 allowed stimulation of A β ₄₂ production, T4 did not. These results reveal a strong A β PP structural requirement for enhanced A β ₄₂ production induced by aftin-5 and triazines, which seems to correspond to the ϵ cleavage sites of A β PP by γ -secretase. At least 10 residues downstream of the A β ₄₂ cleavage site are required for the full effect of aftin-5 and triazines.

Triazines and Aftin-5 shift the cleavage pattern of the γ -secretase substrates alcadeins

Like A β PP, alcadeins/calsyntenins are sequentially cleaved by secretases, first by α -secretase, leading to N- and C-terminal fragments, the latter being then cleaved by γ -secretase to an intracellular domain and the p3-Alcs peptide, in a way similar to A β PP [25, 27] (Fig. 6A) (SI, Fig. S3). We used HEK293 cells stably expressing full length alcadein α to

investigate the effects of triazines on alcadein cleavage. Alcadein α is first cleaved on the N-terminal side (two possible sites) followed by cleavage by γ -secretase leading to p3-Alc α 35 and p3-Alc α 2N+35, the latter representing the major peptide in cultured cells (Fig. 6A). Cleavage at nearby sites (blue arrows) leads to other less abundant peptides. HEK293-alcadein α cells were grown till 60% confluence and treated with 100 μ M aftin-5 or triazines for 24 h. The secreted p3-Alc α peptides were recovered and analyzed by MALDI TOF MS (Fig. 6B).

Quantification of p3-Alc α peptides showed that, compared to the p3-Alc α peptide profile in vehicle treated cells, concentrations of the main alcadein α peptide (p3-Alc α 2N+35) and p3-Alc α 2N+37 peptide remained stable. In contrast, both p3-Alc α 2N+34 and p3-Alc α 2N+36 concentrations dropped by ~50% and p3-Alc α 2N+38 peptide concentration increased massively (up to 28.1 fold for dimethametryn; 16.8 fold for Aftin-5) (Fig. 6C). These results show that, like for A β PP, triazines and aftin induce a shift in the cleavage pattern of Alcadein α , another γ -secretase substrate, suggesting that these products are more likely to interact with γ -secretase rather than with its substrates.

DISCUSSION

Induction of A β ₄₂ production, increase in A β ₄₂/A β ₄₀ ratio

Various drugs (fenofibrate, celecoxib, indomethacin, isoprenoids) [40], DAPT under certain conditions [41, 42], steroids [43], ceramide analogs [44], and SIN-1 (a peroxynitrite donor) [45] have been shown to increase the A β ₄₂/A β ₄₀ ratio, mostly by increasing A β ₄₂ production, though never to the high level seen with aftins [13, 14]. We here show that some, but not all, widely used (though mostly banned nowadays) triazine herbicides induce a massive production of the AD-associated A β ₄₂ peptide in a variety of cell types. Consequently, the A β ₄₂/A β ₄₀ ratio is increased, as observed in both familial EOAD (genetic origin) and LOAD (environmental, epigenetic origin). Detailed analysis of a variety of A β peptides reveals a pattern clearly associated with AD onset, such as increased A β _{1-16/17} [22, 23], A β _{1/5/11-42}, A β ₁₋₄₃ [9-12], and decreased A β _{1-33/37/38} which have been shown previously to be affected in EOAD subjects [46, 47]. While the underlying molecular mechanisms for the observed chemically-induced shift of A β PP cleavage pattern remain unclear, several remarks can be made:

(1) there is a clear structure/activity relationship within triazines, as also observed with aftins: not all products of the chemical class are active. This suggests specific molecular interactions

rather than unspecific effects such as detergent, hydrophobic, membrane or protein structure disrupting actions.

(2) the mechanism of action is more likely to involve an effect on γ -secretase and/or its micro-environment rather than an interaction with its substrates, as shown by the fact that aftins and triazines also induce a shift in the cleavage pattern of alcadeins, another γ -secretase substrate. The A β PP truncation experiments clearly suggest a very specific molecular requirement rather than a global, non-selective effect.

(3) despite extensive proteomics studies (data not shown), we were unable to detect major/significant modifications of protein expression that might be linked to the A β PP cleavage shift induced by triazines, suggesting that RNA or protein synthesis alterations are unlikely involved in the induction of A β ₄₂ production. We were also unable to identify a specific target of triazines through affinity chromatography/proteomics approaches, suggesting that either the lipid raft comprising the γ -secretase or rather hydrophobic domains of γ -secretase might constitute the real targets of triazines (and aftins).

“Alzheimerogens” in the HCE?

The Chemical Abstracts Service (CAS) registry, the world’s largest chemical database, contains more than 101 million organic and non-organic substances. About 15,000 novel substances are registered every day (on average one new substance every 2.5 min. since 50 years!) (www.cas.org). The US EPA Toxic Substances Control Act lists over 84,000 chemicals that are manufactured or imported at levels >10 tons per year, not including pesticides, cosmetics, food stuffs and food additives which are covered by other legislations (www.epa.gov). It is estimated that man is exposed to over 85,000 products. The European REACH initiative aims at regulating all products which are produced/imported at >100 tons/year (>1 ton/year by May 2018). All these products, along with all natural substances to which we are exposed from conception to death, constitute the HCE [17, 18, 48, 49].

The impact of environment on health has been known since antiquity. It is therefore no surprise that a small number of products may enter the human body, cross the blood brain barrier (BBB), alter specific molecular pathways in some of the human brain 10¹¹ neurons and 10¹² glia cells and thereby induce or contribute to specific diseases affecting the central nervous system. Identification of environmental factors involved in neurodegeneration and neurodegenerative diseases is surprisingly still in its infancy [reviews in 50-54]. The nervous system may be exposed to neurotoxic agents acutely (hours, days) or chronically (weeks, years, decades) before disease symptoms appear. Epidemiology studies are particularly

difficult for neurodegenerative diseases since causes and effects are often separated by decades. These studies have, therefore, provided only few examples of environmental agents linked to the onset of neurodegenerative diseases. Pesticides, organic solvents, metals and some natural toxins (cyanobacteria) constitute the most frequently proposed neurotoxic agents. Two recently published books [55, 56] review the impact of early age and even *in utero* exposure to environmental chemical entities on brain development and cognitive abilities.

AD is one of the most prevalent and worrisome neurodegenerative diseases [57]. EOAD is clearly a genetic disease due to specific A β PP or PSEN1/2 mutations leading to overproduction of A β ₄₂ over A β ₄₀. The origin of LOAD (sporadic AD) (>99% of all AD cases) remains a mystery which epidemiological or genome-wide association studies have not solved, the latter having only revealed a few low impact genetic risk factors [58]. The most prominent risk alleles, ApoE ϵ 4 and clusterin/ApoI, link AD to lipid metabolism, and aging, together with several environmental factors, also impose an increased risk. Exposure to numerous industrial and agricultural chemicals correlates with neurotoxicity [50, 51, 52, 53, 54, 59, 60]. Elevated serum pesticides levels, in particular DDE, the major DDT metabolite, are associated with increased risk for AD [61]. DDT increases A β levels [61]. There are epidemiological links between exposure to pesticides and AD [63].

Continuous sub-cutaneous injection of aftin-5 in mice triggers robust dose-dependent increase in brain A β ₄₂ levels (unpublished data). Similar results were obtained with aftin-4 [15] and celecoxib or FT-1 [40]. Although orally administered triazines readily cross the BBB, their short half-life in mice prevented any accumulation *in vivo* (data not shown).

Based on results obtained with products belonging to various chemical classes, we can suspect the presence, in the HCE, of products able to increase the production of the AD-associated A β ₄₂/A β ₄₃ peptides. Such products might be classified as potential “Alzheimerogens” if long-term exposure, slow turn-over, low elimination and BBB permeability allow accumulation in the brain and long-term action on brain cells. It is difficult to predict whether long-term, daily exposures of humans to the widely used triazines described here might have resulted in sustained increase in A β ₄₂ production. We are now investigating other A β ₄₂ inducers which have a longer half-life both in the environment and in man, which accumulate in adipose tissues and which cross the BBB. We believe that such products may contribute to the onset, development and acceleration of sporadic LOAD. It is intriguing that both aftin-5 and triazines were able to stimulate A β ₄₂ production in human cells displaying a pathological A β PP mutation and already showing enhanced A β ₄₂

production. This suggests that environmental factors may synergize with genetic/epigenetic factors in enhancing A β ₄₂ production and triggering AD. Identification of such potential “Alzheimerogens” in the HCE and regulation of human exposure to them should open the way to innovative AD prevention strategies. In addition, some of these products might be turned into pharmacological tools to develop a chemically-induced animal model of AD, with a fundamental and applied potential similar to the MPTP-induced Parkinsonism model [64].

COMPETING INTERESTS

The authors declare that they have no competing interests.

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FIGURE LEGENDS

Fig. 1. Some triazines trigger β - and γ -secretase dependent production of extracellular A β ₄₂. **A.** Effect of 37 triazines on extracellular A β ₄₂ production by N2a-APP695 and CHO-7PA2-APPsw cells. Cells were treated with 100 μ M of each compound for 18 h and cell supernatants were collected for A β ₄₂ levels measurement by ELISA. Aftin-5 was used as a positive control and the corresponding volume of DMSO as a negative control. Levels are expressed as fold change, average \pm SEM of triplicate values, of A β ₄₂ levels over those of control, vehicle-treated cells.(representative of four independent experiments). Horizontal dotted lines indicate levels for 1 and 3 fold changes in A β ₄₂ concentration. **B.** Structure of the six active triazines and of aftin-5. **C.** Extracellular A β ₄₂ production induced by triazines is inhibited by β -secretase inhibitor IV, γ -secretase inhibitors DAPT & BMS 299897 and γ -secretase modulator 'Torrey Pines' compound. N2a-APP695 cells were exposed to 10 μ M of each inhibitor. 1.5 h later, cells were treated with 100 μ M of each active triazine or 50 μ M aftin-5. Extracellular A β ₄₂ levels were measured after 18 h. Average \pm SEM of triplicate values (representative of two independent experiments). **D.** Triazines trigger A β ₄₂ production in primary rat neuron cultures. Cells were exposed to DMSO, 100 μ M of each triazine or aftin-5 for 18 h. Cell supernatants were collected and the levels of A β ₃₈, A β ₄₀ and A β ₄₂ (bottom panel) were determined by ELISA assays (average \pm SEM of triplicate values). The A β ₄₂/A β ₄₀ ratios are shown in the top panel. The horizontal dotted line refers to the basal ratio in control cells.

Fig. 2. Absolute quantification of A β ₃₈, A β ₄₀ and A β ₄₂ using LC-MS/MS. Levels of the three amyloid peptides were determined by mass spectrometry in supernatants of N2a-APP695 (**A**) and CHO-7PA2-APPsw (**B**) cells following 18 h treatment with DMSO, 100 μ M of each triazine or aftin-5. Amyloid levels are expressed as percentage of levels in vehicle-treated cells (bottom panels; average \pm SEM of triplicate values; absolute values in control cell supernatants are indicated under the bottom panels). A β ₄₂/A β ₄₀ ratios are shown in the top panels (horizontal dotted lines refer to the basal ratios in control cells).

Fig. 3. Pattern of amyloid peptides produced by CHO-7PA2-APPsw cells exposed to triazines. Cells were treated for 18 h with DMSO, 100 μ M of each triazine or aftin-5. Cell supernatants were collected and analyzed as described. **A.** Example spectra of supernatants amyloid profiles from CHO-7PA2-APPsw cells exposed to DMSO, aftin-5 or terbutryn. **B.**

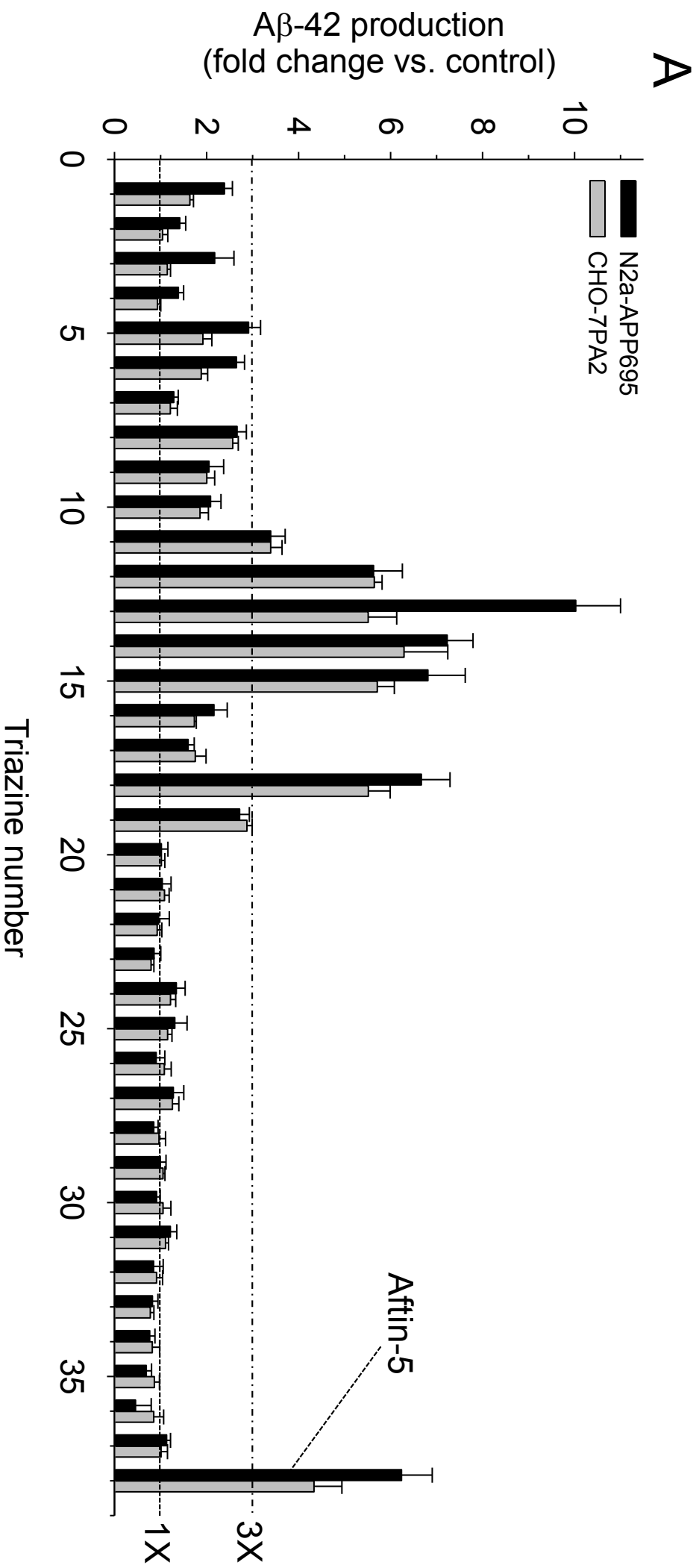
Quantification of all amyloid peptides in CHO-7PA2-APPsw cell supernatants (Log of fold change in triazine or aftin-5 treated cells over control, DMSO-treated cells).

Fig. 4. Triazines trigger enhanced production of A β ₄₂ versus A β ₄₀ in neurons differentiated from human iPSCs. **A.** iPSCs-derived neurons were differentiated for 4 or 10 weeks and then exposed to DMSO or 100 μ M aftin-5 or terbutryn (**14**) for 24 h. **B.** Neurons were derived from iPSCs obtained from a healthy donor (A β PP WT) or from an AD patient (A β PP K724N mutation). They were exposed for 24 h to DMSO, 100 μ M aftin-5 or the six triazines. In both experiments cell supernatants were collected for extracellular A β levels measurement. Levels are expressed as A β ₄₂/A β ₄₀ ratios \pm SEM of triplicate values.

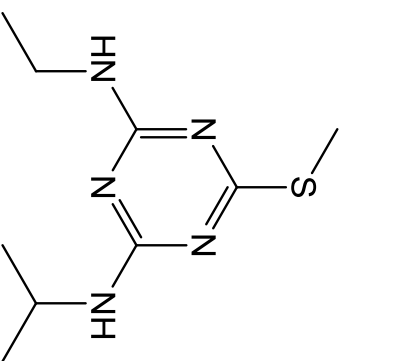
Fig. 5. Effect of A β PP C-terminal truncations on triazines' efficacy. **A.** Only the C-terminal aa sequences of A β PP full length (WT) and C-terminal truncations mutants (T1-T6) are shown. The γ and ϵ cleavage sites are indicated in orange and blue respectively. Numbers indicate the position of the residues involved in those cleavages and refer to the α cleavage site. **B.** Mutants T1 to T6 were expressed transiently in N2a cells which were exposed to DMSO or aftin-5 (100 μ M) for 24 h and the levels of released A β ₄₂ was measured by ELISA (average \pm SEM of triplicate values). **C.** Mutants T1, T3 and T4 expressing N2a cells were exposed for 24 h to DMSO (D), aftin-5 or the six triazines (100 μ M). A β ₄₂ level were measured and are expressed as fold-increase vs. untreated cells (average \pm SEM of triplicate values).

Fig. 6. Triazines alter the cleavage pattern of Alcadein α , leading to increased p3-Alc α 38 production. **A.** Schematic representation of the production of p3-Alc α peptides from Alcadein α . The full length protein is cleaved primarily by α -secretase at His814 or Ala816 (purple arrows). It is then cleaved by γ -secretase at Thr851 (orange arrow) leading to the two main Alcadein α peptides p3-Alc α 35 and p3-Alc α 2N+35 ('2N' denotes the two additional, N-terminal amino acids). Alternative cleavage sites (blue arrows) generate additional p3-Alc α peptides of different sizes. **B.** Immunoprecipitation/mass spectrometry spectra of p3-Alc α peptides produced by HEK-Alcadin α cells exposed to various triazines, aftin-5 or DMSO. Cells were treated for 24 h with 100 μ M of each reagent and p3-Alc peptides were analyzed by MALDI-TOF/MS. Representative profiles for each product (top) and zoom on the p3-Alc α 2N+34, p3-Alc α 2N+35 and p3-Alc α 2N+38 peaks (bottom). **C.** Relative quantification of p3-Alc α peptides produced by cells exposed to all triazines and aftin-5. Levels of each peptide

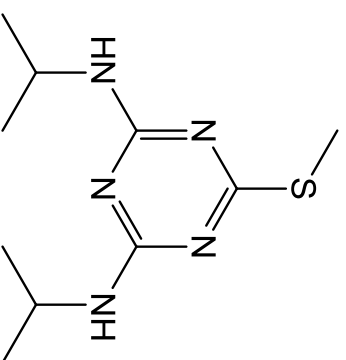
are presented as fold change of ratios over p3-Alc α 2N+35 versus corresponding peptide ratios for DMSO-treated cells (average \pm SEM of triplicate values). Horizontal dotted lines indicate levels for 1 fold change in p3-Alc α /p3-Alc α 35 ratio in treated vs. control cell supernatant. Note the change of scale for p3-Alc α 2N+38/p3-Alc α 2N+35 treated/control ratio.



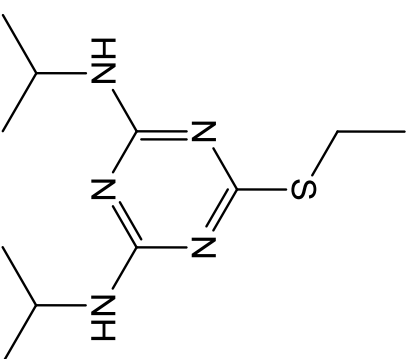
B



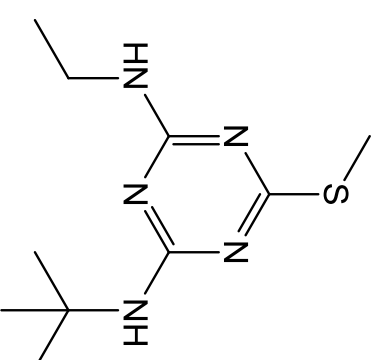
Ametryn (11)



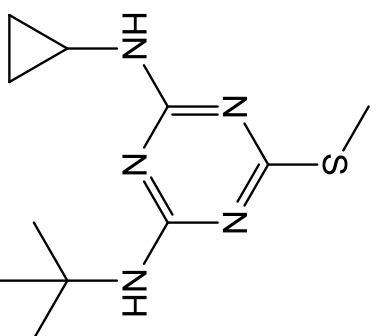
Prometryn (12)



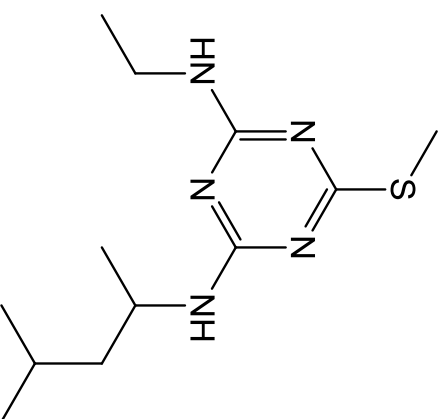
Dipropetryn (13)



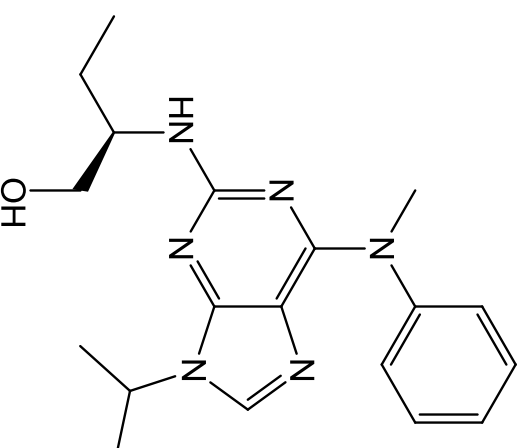
Terbutryn (14)



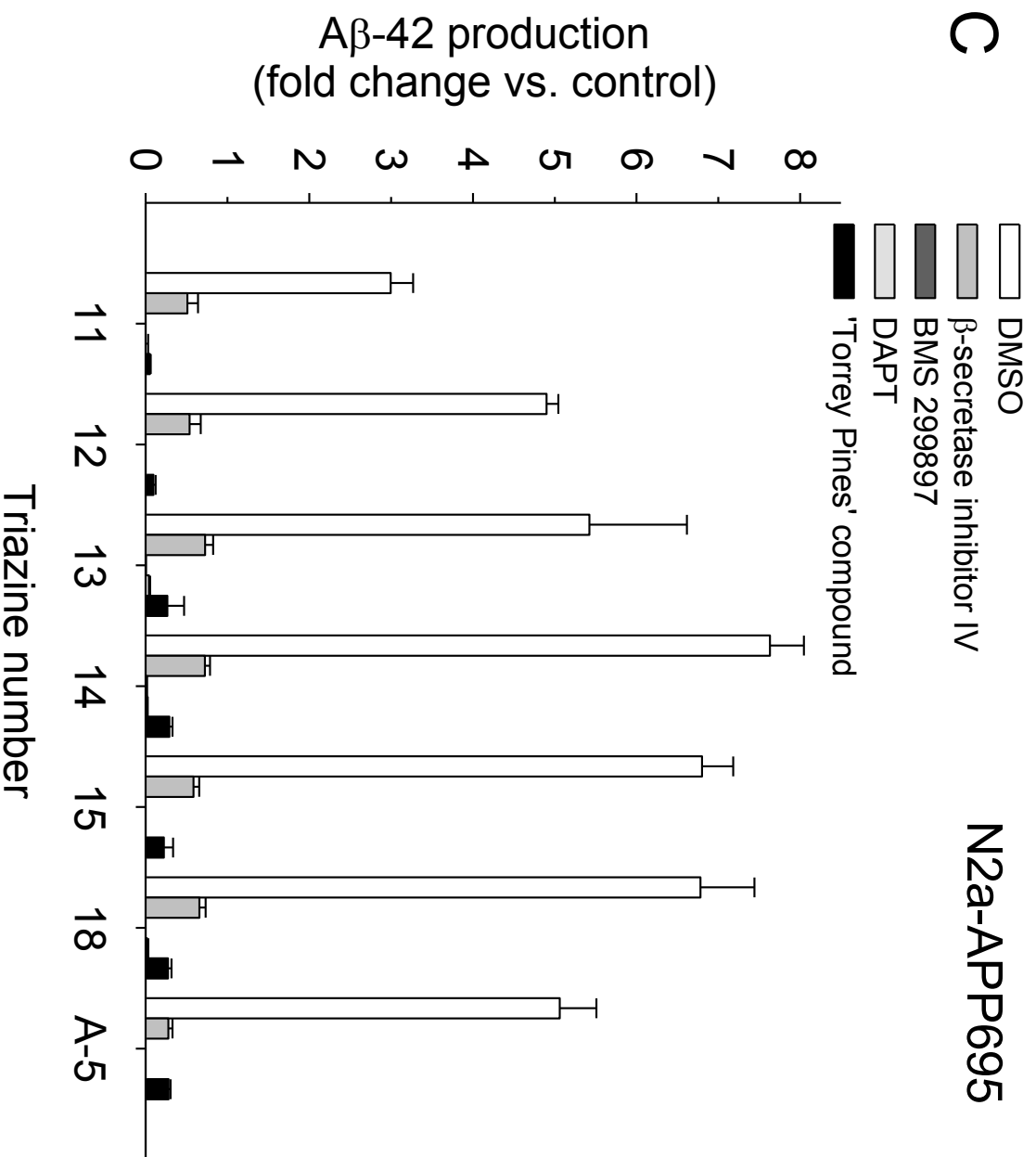
Cybutryne (15)



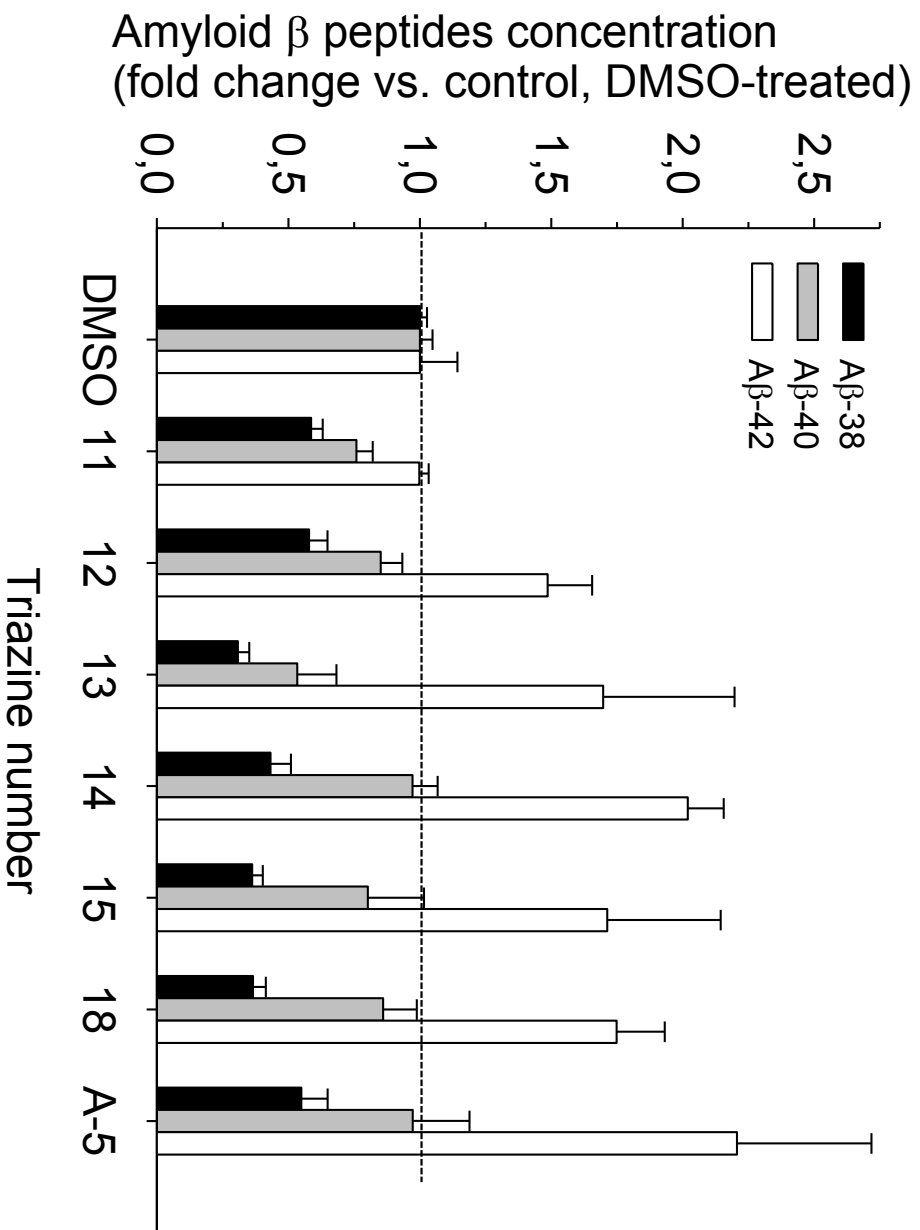
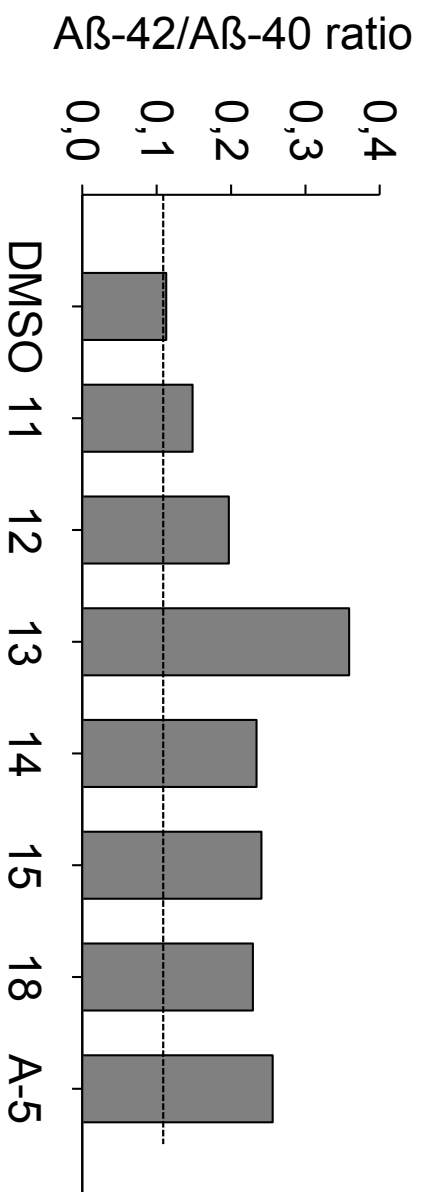
Dimethametryn (18)



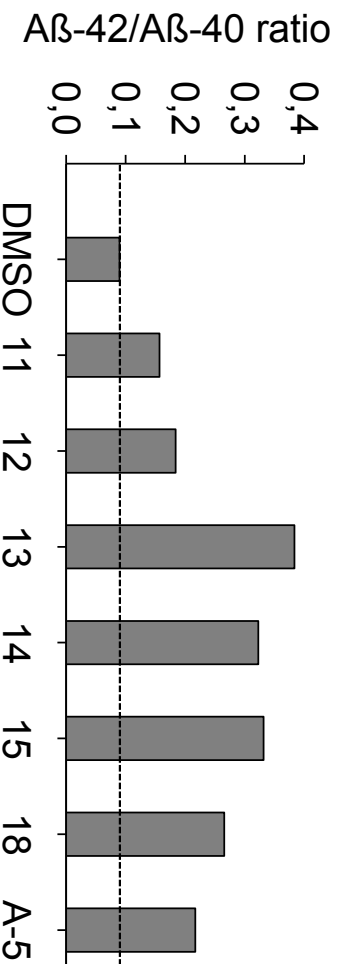
Aftin-5 (38)



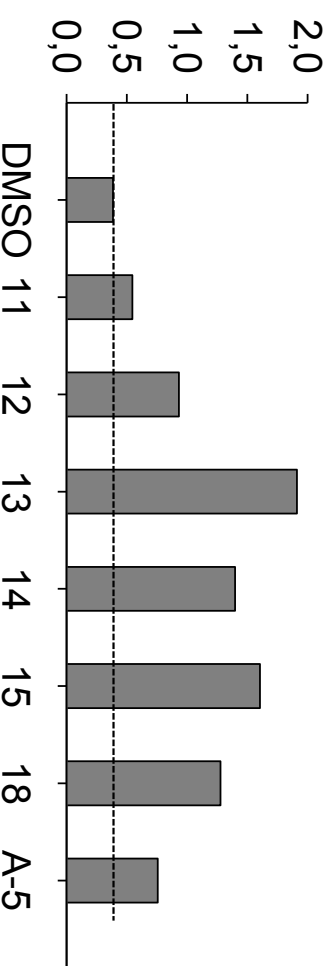
D Primary rat neuron cultures



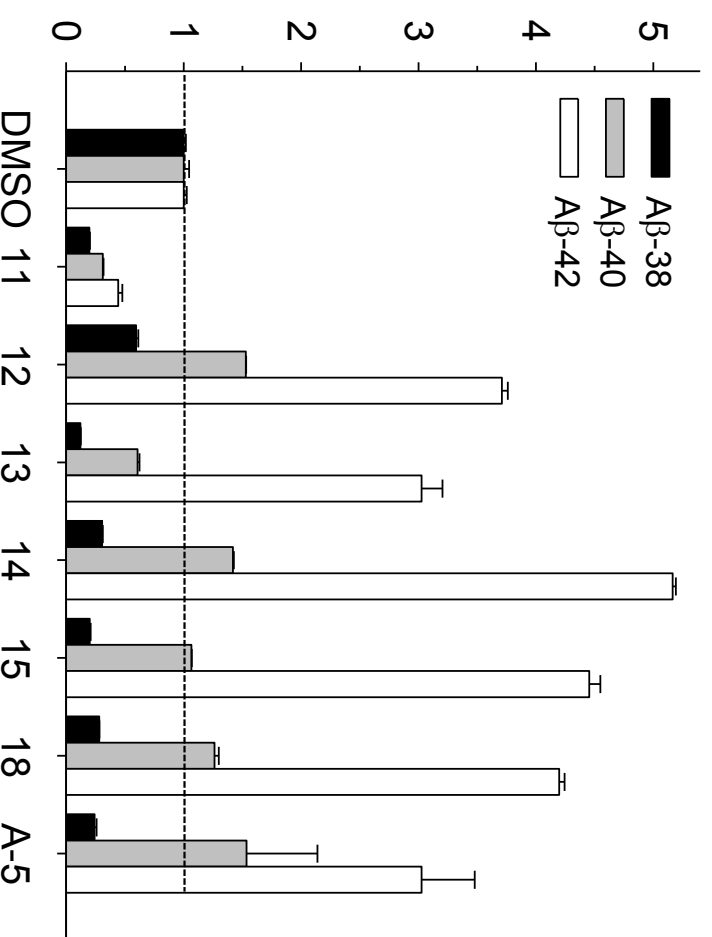
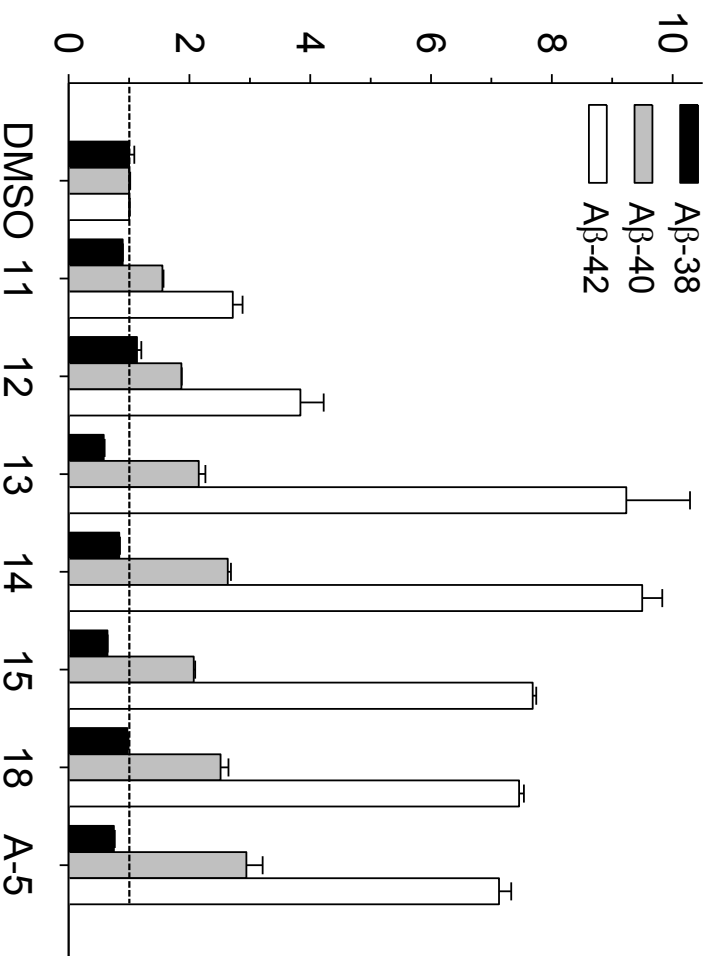
A N2a-APP695



B CHO-7PA2



Amyloid β peptides concentration
(fold change vs. control, DMSO-treated)



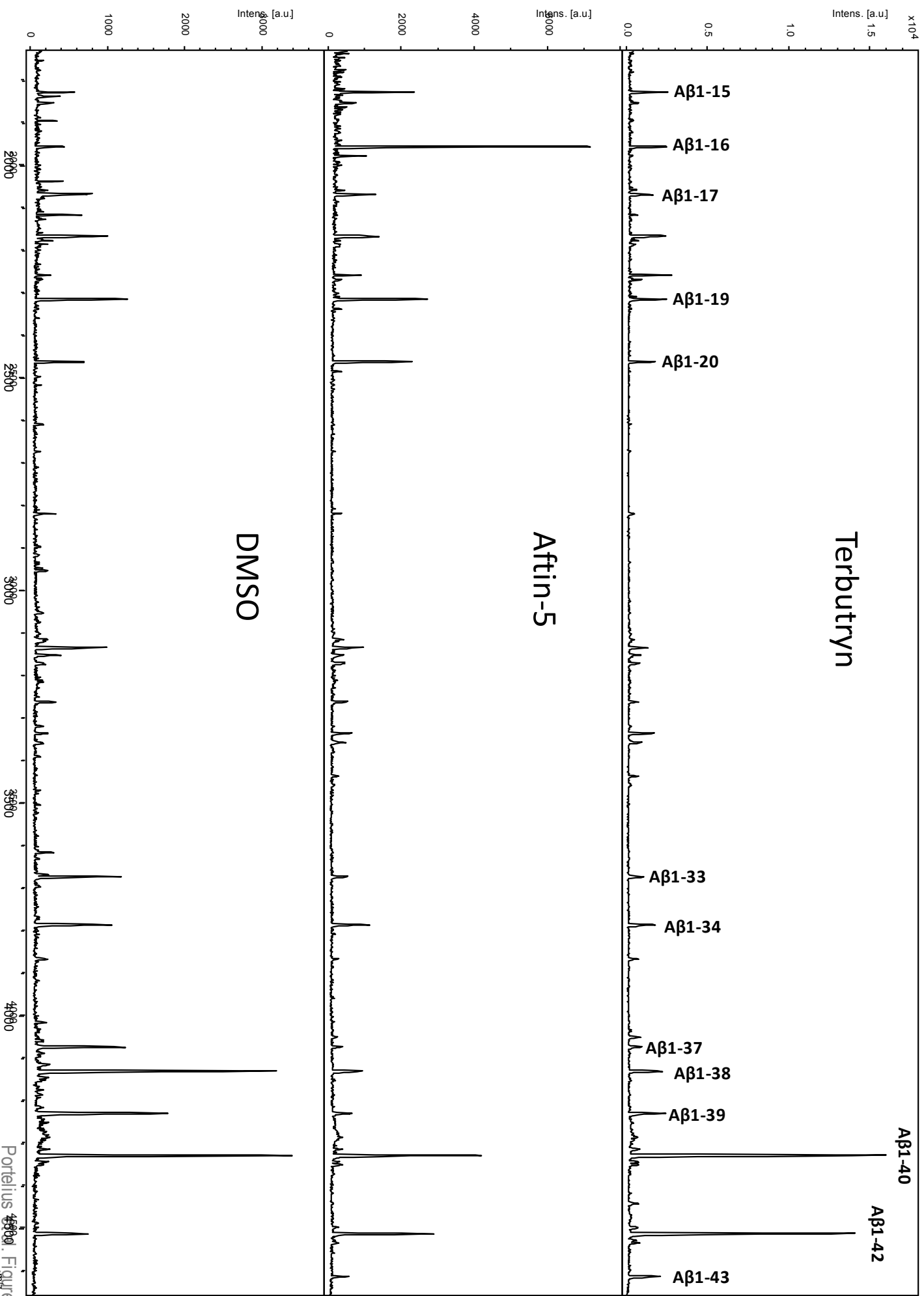
Aβ-38: 524.3 ± 44.8 pg/mL
Aβ-40: 2456.4 ± 40.9 pg/mL
Aβ-42: 219.8 ± 2.6 pg/mL

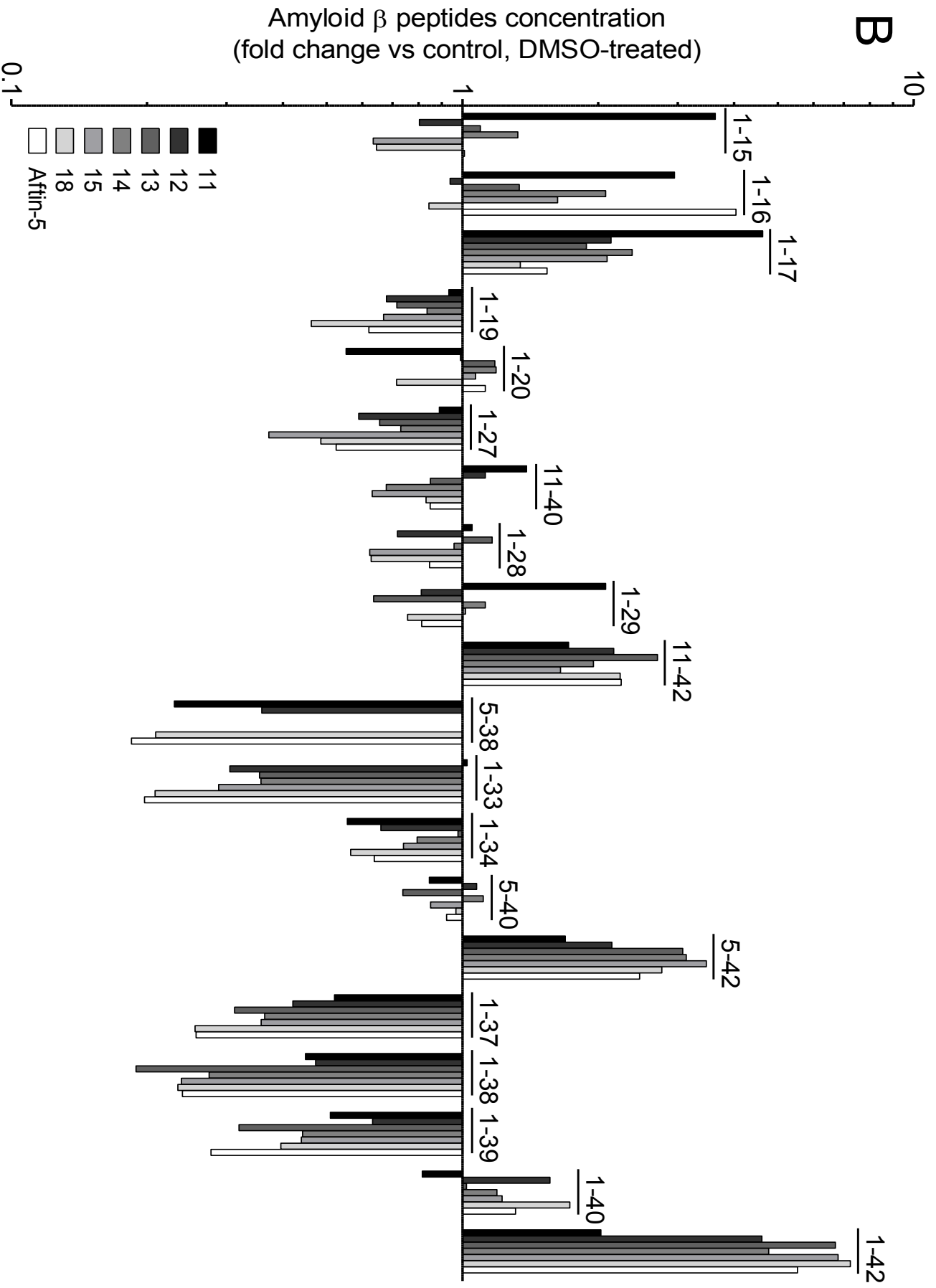
Aβ-38: 908.2 ± 16.7 pg/mL
Aβ-40: 1540.3 ± 71.0 pg/mL
Aβ-42: 591.2 ± 15.3 pg/mL

Triazine number

Triazine number

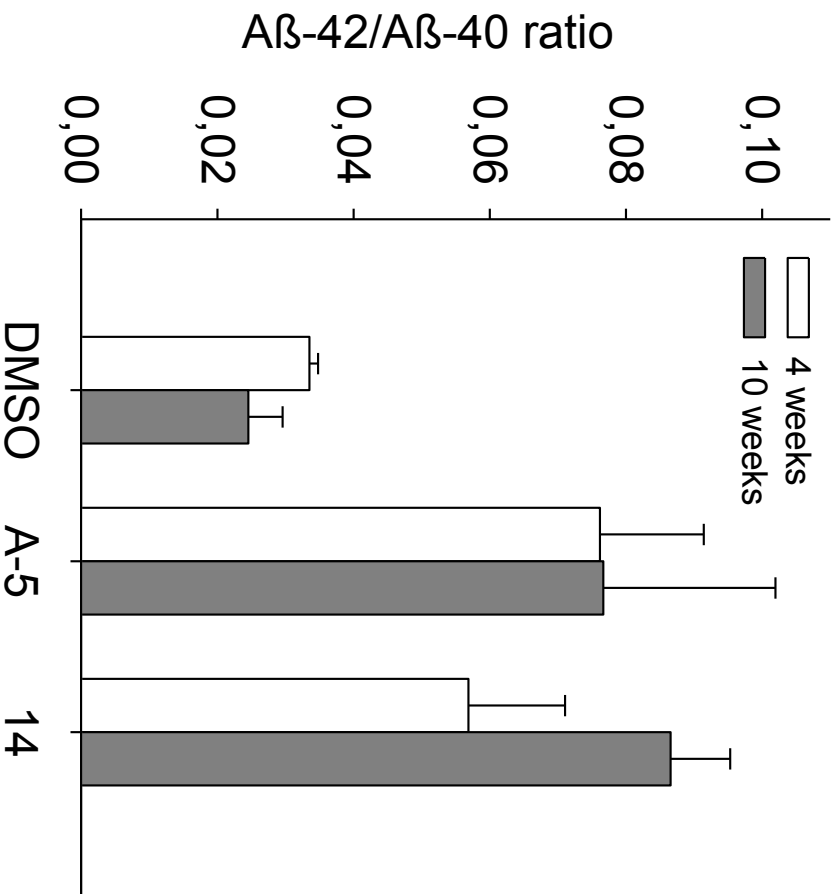
A CHO-7PA2



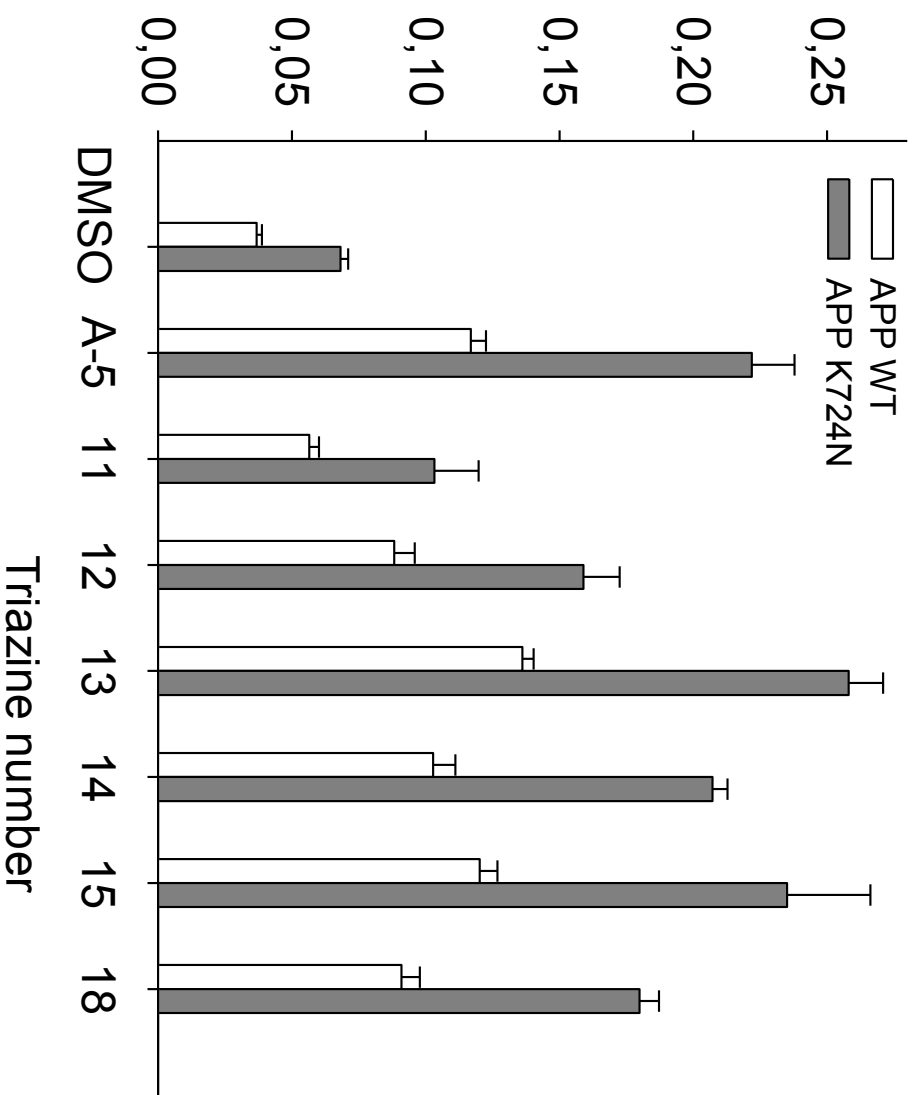


Neurons differentiated from human iPSCs

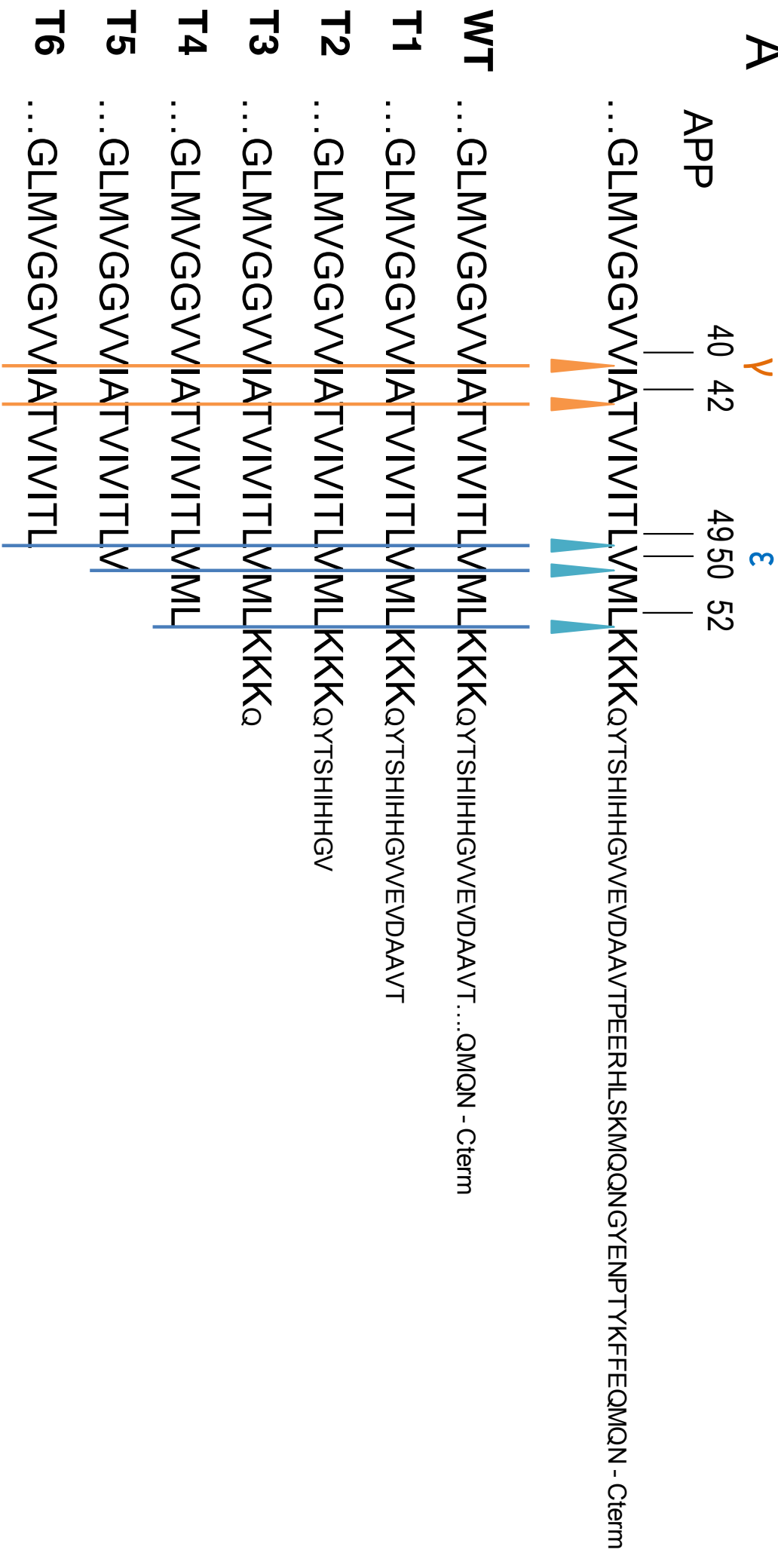
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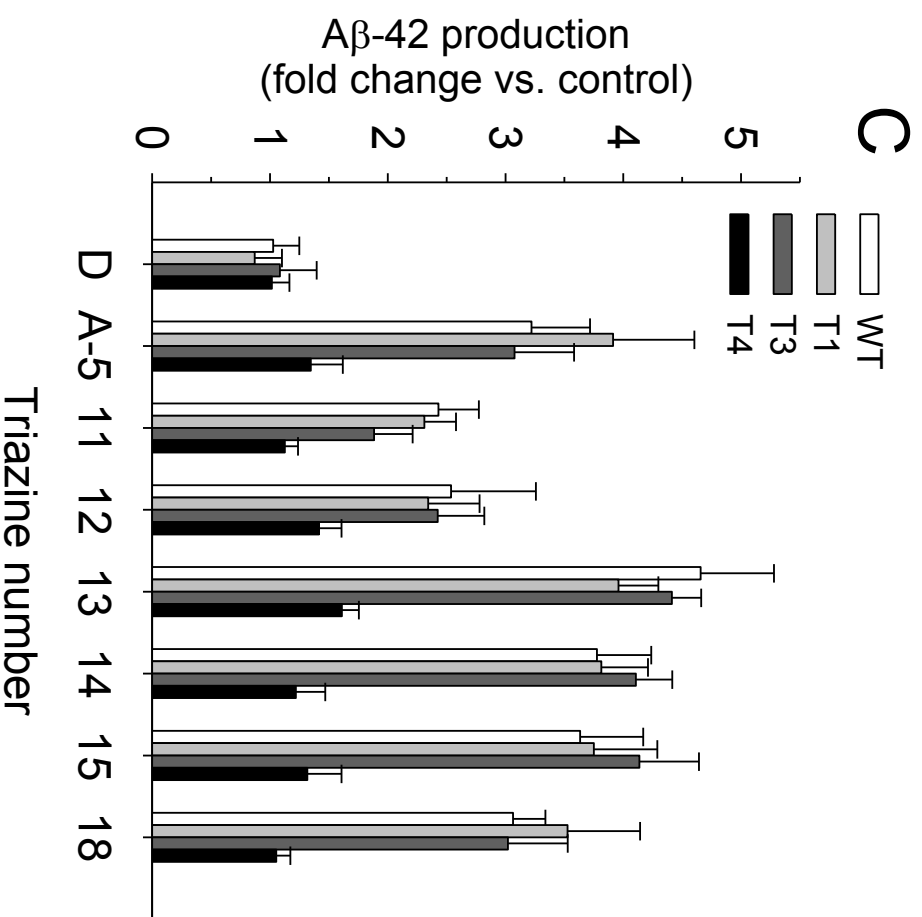
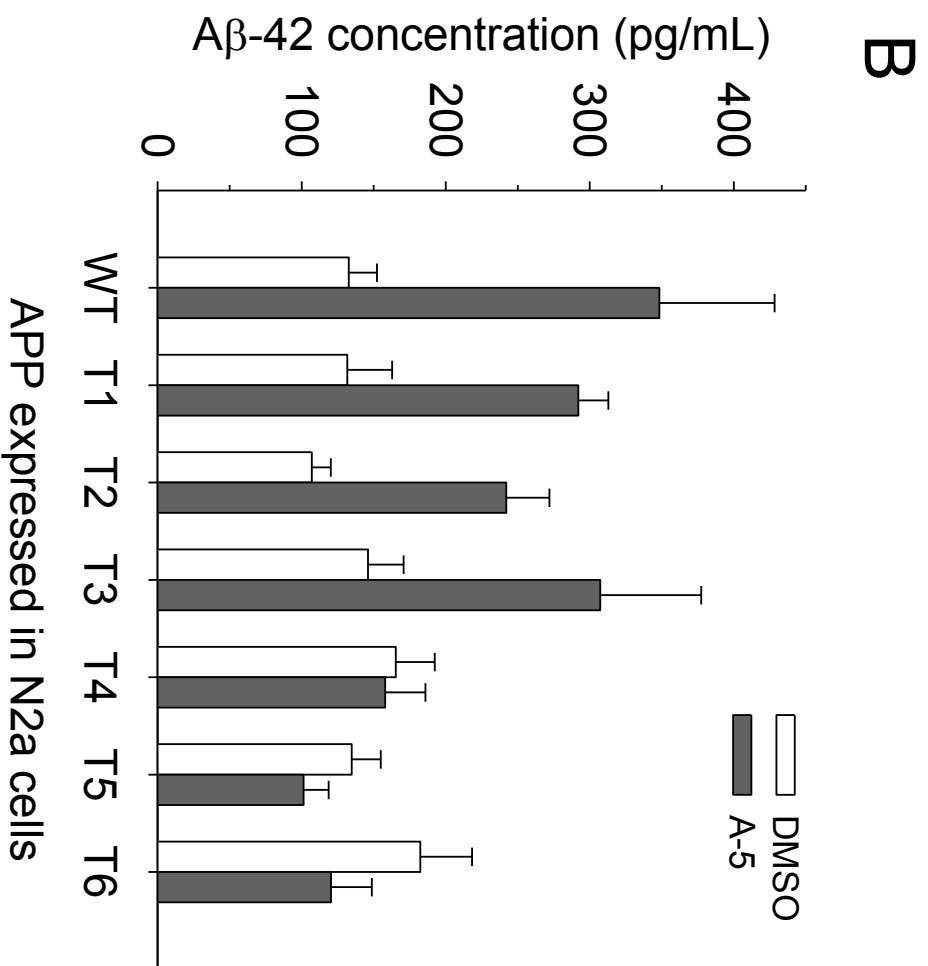


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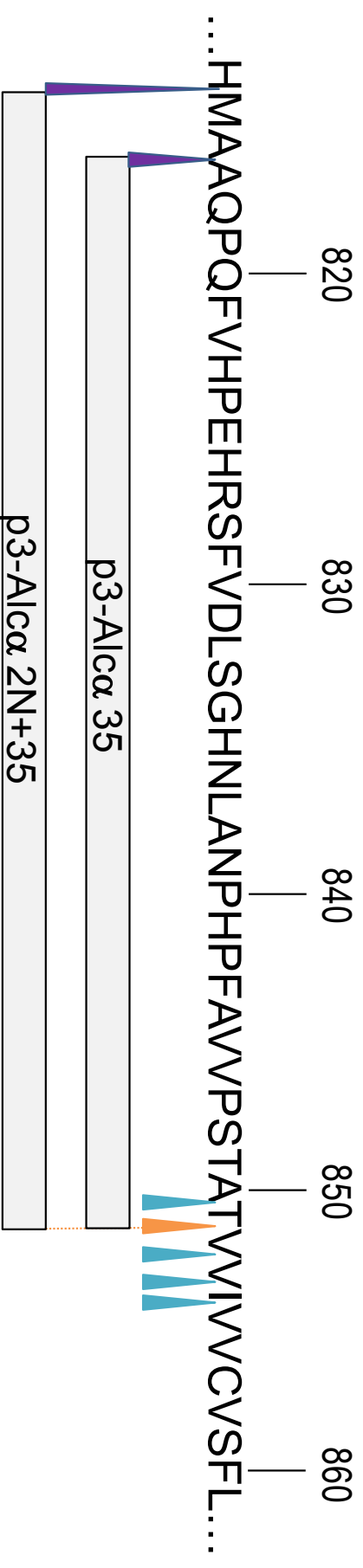
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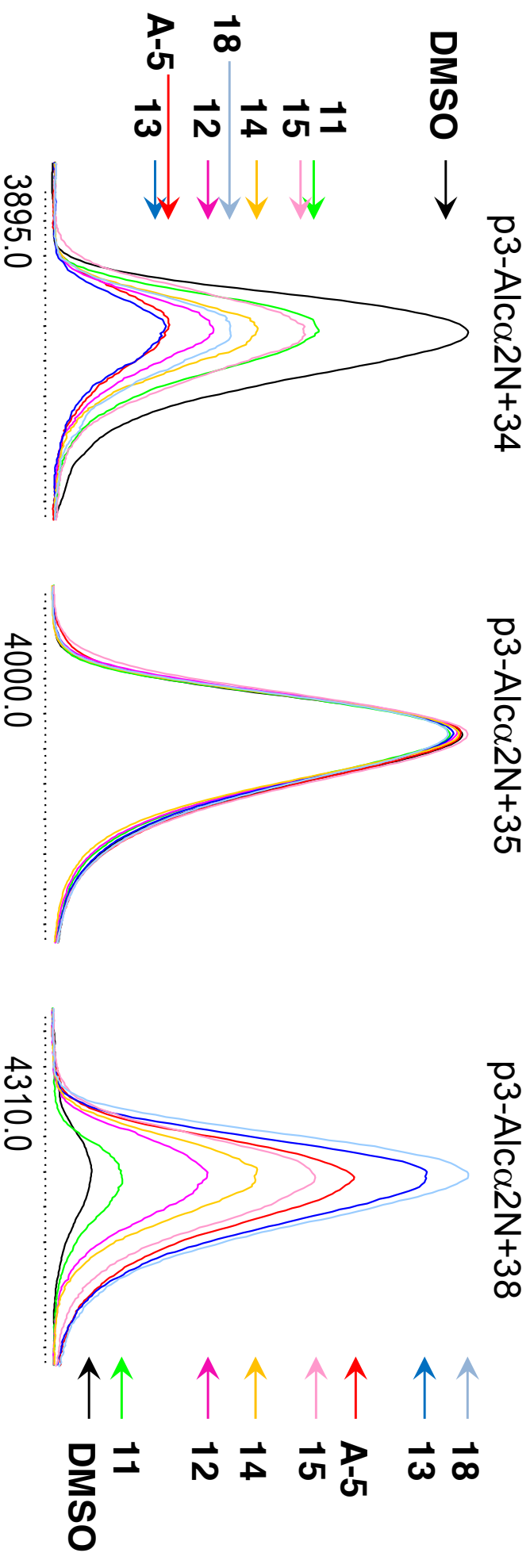
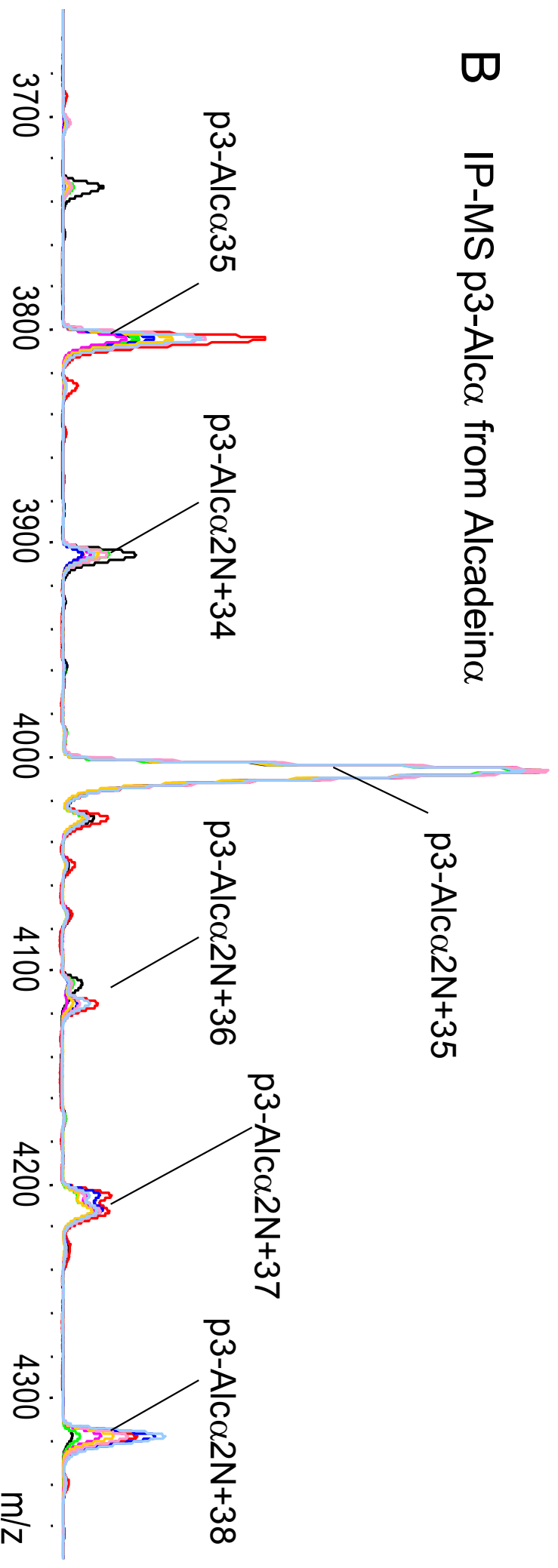


A

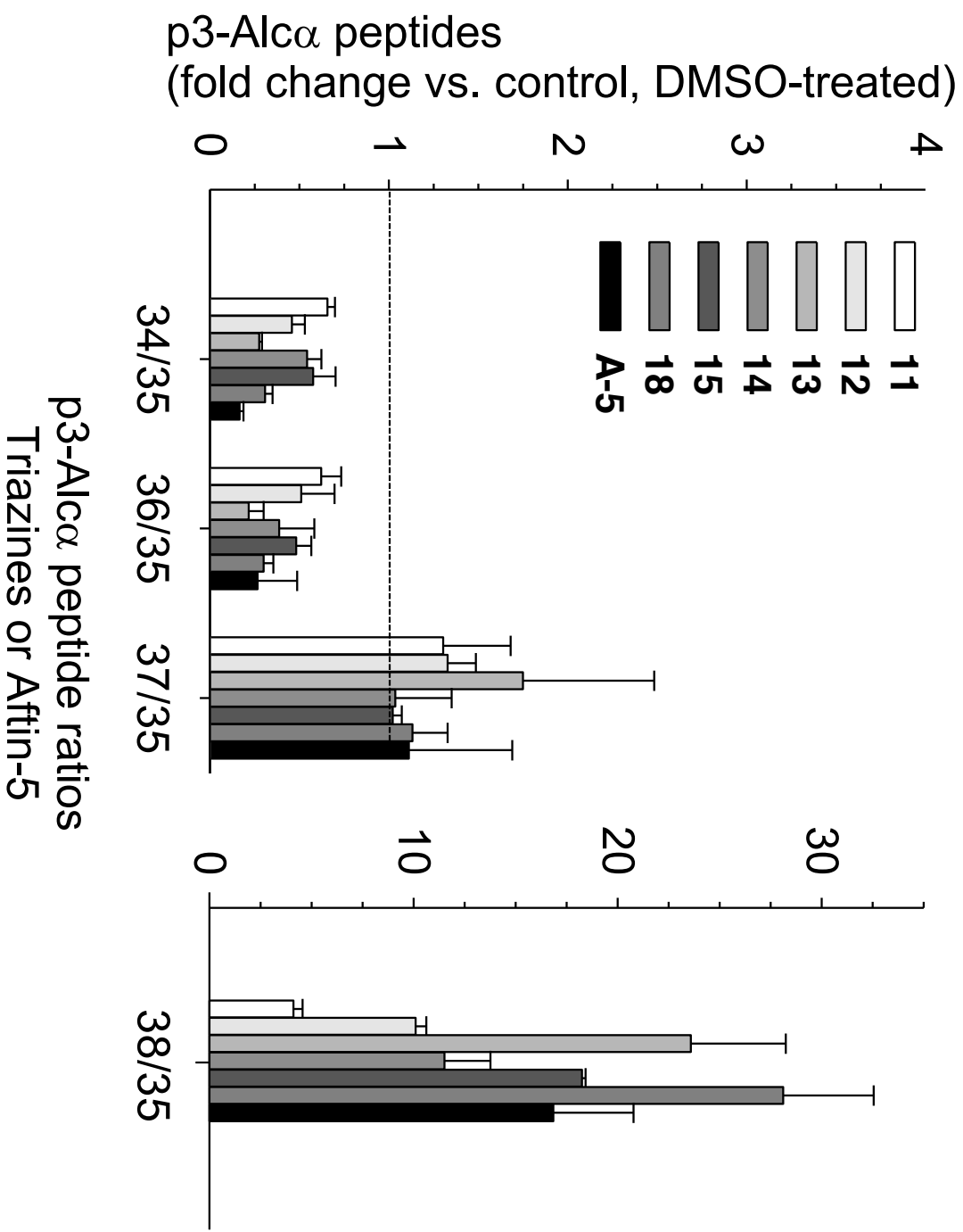
Alcadein α



B IP-MS p3-Alc α from Alcadein α



C



SUPPLEMENTARY INFORMATION

Specific triazine herbicides induce amyloid β 42 production

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1. Supplementary Material & Methods

1.1. Triazines and other reagents

The main triazine herbicides (**1-36**) were obtained commercially from Fluka/ Sigma Aldrich (St. Quentin Fallavier, France). The flame retardant TTBP-TAZ (2,4,6-tris(2,4,6-tribromophenoxy)-1,3,5-triazine) (**37**) was from Sigma Aldrich (St. Quentin Fallavier, France).

Terbutryn and cybutryne metabolites and contaminants, 2-N-tert-butyl-6-(methylsulfanyl)-1,3,5-triazine-2,4-diamine (terbutryn impurity) (**39**), terbutylazine-2-hydroxy (**41**), 4-(tert-butylamino)-6-(ethylamino)-1,3,5-triazine-2-thiol (**42**), 4-(tert-butylamino)-6-(ethylamino)-1,3,5-triazine-2-thiol (**43**) and N,N'-dicyclopropyl-1,3,5-Triazine-2,4,6-triamine (cyromazine impurity) (**44**) were purchased from MicroCombiChem (Wiesbaden, Germany). Metabolite M13 (**40**) was synthesized by JL and YTC (not shown).

A panel of 236 triazines was selected in a large triazines library constructed as described previously [30, 31]. Twenty products displayed some activity (**45-65**) (Supplementary Table S5). Five triazines (**49, 66-69**) bearing a poly-ethylene glycol linker (2, 3 or 4 ethylene glycol moieties) were synthesized by JL and YTC (not shown).

Aftin-5 (**38**) (Adipogen International, San Diego, CA, USA) was synthesized as previously described [14]. Aftin-5 bearing a poly-ethylene glycol linker (5 ethylene glycol moieties) (**70**) was synthesized by NO and HG (not shown).

All compounds were solubilized as 100 mM stock solutions in 100% dimethylsulfoxide (DMSO) and diluted just prior use.

The γ -secretase modulator ('Torrey Pines' compound) was synthesized as previously described [14, 30, 31]. DMSO, Nonidet P-40, Tween-20, CHAPSO (3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate), dithiothreitol (DTT), bovine serum albumin (BSA), Na₂CO₃, NaHCO₃, citric acid monohydrate, Na₂HPO₄·2H₂O, H₂O₂, digitonin were purchased from Sigma Aldrich. Protease inhibitors mix (Complete) was obtained from Roche (Boulogne-Billancourt, France). Streptavidin-horseradish peroxidase (HRP) conjugate was purchased from Thermo Scientific Pierce (Brebieres, France). o-Phenylenediamine dihydrochloride (OPD) tablets were from Invitrogen (St Aubin, France). γ -Secretase inhibitors BMS299897 and DAPT (N-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-

phenyl]glycine-1,1-dimethylethyl ester) were obtained from Tocris Bioscience (Lille, France). β -secretase inhibitor IV was from Calbiochem (Molsheim, France).

1.2. Cell cultures: cell lines, primary neuron cultures, iPSCs-derived neurons & HEK293-alcadein α cells

N2a-APP695 and CHO-7PA2 cell lines

N2a-APP695 cells (ECACC n° 89121404, lot 07/D/032) were cultured in Dulbecco's minimal essential medium (DMEM) / OptiMEM (1:1 v/v) (Invitrogen, Saint Aubin, France) containing 0.2 mg/mL Geneticin (Gibco, c/o Invitrogen) and 0.5% fetal bovine serum (FBS, Gibco) in a humidified, 5% CO₂ incubator. CHO-7PA2 cells (CHO cells stably expressing APP751 with the Val717Phe familial AD mutation, obtained from D.J. Selkoe, Harvard Medical School, Boston, USA) were cultured in DMEM/F12 (Invitrogen) + 0.5% FBS with Glutamax, Phenol Red and without HEPES. Cells were split routinely every 4/5 days. They were first rinsed with phosphate buffered saline (PBS) (Gibco) and detached from the plate bottom using 4 mL Versene (Gibco) at room temperature (RT) for 3-4 min. Eight mL of fresh medium were added to the cell suspension, and the mix was centrifuged for 3 min at 1,000 rpm. The cell pellet was resuspended in fresh medium before seeding (1/10 dilution) in new flasks.

Primary Rat Neuronal Cultures

Primary neuronal cultures were prepared from E18 OFA rats embryos brains (Charles River laboratories, L'Arbresle, France). All procedures were performed according to European law (EEC 86/609) and were approved by the local veterinary authorities (N° 12037). Primary cultures of cortical neurons were prepared according as described [65] with minor modifications. Dissociated neurons were plated onto poly-D-lysine (50 μ g/mL) coated coverslips (14 mm diameter, Marienfeld GmbH, Lauda-Königshofen, Germany) into 12 wells culture plates (Costar, Corning NY, USA) at 10⁵ cells/cm² for cortical neurons. All experiments were performed at 15 days *in vitro* (DIV). Briefly, cortices were dissected in Hank's Balanced Salt Solution (HBSS) containing 5 mM glucose (dissection medium). The medium was replaced 0.05% trypsin in 0.5 mM EDTA (Life Technologies, Grand island, NY, USA). Cortices were digested separately for 15 min at 37°C. After removing the trypsin, cortices were washed in dissection medium, and incubated in HBSS containing trypsin inhibitor (2 mg/mL, Sigma Aldrich, St Quentin Fallavier, France) and DNase I (0.05%, Sigma) for 3 minutes at 37°C. Cortices were washed in dissection medium, taken up in few mL of dissection medium and gently triturated by passing through a 1 mL ART® filter tip. Cells were counted and seeded at a density of 10⁵ cells/cm² into 12 wells culture plates (Costar) in plating medium (DMEM medium containing 10% heat-inactivated horse serum (Life Technologies)). Wells contained acid-washed glass coverslips (14 mm diameter, Marienfeld) which had been precoated with 50 μ g/mL poly-D-lysine (Sigma). Cultures were left to attach at 37°C with 5% CO₂. After 2 h, the plating medium was replaced with Neurobasal containing 2 mM glutamine, 1 mM sodium pyruvate, 10 μ g/mL penicillin and streptomycin, and 2% B27 supplement (Life Technologies) (culture medium). After 4 days, cytosine arabinoside (Sigma, 3 μ M final concentration) was added to suppress glial cell proliferation. Cultures were fed twice a week by adding a quarter of the volume of fresh culture medium (after removal of the same volume). At DIV 15th, neurons were incubated in culture medium (MEM 1X (Life Technologies), HEPES buffer 1M 1,5%

(Life Technologies) and NaOH (Sigma) up to pH 7,3) containing 100 μ M herbicides or 100 μ M affin-5 (ManRos Therapeutics, Roscoff, France) or vehicle (0,1% sterile DMSO, Sigma) for 18 hours. At the end of the treatment, secreted A β ₃₈, A β ₄₀ and A β ₄₂ peptides in conditioned medium were quantified by a sandwich immunoassay using the Meso Scale Discovery SECTOR Imager 2400. All reagents were from Meso Scale Discovery (Rockville MD, USA).

Human iPSCs-derived neuronal cultures

Induced pluripotent stem cells (iPSCs) from 33-year old healthy male (A β PP WT, wild-type) and 55-year old female harboring a lysine 724 to asparagine mutation in A β PP (A β PP K724N mutation) have been generated and characterized previously [37, 38]. From iPSCs we generated long-term self-renewing neuroepithelial stem cells (It-NES cells), a homogeneous population which can be continuously propagated in the presence of FGF2 and EGF. To that end, 4-day-old embryoid bodies were transferred to polyornithine-coated tissue culture dishes and propagated in N2 medium (DMEM/F12 high glucose; N2 supplement, both Life Technologies, Darmstadt, Germany). Within 10 days, neural island consisting of neural rosettes and neural tube-like structures developed in the embryoid body outgrowth. These islands were mechanically isolated by separation from the surrounding cells with a scalpel. Isolated clusters were further propagated for 2 days as free-floating neurospheres in N2 medium containing 10 ng/mL FGF2 and 10 ng/mL EGF (both R&D Systems, Wiesbaden, Germany) and 1 μ L/mL B27 Supplement (Life Technologies; short: N2 FEB medium). Spheres were triturated into single cells by incubating the spheres with trypsin/EDTA for 10 min followed by gentle dissociation with a 1.000 μ L pipette tip. Cells were plated on polyornithine (Sigma Aldrich) and laminin (Life Technologies) precoated plastic dishes. Passaging of the cells was performed when cells had reached full confluence (normally every 3-4 days) at a 1:2 – 1:3 ratio using trypsin/EDTA. Medium was changed every other day while growth factors were added on a daily basis.

Neuronal differentiation was performed by plating 300,000 and 1,000,000 cells per well of a Geltrex (Life Technologies; diluted 1:50)-precoated 12-well plate or 6-well plate, respectively, in N2 FEB medium. The following day, the medium was changed to terminal differentiation medium containing DMEM/F12 and MACS Neuro Medium (Miltenyibiotec, Bergisch Gladbach, Germany) mixed at a 1:1 ratio and supplemented with 1:200 N2 supplement, 1:100 B27 supplement and 300 ng/mL cAMP (Sigma Aldrich). Medium was changed every other day.

Compound treatment was performed on neuronal cultures differentiated for at least 4 weeks. Cells were pretreated for 16 h with compound containing medium (all compounds at 100 μ M in 500 μ L medium/ 12-well plate and 1,5 ml medium/ 6-well plate). Solvent only (DMSO) was used as control. Medium was replaced (same volume with freshly added compounds) and supernatants and lysates were harvested 24 h later. Supernatants were directly frozen in liquid nitrogen until analyzed. A β ₄₂ and A β ₄₀ in the supernatants were analyzed by ELISA (Meso Scale Discovery, Rockville MD, USA) and normalized to the protein content of the cell pellet.

HEK293-alcadeina cell culture and Alcadein fragments analysis

The full-length human Alcadeina1 (Alca) open reading frame [24] was subcloned into the HindIII and XbaI sites of pcDNA3.1 (Hygro+) vector (Invitrogen/Thermo Fisher Scientific,

Carlsbad, CA), transfected into HEK293 cells with Lipofectamine 2000 (Invitrogen/Thermo Fisher Scientific, Carlsbad, CA), and cells stably expressing Alca were cloned. The cells cultured in dish coated with poly-L-lysine were treated with Aftin-5 or triazines (100 μ M) for 24 h. The secreted p3-Alca were recovered from the cultured medium by immunoprecipitation with anti-p3-Alca UT175 antibody, an antibody raised to an antigen peptide composed of Cys plus the human Alca1 839-851 sequence, using Protein G-Sepharose beads (GE Healthcare, Little Chalfont, UK). The beads were sequentially washed and samples were eluted with trifluoroacetic acid/acetonitrile/water (1:20:20) saturated with sinapinic acid, and subject to MALDI-TOF-MS analysis using an Ultraflex II TOF/TOF (Bruker Daltonics, Bremen, Germany). Molecular masses were calibrated using the peptide calibration standard (Bruker Daltonics) [25].

1.3. Transient transfections with A β PP truncation mutants

We used the human APP cDNA for all the experiments presented and the truncation mutants were generated by PCR using standard molecular techniques.

Briefly, full-length human APP cDNA was previously subcloned by using standard PCR and TA cloning molecular techniques, into the pcDNA3.1(+) plasmid (Invitrogen, Carlsbad, CA) at BglII/BstXI restriction sites. Truncated APP mutants were subcloned from TA by EcoRI/EcoRI restriction sites into APP containing pcDNA3.1 (+) plasmid.

Forward oligonucleotide for:

T1-T6: 5'-TAT ATA AGA TCT CTG AAG TGA AGA TGG ATG C-3'

Reverse oligonucleotides for:

T1: 5'-TAT ATA CCA GAC TAC TGG CTA GGT GAC AGC GGC GTC AAC-3'

T2: 5'-TAT ATA CCA GAC TAC TGG CTA CAC ACC ATG ATG AAT GGA-3'

T3: 5'-TAT ATA CCA GAC TAC TGG CTA CTG TTT CTT CTT CAG CAT -3'

T4: 5'-TAT ATA CCA GAC TAC TGG CTA CAG CAT CAC CAA GGT GAT-3'

T5: 5'-TAT ATA CCA GAC TAC TGG CTA CAC CAA GGT GAT GAC GAT -3'

T6: 5'-TAT ATA CCA GAC TAC TGG CTA CAA GGT GAT GAC GAT CAC -3'

N2a cells were maintained in growing medium (50% DMEM, 50% OPTI-MEM) supplemented with 5% fetal bovine serum (vol/vol). Transfections were carried using Fugene6 according to the manufacturer instructions. For experiments, cells were plated in 6-well plates at a density of 4×10^5 cells per well. Cells were grown to about 40% confluence and then transfected with relevant plasmid constructs using FuGENE6 (Roche, Indianapolis). 48 h after transfection, cells were treated for 24 h with various triazines. Supernatants were collected for A β 42 measurements and cells were harvested for protein quantification.

1.4. Amyloids assays

Amyloids were measured by different methods according to the different cell types:

- Primary rat neuronal cultures and human iPSCs-derived neuronal cultures: Meso Scale Discovery sandwich ELISA immunoassay.
- N2a cells transfected with various APP C-terminal truncated mutants: BioSource International ELISA assay (see below).

- N2a-APP695 and CHO-7PA2 cell lines: an in-house sandwich ELISA assay described below, as well as tandem mass spectrometry (MS/MS) (see below) and immunoprecipitation / mass spectrometry (IP-MS) (see below).

In-house ELISA capture assay [14]

N2a-APP695 or CHO-7PA2 cells were seeded at 10,000 cells/well in a 96 well plate with modified media (0.5% FBS) and incubated overnight. Cells were treated with fresh media and different compounds (equal quantity of DMSO), then incubated for 18 h in a humidified, 5% CO₂ incubator. The plate was finally centrifuged to remove cell fragments before collecting supernatant samples for amyloids levels determination (see below). To determine the amount of secreted A β peptides in the primary rat neuron culture medium, 15 days culture medium was discarded and replaced by fresh media which was collected after 18 h incubation.

A β ₃₈, A β ₄₀, A β ₄₂ levels were measured in a double antibody sandwich ELISA using a combination of monoclonal antibody (mAb) 6E10 (Covance c/o Ozyme, Montigny-le-Bretonneux, France) and biotinylated polyclonal A β ₃₈ [66], A β ₄₀ or A β ₄₂ [67] antibodies (provided by Dr. P.D. Mehta, Staten Island, USA). Briefly, 100 μ L mAb 6E10 diluted in carbonate-bicarbonate buffer (buffer (0.015 M Na₂CO₃ + 0.035 M NaHCO₃) pH 9.6), was coated in the wells of microtiter plates (Maxisorp, Nunc, ThermoFisher Scientific, Illkirch, France) and incubated overnight at 4°C. The plates were washed with PBST (PBS containing 0.05% Tween-20) and blocked for 1 h with 1% BSA in PBST to avoid non-specific binding. Each of the A β ₃₈, A β ₄₀ or A β ₄₂ antibodies did not cross-react with the other amyloid peptides (data not shown). Standard curves were prepared with synthetic amyloids and each of the three antibodies. Fitting was performed using a 4 parameters sigmoid equation (SigmaPlot, Systat, Macon, France).

Typically, the first day, the cells were detached of the flask with Versene, centrifuged during 3 minutes at 1000 rpm and suspended in 10 mL of medium. The cells were counted and seeded in 6 well plates at 200,000 cells/well. The medium used were DMEM/F12 supplemented with 0.5% FBS for the CHO-7PA2 cells and DMEM/Optimem (1:1) supplemented with 0.5% FBS for N2aAPP695 cells. The second day, the cells were treated for 24 h with 100 μ M Aftin 5 or Triazine in a final concentration of DMSO of 0.1%. Control cells were also exposed to 0.1% DMSO. The volume final of medium was 2 mL. The third day, after the 24 h treatment, the plates were agitated slowly and 2 mL of medium were collected in one tube (3 tubes per condition). The tubes were inverted and 200 μ L were removed for ELISA analysis. The remaining 1.8 mL were frozen at -80°C.

Following a washing step, experimental samples were added into the wells and incubation was carried out for 2 h at RT and overnight at 4°C. Plates were washed before incubation with biotinylated polyclonal antibody diluted in PBST + 0.5% BSA at RT for 75 min. After a washing step, streptavidin-HRP conjugate, diluted in PBS + 1% BSA, was added and incubation was carried out for 45 min at RT. After washing, 100 μ L OPD in citrate buffer (0.049 M citric acid monohydrate + 0.1 M Na₂HPO₄.2H₂O + 1 mL H₂O₂ 30%/L) pH 5.0 were added. The reaction was stopped after 15 min with 100 μ L 1 N sulfuric acid. The optical density was measured at 490 nm in a microELISA reader (BioTek Instrument, El 800, Gen 5 software).

BioSource International ELISA assays and data analysis

β Amyloid 1-40 or 1-42 Colorimetric Elisa kits were used for cells transfected with human full length APP and its truncation mutants. After immobilization of total A β from media, A β 40/42 peptide determinations were made by sandwich ELISA (BioSource International, Camarillo, CA). A β levels were normalized to total protein levels after cell recovery. Cells were recovered with 100 μ L of RIPA buffer (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS), incubated for 30 min on ice, and centrifuged at $10,000 \times g$ for 20 min at 4°C. A β 40 and A β 42 standard curves were plotted as a sigmoidal dose-response curve (variable slope) by using GraphPad Prism ver. 4.0. Data presented are the results of at least three independent experiments done in triplicate.

Absolute quantification of amyloids by tandem mass spectrometry (MS/MS)

Solid phase extraction, liquid chromatography (LC) and MS/MS analysis of A β species was performed as described previously [34, 35] with the following modifications. Standard curves for A β 38 and A β 42 were prepared at 0.15, 0.5, 1, 2, 3 and 4 ng/mL while A β 40 was prepared at 15, 50, 100, 200, 300 and 400 ng/mL using unlabeled peptides (rPeptide) in DMEM/F12 supplemented with 0.5% FBS. Uniformly labeled ^{15}N -A β 38, A β 40 and A β 42 peptides (rPeptide) were added to a final concentration of 1.6 ng/mL in calibrators and unknown samples as internal standards. Standard curves were constructed using the unlabeled to ^{15}N -A β peak area ratios and fitted using linear regression. All standard curves were linear and had an R^2 value greater than 0.998. Concentrations of unknowns were extrapolated from the standard curves using the peak area ratio of endogenous to ^{15}N -A β .

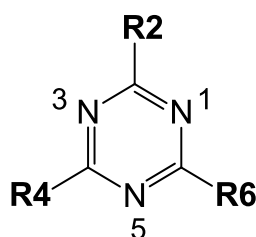
Amyloids profile analysis by immunoprecipitation / mass spectrometry (IP-MS)

Immunoaffinity capture of A β species was combined with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS for analyzing a variety of A β peptides in a single analysis as described [68]. In brief, the anti-A β antibodies 6E10 and 4G8 were separately coupled to magnetic beads. After washing of the beads, the 4G8 and 6E10-coated beads were used in combination for immunoprecipitation. After elution of the immune-purified A β peptides, analyte detection was performed on an UltraFLEXtreme MALDI TOF/TOF instrument (Bruker Daltonics). For each peak the areas were normalized against the sum for all the A β peaks in the spectrum followed by averaging of results for separately determined duplicate samples [36].

1.5. Cell viability

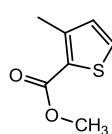
To measure cell viability, the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Charbonnières-les-Bains, France) was used in the same wells as the capture ELISA assay. Briefly, 20 μ L of MTS reagent was added in each well containing 100 μ L of media and incubation then proceeded for 3 h (37°C, 5% CO₂ and 95% humidity). Measurements were made at OD 490 Δ 630 nm using a microELISA reader.

2. Supplementary Tables

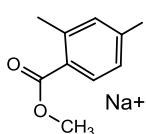


#	Triazines	2	4	6
1	Atrazine	-Cl	-NH-C ₂ H ₅	-NH-CH(CH ₃) ₂
2	Simazine	-Cl	-NH-C ₂ H ₅	-NH-C ₂ H ₅
3	Propazine	-Cl	-NH-CH(CH ₃) ₂	-NH-CH(CH ₃) ₂
4	Cyanazine	-Cl	-NH-C ₂ H ₅	-NH-C(CH ₃) ₂ CN
5	Terbuthylazine	-Cl	-NH-C ₂ H ₅	-NH-C(CH ₃) ₃
6	Sebuthylazine	-Cl	-NH-C ₂ H ₅	-NH-CH(CH ₃)-C ₂ H ₅
7	Atraton	-OCH ₃	-NH-C ₂ H ₅	-NH-CH(CH ₃) ₂
8	Terbumeton	-OCH ₃	-NH-C ₂ H ₅	-NH-C(CH ₃) ₃
9	Prometon	-OCH ₃	-NH-CH(CH ₃) ₂	-NH-CH(CH ₃) ₂
10	Simetryn	-SCH ₃	-NH-C ₂ H ₅	-NH-C ₂ H ₅
11	Ametryn	-SCH ₃	-NH-C ₂ H ₅	-NH-CH(CH ₃) ₂
12	Prometryn	-SCH ₃	-NH-CH(CH ₃) ₂	-NH-CH(CH ₃) ₂
13	Dipropetryn	-SC ₂ H ₅	-NH-CH(CH ₃) ₂	-NH-CH(CH ₃) ₂
14	Terbutryn	-SCH ₃	-NH-C ₂ H ₅	-NH-C(CH ₃) ₃
15	Cybutryne	-SCH ₃	-NH-cycloC ₃ H ₅	-NH-C(CH ₃) ₃
16	Aziprotryn	-SCH ₃	-N=N ⁺ =N ⁻	-NH-CH(CH ₃) ₂
17	Desmetryn	-SCH ₃	-NH-CH ₃	-NH-CH(CH ₃) ₂
18	Dimethametryn	-SCH ₃	-NH-C ₂ H ₅	-NH-CH(CH ₃)-CH(CH ₃) ₂
19	Methoprotryn	-SCH ₃	-NH-CH(CH ₃) ₂	-NH-(CH ₂) ₃ -OCH ₃
20	Hydroxyatrazine	=O	-NH-C ₂ H ₅	-NH-CH(CH ₃) ₂
21	Desethylhydroxy-atrazine	-OH	-NH ₂	-NH-CH(CH ₃) ₂
22	Ammeline	-OH	-NH ₂	-NH ₂
23	Didealkylatrazine	-Cl	-NH ₂	-NH ₂
24	Desethylatrazine	-Cl	-NH ₂	-NH-CH(CH ₃) ₂
25	Deisopropylatrazine	-Cl	-NH-C ₂ H ₅	-NH ₂
26	Hexazinone *	=O	=O	-N(CH ₃) ₂
27	Terbumeton-desethyl	-OCH ₃	-NH ₂	-NH-CH(CH ₃)-C ₂ H ₅
28	Thifensulfuron-methyl	-OCH ₃	-CH ₃	-NH-CO-NH-SO ₂ -X1
29	Iodosulfuron-methyl-sodium	-OCH ₃	-CH ₃	-NH-CO-NH-SO ₂ -X2
30	Chlorosulfuron	-OCH ₃	-CH ₃	-NH-CO-NH-SO ₂ -X3
31	Tribenuron-methyl	-OCH ₃	-CH ₃	-N(CH ₃)-CO-NH-SO ₂ -X4
32	Triasulfuron	-OCH ₃	-CH ₃	-NH-CO-NH-SO ₂ -X5
33	Triflusulfuron-methyl	-OCH ₂ -CF ₃	-N(CH ₃) ₂	-NH-CO-NH-SO ₂ -X6
34	Cinosulfuron	-OCH ₃	-OCH ₃	-NH-CO-NH-SO ₂ -X7
35	Metsulfuron-methyl	-OCH ₃	-CH ₃	-NH-CO-NH-SO ₂ -X8
36	Trietazine	-Cl	-N(C ₂ H ₅) ₂	-NH-CH(CH ₃) ₂
37	TTBP-TAZ	2,4,6-tribromophenoxy	2,4,6-tribromophenoxy	2,4,6-tribromophenoxy

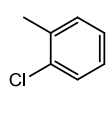
* N1 = - phenyl ; N3 = - CH₃



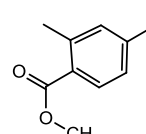
X1



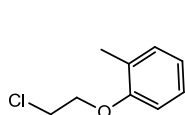
X2



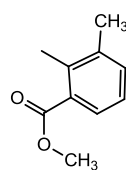
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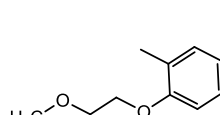
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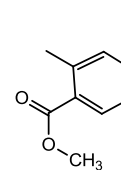
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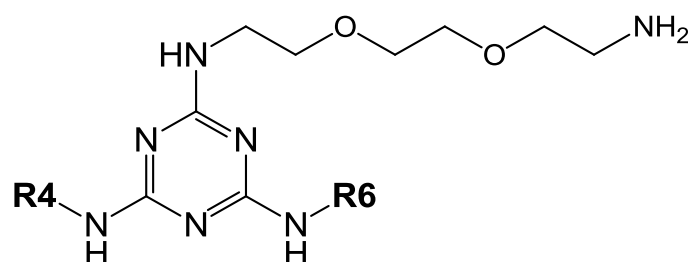


X8

Table S1. Structure of the triazines tested.

#	Triazines	N2a-APP695 (A β ₄₂ fold change)	CHO-7PA2 (A β ₄₂ fold change)
1	Atrazine	2.39 ± 0.17	1.64 ± 0.08
2	Simazine	1.42 ± 0.13	1.04 ± 0.12
3	Propazine	2.18 ± 0.42	1.15 ± 0.07
4	Cyanazine	1.39 ± 0.11	0.93 ± 0.07
5	Terbuthylazine	2.91 ± 0.26	1.92 ± 0.19
6	Sebuthylazine	2.65 ± 0.17	1.89 ± 0.14
7	Atraton	1.29 ± 0.10	1.21 ± 0.15
8	Terbumeton	2.66 ± 0.20	2.57 ± 0.12
9	Prometon	2.05 ± 0.32	2.00 ± 0.17
10	Simetryn	2.09 ± 0.23	1.86 ± 0.18
11	Ametryn	3.39 ± 0.31	3.39 ± 0.25
12	Prometryn	5.63 ± 0.63	5.65 ± 0.17
13	Dipropetryn	10.03 ± 0.97	5.51 ± 0.62
14	Terbutryn	7.23 ± 0.56	6.29 ± 0.95
15	Cybutryne	6.81 ± 0.81	5.71 ± 0.37
16	Aziprotryn	2.16 ± 0.29	1.73 ± 0.04
17	Desmetryn	1.60 ± 0.13	1.76 ± 0.23
18	Dimethametryn	6.67 ± 0.62	5.52 ± 0.48
19	Methoprotryn	2.72 ± 0.21	2.88 ± 0.11
20	Hydroxyatrazine	1.02 ± 0.14	1.02 ± 0.07
21	Desethylhydroxy-atrazine	1.04 ± 0.19	1.09 ± 0.10
22	Ammeline	0.96 ± 0.24	0.93 ± 0.10
23	Didealkylatrazine	0.86 ± 0.15	0.79 ± 0.06
24	Desethylatrazine	1.34 ± 0.19	1.22 ± 0.11
25	Desisopropylatrazine	1.31 ± 0.26	1.15 ± 0.09
26	Hexazinone	0.90 ± 0.19	1.08 ± 0.15
27	Terbumeton-desethyl	1.28 ± 0.22	1.26 ± 0.14
28	Thifensulfuron-methyl	0.85 ± 0.09	0.97 ± 0.14
29	Iodosulfuron-methyl-sodium	0.99 ± 0.12	1.05 ± 0.04
30	Chlorosulfuron	0.91 ± 0.08	1.05 ± 0.17
31	Tribenuron-methyl	1.21 ± 0.14	1.11 ± 0.06
32	Triasulfuron	0.85 ± 0.21	0.92 ± 0.13
33	Triflusulfuron-methyl	0.82 ± 0.12	0.78 ± 0.08
34	Cinosulfuron	0.77 ± 0.11	0.82 ± 0.16
35	Metsulfuron-methyl	0.69 ± 0.11	0.86 ± 0.11
36	Trietazine	0.46 ± 0.34	0.85 ± 0.21
37	TTBP-TAZ	1.13 ± 0.09	1.01 ± 0.14
38	Aftin-5	6.24 ± 0.67	4.34 ± 0.61

Table S2. Effect of 37 triazines on extracellular amyloid A β ₄₂ production by N2a-APP695 and CHO-7PA2 cells. Cells were treated with 100 μ M of each compound for 18 h and cell supernatants were collected for extracellular A β ₄₂ levels measurement by an ELISA assay. Aftin-5 was used as positive control and corresponding volume of vehicle (DMSO) was used as a negative control. Levels are expressed as fold change \pm standard error of A β ₄₂ level over the A β ₄₂ level of control, vehicle-treated cells. Average of two experiments performed in triplicate (representative of four independent experiments). In bold, products inducing >3 fold increase of A β ₄₂ production. TTBP-TAZ, 2,4,6-tris(2,4,6-tribromophenoxy)-1,3,5-triazine. Products were tested at 1, 10 and 100 μ M, but only results with 100 μ M are shown here to also show the inactive products. A dose-dependent effect was often seen with the active compounds.



#	Triazines	R ₂	R ₄	R ₆	N2a-APP695 (A β ₄₂ fold change)
45	AA9-E02 *	-NH-(CH ₂) ₂ - (OCH ₂ CH ₂) ₂ -NH ₂	-(CH ₂) ₂ -Ph	-NH- <i>i</i> -Pr	1.29 ± 0.01
46	AA9-E03	"	-(CH ₂) ₂ -Ph	-NH-C ₄ H ₉	3.14 ± 0.15
47	AA9-E04	"	-(CH ₂) ₂ -Ph	-N-(C ₄ H ₉) ₂	1.59 ± 0.06
48	AA9-E05	"	-(CH ₂) ₂ -Ph	-NH-cyclohexyl	2.31 ± 0.15
49	AA9-F03	"	-CH ₂ -Bn- <i>p</i> -OCH ₃	-NH-C ₄ H ₉	5.63 ± 0.16
50	AA9-F08	"	-CH ₂ -Bn- <i>p</i> -OCH ₃	-piperidine	3.37 ± 0.17
51	AA9-G03	"	-Ph- <i>p</i> -OCH ₃	-NH-C ₄ H ₉	4.29 ± 0.32
52	AA9-G04	"	-Ph- <i>p</i> -OCH ₃	-N-(C ₄ H ₉) ₂	2.20 ± 0.13
53	AA9-G05	"	-Ph- <i>p</i> -OCH ₃	-NH-cyclohexyl	2.74 ± 0.05
54	AA9-G06 *	"	-Ph- <i>p</i> -OCH ₃	-NH-CH ₂ -CH=CH ₂	2.34 ± 0.38
55	AA9-G07	"	-Ph- <i>p</i> -OCH ₃	-NH-CH ₂ -(2- tetrahydrofuran)	3.07 ± 0.39
56	AA9-G08	"	-Ph- <i>p</i> -OCH ₃	-piperidine	2.63 ± 0.43
57	AA9-H03	"	-CH ₂ -CH(OH) ₂	-NH-C ₄ H ₉	2.68 ± 0.40
58	AA10-B02	"	-CH ₂ -Bn- <i>p</i> -OCH ₃	-NH-C ₈ H ₁₇	2.88 ± 0.12
59	AA10-B10	"	-CH ₂ -Bn- <i>p</i> -OCH ₃	-NH- <i>p</i> -F-Bn	4.43 ± 0.11
60	AA10-C10	"	-Ph- <i>p</i> -OCH ₃	-NH- <i>p</i> -F-Bn	2.28 ± 0.36
61	AA11-A09	"	-CH ₂ -Bn	-NH-CH(isopropyl)- CO-OCH ₃	3.29 ± 0.58
62	AA11-B08	"	-CH ₂ -Bn- <i>p</i> -OCH ₃	-NH-CH(benzyl)-CO- OCH ₃	2.71 ± 0.47
63	AA11-B09	"	-CH ₂ -Bn- <i>p</i> -OCH ₃	-NH-CH(<i>i</i> -Pr)-CO- OCH ₃	3.03 ± 0.17
64	AA11-E08	"	-CH ₂ -Bn	- <i>p</i> -tBut-Ph	2.27 ± 0.15
65	AA11-F08	"	-CH ₂ -Bn- <i>p</i> -OCH ₃	- <i>p</i> -tBut-Ph	2.14 ± 0.10
66	A1	-S-(CH ₂) ₂ - (OCH ₂ CH ₂) ₃ -NH ₂	-NH- <i>i</i> -Pr	-NH- <i>i</i> -Pr	8.17 ± 0.37
67	B1	-S-(CH ₂) ₂ - (OCH ₂ CH ₂) ₄ -NH ₂	-NH- <i>i</i> -Pr	-NH- <i>i</i> -Pr	4.17 ± 0.89
68	D1	-NH-(CH ₂) ₂ - (OCH ₂ CH ₂) ₂ -NH ₂	-NH- <i>i</i> -Pr	-NH- <i>i</i> -Pr	2.22 ± 0.15
69	C1	-NH-(CH ₂) ₂ - (OCH ₂ CH ₂) ₃ -NH ₂	-NH- <i>i</i> -Pr	-NH- <i>i</i> -Pr	2.63 ± 1.08
70	Aftin-5L				5.10 ± 0.57

Table S3. Effect of 21 products selected from a triazine library on extracellular Amyloid A β ₄₂ production by N2a-APP695. N2a-APP695 cells were first treated with 10 and 100 μ M of 236 triazines for 18 h and cell supernatant was collected for extracellular A β ₄₂ levels measurement by an ELISA assay. The library was screened in triplicate, and active products (> 3 fold increase in A β ₄₂ production) were tested at 100 μ M on N2a-APP695 cells in three independent experiments. Levels are expressed as fold change of A β ₄₂ level over the A β ₄₂ level of control, vehicle-treated cells. Average \pm errors bars represent standard error of triplicate values. *, 33 μ M. Aftin-5 was used as positive control, and corresponding volume of vehicle (DMSO) was used as a negative control.

3. Supplementary figures

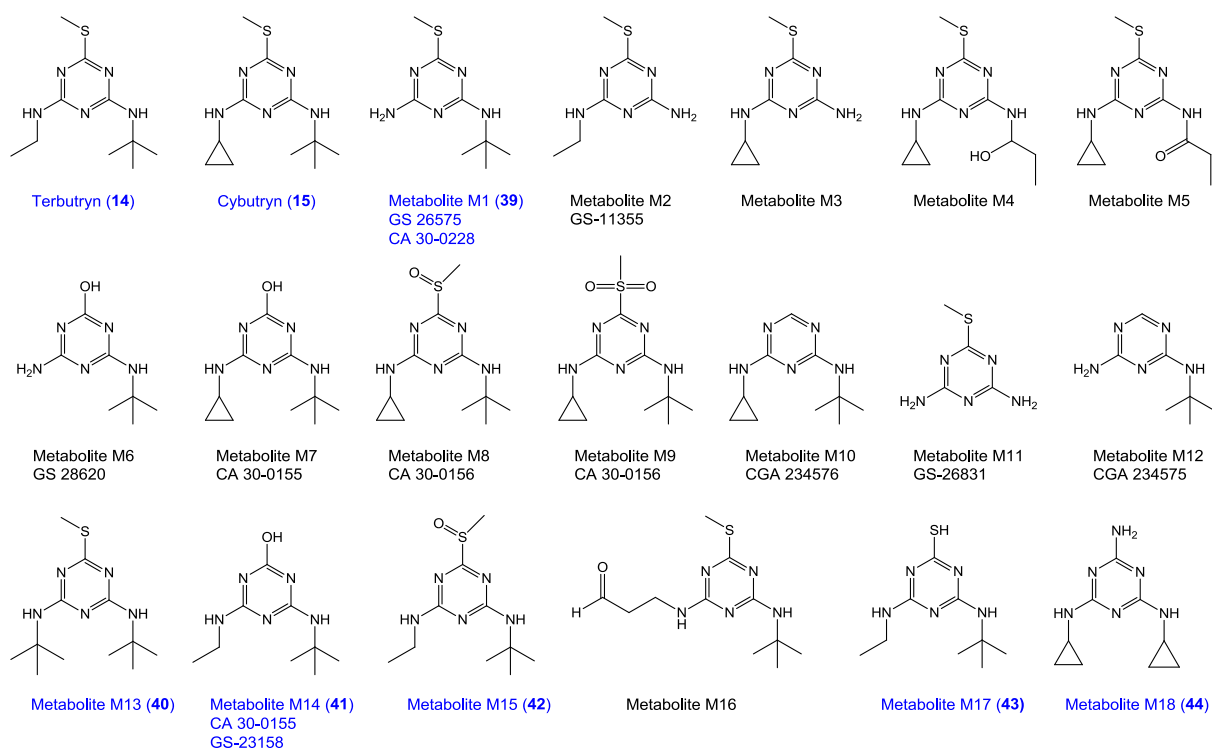
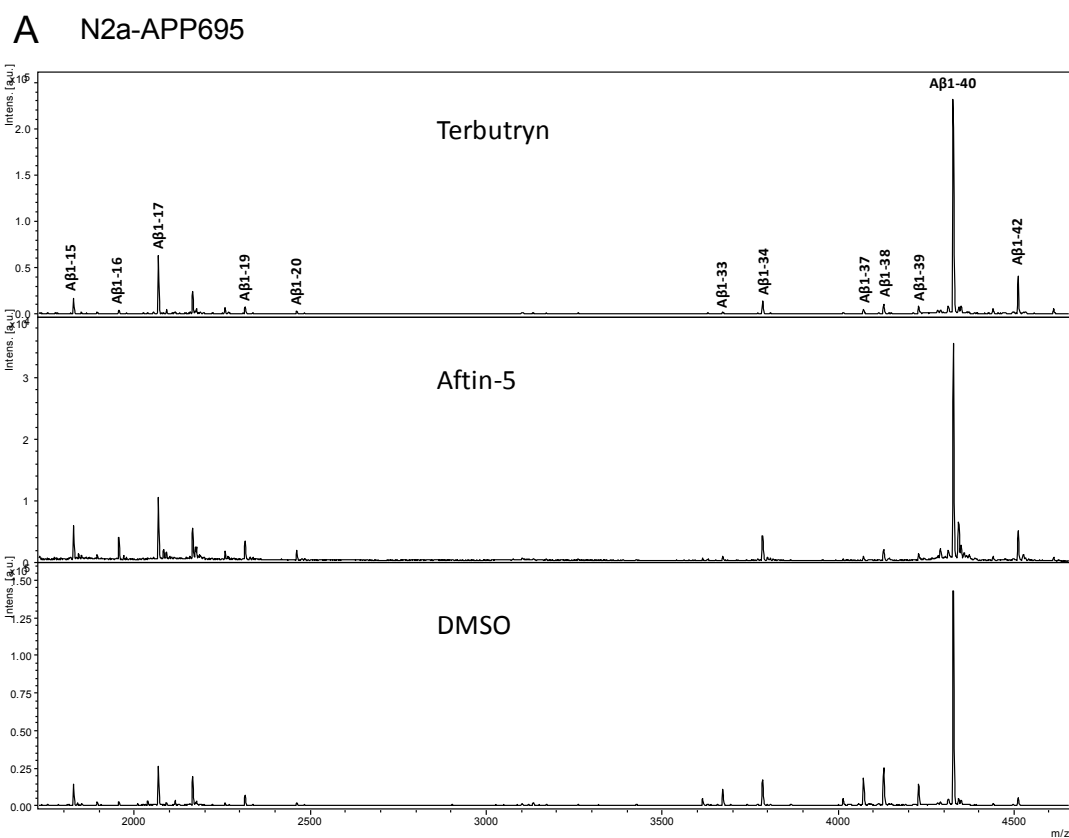


Figure S1. Structure of main terbutryn and cybutryn metabolites. Blue, products which were available for evaluation in the cellular $A\beta_{42}$ production assay.



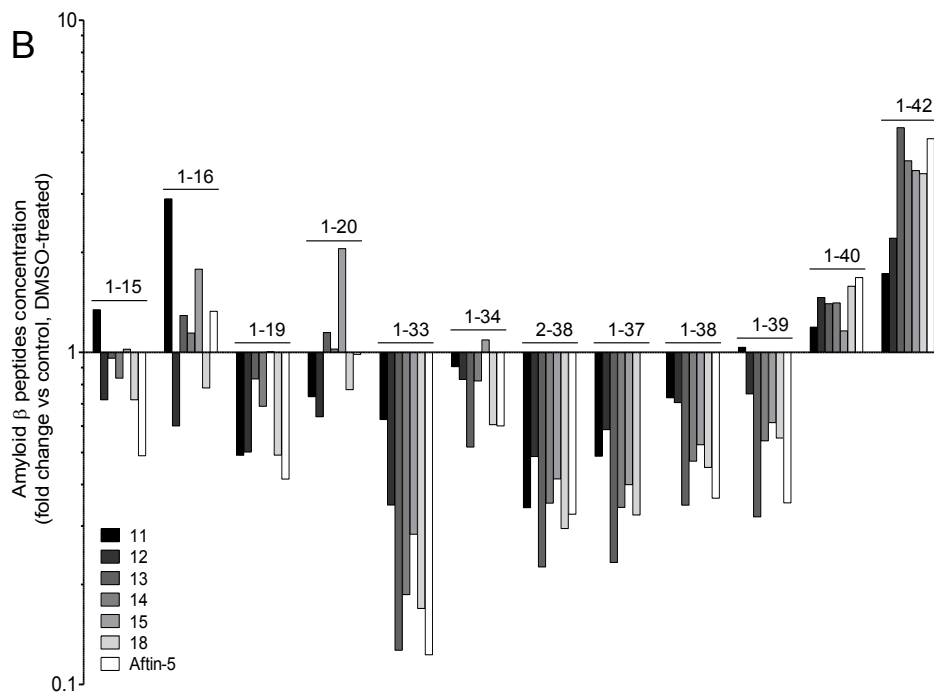


Figure S2. Pattern of amyloid peptides produced by N2a-APP695 cells exposed to triazines. Cells were treated for 18 h with DMSO, 100 μ M of each triazine or aftin-5. Cell supernatants were collected and analyzed as described. **A.** Example spectra of supernatants amyloid profiles from N2a-APP695 cells exposed to DMSO, aftin-5 or terbutryn. **B.** Quantification of all amyloid peptides in N2a-APP695 cell supernatants (Log of fold change in triazine or aftin-5 treated cells over control, DMSO-treated cells).

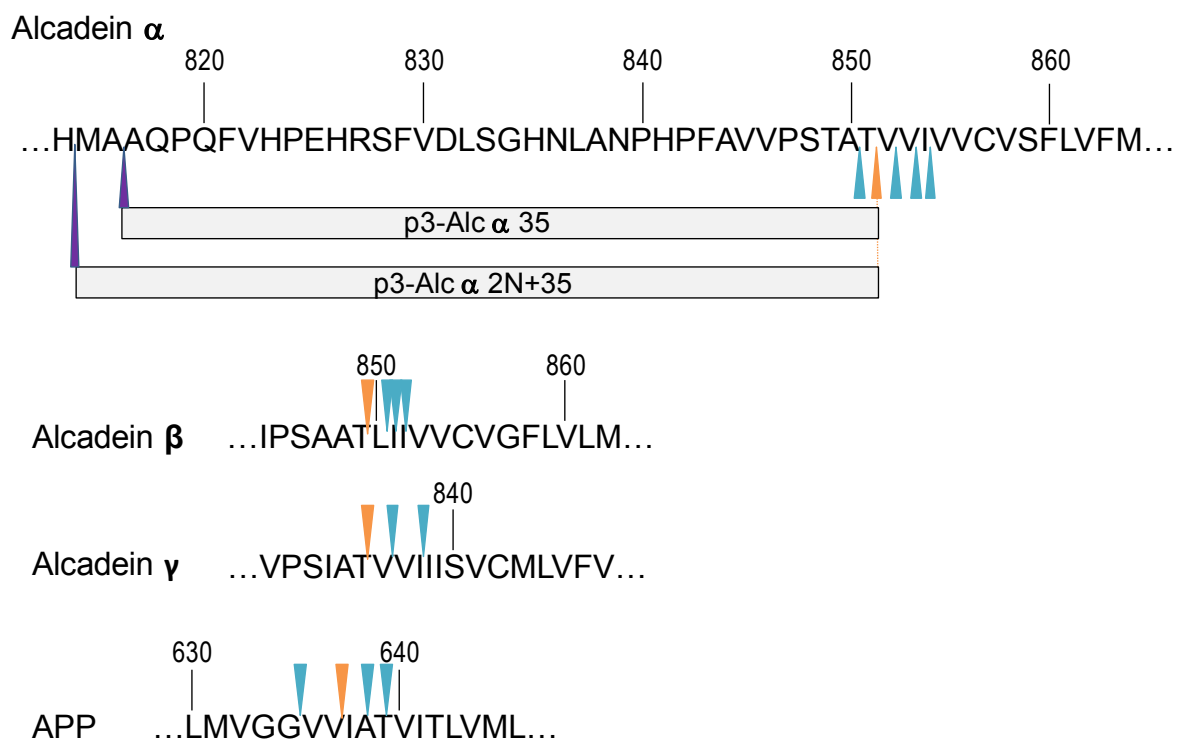


Figure S3. γ -Secretase cleavage sites in Alcadeins and A β PP.