

# Blockade of the Interaction of Calcineurin with FOXO in Astrocytes Protects Against Amyloid- $\beta$ -Induced Neuronal Death

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## **ABSTRACT**

Astrocytes actively participate in neuro-inflammatory processes associated to Alzheimer's disease (AD), and other brain pathologies. We recently showed that an astrocyte-specific intracellular signaling pathway involving an interaction of the phosphatase calcineurin with the transcription factor FOXO3 is a major driver in AD-associated pathological inflammation, suggesting a potential new druggable target for this devastating disease. We have now developed decoy molecules to interfere with calcineurin/FOXO3 interactions, and tested them in astrocytes and neuronal co-cultures exposed to amyloid- $\beta$  (A $\beta$ ) toxicity. We observed that interference of calcineurin/FOXO3 interactions exerts a protective action against A-induced neuronal death and favors the production of a set of growth factors that we hypothesize form part of a cytoprotective pathway to resolve inflammation. Furthermore, interference of the A-induced interaction of calcineurin with FOXO3 by decoy compounds significantly decreased amyloid- $\beta$  protein precursor (A $\beta$ PP) synthesis, reduced the A $\beta$ PP amyloidogenic pathway, resulting in lower A levels, and blocked the expression of pro-inflammatory cytokines TNF $\alpha$  and IL-6 in astrocytes. Collectively, these data indicate that interrupting pro-inflammatory calcineurin/FOXO3 interactions in astrocytes triggered by A $\beta$  accumulation in brain may constitute an effective new therapeutic approach in AD. Future studies with intranasal delivery, or brain barrier permeable decoy compounds, are warranted.

## INTRODUCTION

An intense search for new druggable targets to treat Alzheimer's disease (AD) is underway, as no effective treatment is yet in sight. While analyzing the insulin-like growth factor I (IGF-I) pathway as a paradigm of endogenous neuroprotective signalling underlying responses to brain disease, we recently unveiled an astrocyte-specific pathway involved in resolution of inflammation triggered by diverse types of brain injuries, including AD [1, 2]. This pathway recruited the phosphatase calcineurin and the transcription factor FOXO, both already proposed to be involved in AD pathology [3, 4]. Specifically, we observed that IGF-I disrupts the association of calcineurin with FOXO3 that takes place in response to pro-inflammatory stimuli such as TNF $\alpha$  or amyloid- $\beta$  (A $\beta$ ). Indeed, calcineurin/FOXO3 complexes were found in the brain of AD patients, but not in age-matched non-demented controls [2]. In this way, IGF-I promotes resolution of the neuro-inflammatory cascade by halting downstream activation of the canonical pro-inflammatory transcription factor NF $\kappa$ B [2]. We hypothesized that inhibition of the association of calcineurin with FOXO3 may constitute a feasible target to prevent neuro-inflammation associated to AD and other diseases, and in this way halt or delay neuronal demise.

Using computer-assisted drug design, we discovered a series of decoy compounds that interfere with the association of calcineurin with FOXO3 in response to pro-inflammatory stimuli. Two of these peptide-mimetics showed good neuroprotective profile in an in vitro system mimicking A-induced neuronal injury.

## MATERIALS AND METHODS

### Materials and animals

C57BL6/J (wild type, wt) and APP/PS1 with C57BL6/J background (a kind gift from P. Mouton, NIH) mice were used. Animals were used according to European (86/609/EEC & 2003/65/EC, European Council Directives) guidelines and studies were approved by the Madrid Government Bioethics Committee.

Bacterial lipopolysaccharide (LPS) was from Sigma (St. Louis, USA), and A $\beta$ 40 was from Polypeptide (Strasbourg, France). Antibodies against activated caspase 3 (Cell Signaling, USA, a marker of apoptotic cells), A $\beta$  (6E10 from BioLegend (1/6000), and 82E1 from IBL (1/5000) were used in combination),  $\beta$ 3-tubulin (1/5000; Promega, USA, a neuronal marker), GFAP (Sigma, an astrocyte marker),  $\beta$ -tubulin (1/2000; Sigma), calcineurin (Cell Signaling), FOXO3 (Cell Signaling), IGF-I (Abcam, UK), IGF-II (Abcam), FGF-8 (Santa Cruz, USA), sA $\beta$ PP $\alpha$  (IBL-America, USA), and BSA (Santa Cruz) were all used at a 1/1000 dilution except when indicated otherwise. Secondary antibodies were goat anti-

rabbit or mouse HRP- (1/20000, BioRad, USA) or Alexa Fluor 488 or 594 donkey anti-mouse or rabbit (1/1000, Molecular Probes, USA).

#### Protein expression and purification

Vectors pGEX-6P-1-human Calcineurin and pGEX-4T3-human FOXO3 were purchased from Addgene (plasmids #13251 and #1790). Recombinant calcineurin and FOXO3 GST-fusion proteins were expressed in *E. coli* BL21(DE3) (Invitrogen) strain [5]. Cultures were grown at 280 rpm and 37°C until they reached an OD<sub>595</sub> of 0.5–0.8, and expression was induced by addition of 1 mM IPTG for 4 h. Bacteria cells were then lysed with 1 mg/ml lysozyme, 1% Triton X-100, as reported previously [6] with modifications, such as the addition of 0.5% Tween-20 and sonication pulses. Recombinant proteins were purified by GST-affinity chromatography using GSTrap HP columns (GE Healthcare, USA) on an FPLC apparatus (ÄKTA Purifier, GE Healthcare), with a buffer system consisting of 10 mM sodium phosphate, 0.14 M NaCl, pH 7.4 and 50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0 for binding and elution, respectively. A size-exclusion chromatography on a HiLoad 16/60 200 PG column (GE Healthcare) using PBS pH 7.4 was performed after GST-affinity chromatography purification. For both proteins, ~90% purity at the end of the purification procedures was reached, as estimated by SDS-PAGE electrophoresis. Finally, the purified fractions were concentrated (up to 50 M) and the buffer exchanged to 1 mM pH 7.4 by ultrafiltration using Amicon Ultra-15 10K filters (Millipore, Germany), prior to performing the pull-down assays. Protein concentration was determined by absorbance at 280 nm using its calculated molar extinction coefficient.

#### Pull-down assays

GST fused to wild type calcineurin or wild type FOXO3 proteins were employed as baits in pull-down experiments. Glutathione Sepharose 4B prepack columns (GE Healthcare) were washed with binding buffer (PBS pH 7.4), and equilibrated with PBS, 1% Triton X-100 for 30 min at room temperature. One ml of lysates of CHO K1 cells transfected with calcineurin (a kind gift of JM Redondo, CNIC) or FOXO3 (Addgene) mutant constructs was added to the GST fusion protein columns for 30 min. Columns were then washed five times with binding buffer, and the bound protein was eluted in 50 mM Tris-HCl, 10 mM reduced glutathione pH 8.0, dissolved in 5% β-mercaptoethanol Laemli buffer and analyzed by western blotting.

#### Virtual screening

Our strategy for the discovery of new hits was carried out by virtual screening, using as template a pharmacophore defined from the analysis of the crystal structure of the complex

of calcineurin with the calcineurin-inhibiting domain of the African Swine Fever Virus Protein A238L (entry 4F0Z of the pdb)[7]. In this structure, the A238L peptide competitively inhibits calcineurin by occupying the critical substrate recognition site  $\phi$ LxVP, while leaving the catalytic center fully accessible (Fig. 1A).

Analysis of the crystal structure allowed identifying several chemical groups putatively critical for the peptide-calcineurin interaction. Accordingly, we defined a five point pharmacophore that consisted in two hydrogen bond acceptors points (F1 and F2), one hydrogen bond donor (F3), and two hydrophobic or aromatic points (F4 and F5). An exclusion volume was added to simulate the receptor surface, in order to avoid molecules clashing with the receptor. The pharmacophore was used as query for a search on the lead-like compound database of the MOE program (ca. 650.000 compounds) [8]. The search yielded a total of 3285 hits that fulfilled the pharmacophore hypothesis. These molecules were subsequently submitted to a docking process onto the  $\phi$ LxVP site, using the program Glide with the SP scoring function [9]. Molecules in their bioactive conformations were subsequently analyzed for pharmacophore fulfillment, revealing that only 509 molecules fulfilled at least four pharmacophoric points. Of this subset, only those with a docking score (SP) greater than 5 were subsequently grouped into 20 clusters, following a hierarchical clustering analysis. For this purpose, molecules were classified by means of a three-point pharmacophore fingerprint, and their distance measured by the Tanimoto coefficient, using the program Canvas of Schrödinger software [10]. From the cluster analysis we selected one representative of each cluster for testing. However, only 11 molecules could be acquired and tested (compounds A CN 001, A CN 002, A CN 003, A CN 005, A CN 006, A CN 008, A CN 009, A CN 010, A CN 011, A CN 013, and A CN 014). Based on the results, another 10 compounds were selected from the clusters where active compounds had been found (compounds A CN 005 and A CN 006): A CN 15 to A CN 24. Finally, we selected 6 additional compounds structurally related to the most active compound found (A CN 006): A CN 027 to A CN 32.

#### In vitro cultures

Pure astroglial cultures (>95% GFAP+, OX-42-, A2B5- cells) were prepared as described [1]. Briefly, postnatal (day 3–4) mice were rapidly decapitated to minimize suffering, and their brains dissected and immersed in ice-cold DMEM/F-12 (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Cortex and hippocampus were removed and cut into 1 mm pieces. Tissue fragments were dissociated mechanically, and the resulting cell suspension was centrifuged (1000 rpm/10 min) and plated in DMEM/F-12 with 10% FBS,  $5 \times 10^5$  cells per well. Cultures were allowed to grow for 2 days. Pure neuronal cultures

were obtained as described [1]. Astrocyte-neuron co-cultures were prepared as follows: Astrocytes were plated, and when they were 70% confluent, the medium was removed and neurons were plated onto them with freshly added medium (Neurobasal+B27 (Invitrogen), 4 mM glutamine, and 25 mM KCl). Cultures were allowed to grow for 7 days. In experiments using wild type (wt) cultures, cells were exposed for 16 h to 10 nM LPS or 2  $\mu$ M A $\beta$  prior to adding decoy compounds, and cell death was assessed 2 h later. Cytotoxicity was determined with the MTT assay following the manufacturer's instructions.

#### Immunofluorescence

Immunocytochemistry was performed as described [11]. Cells were plated on 20 mm coverslips and fixed. Coverslips were blocked with 5% normal horse serum, and incubated overnight at 4°C with the respective primary antibody in phosphate buffer (PB) containing 0.1% bovine albumin, 3% horse serum, and 0.2% Triton X-100. After several washes in PB, sections were incubated with an Alexa Fluor 488 or 594 donkey anti-mouse or rabbit (1/1000, Molecular Probes). Omission of primary antibody was used as control. Neuronal death was determined by a single investigator in blinded cultures, as described [12]. Confocal analysis was performed in a Leica microscope (Leica Microsystems, Germany).

#### Immunoassays

Cultured cells were homogenized in ice-cold buffer consisting of 10 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP40, 1 mM sodium orthovanadate, and a protease inhibitor cocktail (Sigma) plus 2 mM PMSF. Western blot and immunoprecipitation were performed as described [1]. Western blot membranes were reblotted with either the same antibody used for immunoprecipitation, or with anti- $\beta$ -tubulin or anti-bovine serum albumin as internal standards, and to normalize for protein load. Levels of the protein under study were expressed relative to protein load in each lane. Densitometric analysis was performed using Quantity One software (BioRad).

#### Quantitative PCR

The first strand cDNA was synthesized from RNA using the RT<sup>2</sup> First Strand Kit (SABiosciences). The  $\Delta\Delta$ Ct method was used to analyze the expression level of each gene using the Data Analysis Template provided by SABiosciences. After PCR, the dissociation curve for each gene was examined to exclude those with nonspecific amplification, or with undetectable expression. Quantitative real-time PCR was performed using an ABI Prism 7500 detection System (Applied Biosystems).

#### Statistics

Statistical analyses were performed with a t-test when comparing two groups, and a two-way ANOVA for comparing multiple groups. Statistical significance was established when  $p < 0.05$ . Results shown are mean  $\pm$  SEM.

## RESULTS

Using pull-down assays with several FOXO3 and calcineurin mutants, we mapped regions involved in FOXO3-calcineurin interaction at residues 468–673 of FOXO3 and 347–521 of calcineurin (Supplementary Figure 1A). We then used computer-assisted design to discover a series of decoy compounds directed to interfere with the binding region of calcineurin with FOXO3. Different series of such compounds (ACN01–ACN032) were tested in cultured astrocytes under inflammatory challenge, using LPS (10 nM) to promote association of calcineurin with FOXO3 [1]. Using this assay, we identified compounds ACN018 and ACN030, -two ((1,3,4-oxadiazol-2-yl) methyl) carbamate analogs with diverse substituents, displaying the greatest inhibition on calcineurin/ FOXO3 interactions (Fig. 1B), without affecting cell viability (Supplementary Figure 1B).

Next, we tested the capacity of these compounds to interfere with A $\beta$ -induced neuronal death. We hypothesized that if they are able to counteract pro-inflammatory signaling in astrocytes and re-direct them toward resolution of inflammation, treated astrocytes would become more neuroprotective. Importantly, under culture conditions only neurons die upon A $\beta$  challenge, as indicated by neuronal-specific immunostaining of activated caspase 3, a marker of apoptosis (Fig. 1C). On the other hand, neurons exposed to A $\beta$  do not show calcineurin/FOXO complexing (Fig. 1D). The latter indicates that calcineurin/FOXO3 complexes in co-cultures exposed to pro-inflammatory challenge originate only in astrocytes, confirming previous observations of an essential role of astrocytes in mediating A $\beta$  neurotoxicity in culture [13]. When either compound 018 or 030 were added to co-cultures exposed to A $\beta$  (2  $\mu$ M), levels of activated caspase 3 drastically decreased, as compared to control co-cultures (Fig. 1E). Furthermore, protection against A $\beta$ -induced neuronal death provided by these compounds was linked to a decrease in the reported A $\beta$ -induced increase in A $\beta$ PP [14], and in its resultant increased amyloidogenic processing. Thus, as shown in Fig. 2, A $\beta$ PP mRNA and A $\beta$  levels were reduced after addition of 018 or 030. To confirm an effect of 018 and 030 on A $\beta$ PP/A $\beta$  levels, we treated astrocyte-neuronal co-cultures obtained from APP/PS1 mice, that produce abnormally high levels of A $\beta$ PP [15], with these compounds. We confirmed that levels of A $\beta$ PP mRNA and A $\beta$  were also significantly reduced (Fig. 2).

When calcineurin/FOXO3 interactions are interfered in vivo after inflammatory injury, brain levels of different neurotrophic factors greatly increased [2]. As indicated above, we

consider that this neurotrophic response forms part of the resolution of inflammation. Thus, we tested in cultured astrocytes whether a similar neurotrophic effect is observed when calcineurin/FOXO3 interactions are interfered with decoy compounds. We measured IGF-I, IGF-II, and FGF-8 because they were previously found to show highest increases after calcineurin/FOXO3 uncoupling [2]. Since interfering with calcineurin/FOXO3 interactions modulates A $\beta$ PP processing (Fig. 2), we also determined the effect of compounds 018 and 030 on sA $\beta$ PP $\alpha$  levels, a neurotrophic derivative of A $\beta$ PP [16] that modulates A $\beta$ PP processing [17]. As shown in Fig. 3, we found that cultured astrocytes exposed to A $\beta$  showed increased levels of these neurotrophic proteins when either decoy compound was added. Moreover, in astrocytes from APP/PS1 mice, these neurotrophic factors also increased in response to compounds 018 and 030 (Fig. 3C). Accordingly, expression of pro-inflammatory cytokines TNF $\alpha$  and IL-6 in astrocytes, that was increased in response to A $\beta$  challenge, was also attenuated. The inhibitory action of these compounds was seen both in wild type astrocytes exposed to A, and in astrocytes from APP/PS1 mice (Fig. 4).

## DISCUSSION

The present results indicate that interfering calcineurin/FOXO interactions, that take place during pro-inflammatory activation in astrocytes, using decoy molecules, may represent a feasible new approach to treat neuro-inflammatory damage associated to brain amyloidosis in AD. An advantage of this approach is that association of calcineurin with FOXO3 in response to inflammatory stimuli does not take place in neurons, which reduces possible off-target effects. Our observations lend support to future studies using either intranasal delivery of this type of decoy compounds or, alternatively, develop similar compounds able to cross the blood-brain barrier for systemic administration. Because previous *in vivo* models showed that inhibition of calcineurin/FOXO interactions is sufficient to significantly delay brain amyloidosis, and prevent, or even revert, cognitive decline in AD mice [2], we anticipate similar therapeutic actions for these type of decoy compounds when used *in vivo*. Furthermore, the present results indicate that the interaction between calcineurin and FOXO3 in astrocytes is also instrumental in neuronal death elicited by A $\beta$ , which opens the possibility that the use of similar compounds in AD patients may also halt neuronal death. However, this possibility will require additional studies.

Until now, anti-inflammatory approaches to treat AD have failed [18]. This strategy was initiated as a result of epidemiological observations supporting a role of inflammation in AD, as patients receiving non-steroidal anti-inflammatory compounds showed protection against the disease [19]. Diverse short-comings may account for a lack of effect of anti-inflammatory therapies in AD patients. Thus, depending on the anti-inflammatory used,



very different outcomes on inflammatory pathways may be expected. Further, regulation of inflammatory cytokines is context- and stage-dependent, so established tauopathy or functional disturbances related to neuronal loss may not be counterbalanced [20]. However, the strategy that we propose to use is based on cell-specific (astrocytes) pro/anti-inflammatory pathways, which until now has not been considered. Activation of calcineurin is important in the response to pro-inflammatory stimuli such as TNF $\alpha$  [21], and we previously documented that calcineurin activation participates in both progression and resolution of inflammation, depending on upstream activators such as TNF $\alpha$  or IGF-I, respectively [1]. By inhibiting the interaction of calcineurin with FOXO3, this pathway is re-directed towards resolution of inflammation. Lowering inflammation will reduce A $\beta$ PP synthesis [22, 23], as we now observed. In turn, an increased output of neuroprotective mediators will probably reduce amyloidogenic A $\beta$ PP processing [2, 22]. Indeed, increased levels of sA $\beta$ PP $\alpha$  together with decreased production of A $\beta$  peptides were detected. sA $\beta$ PP $\alpha$  also inhibits BACE-1 activity, which in turns will reduce A $\beta$  levels [24], as we observed now in cultured astrocytes.

In summary, pharmacological manipulation of an astrocyte-specific pro-inflammatory pathway that interferes with the association of the phosphatase calcineurin with the transcription factor FOXO3, protects neurons against A-induced cell death. Cell-specific anti-inflammatory drugs may open a new avenue to treat neuro-inflammation associated to AD and other neurodegenerative diseases.

#### ACKNOWLEDGMENTS

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## CAPTIONS TO FIGURES

Figure 1. Decoy compounds that interfere with calcineurin and FOXO3 interactions. A) 3D model of the complex of calcineurin with the calcineurin-inhibiting domain of the African swine fever virus protein A238L. The position of the substrate recognition site  $\phi$ LxVP is shown. Calcineurin A (CNA) and B (CNB) moieties are indicated. B) Example of the screening system used to identify compounds that interfere with the association of calcineurin with FOXO3 upon stimulation of astrocytes with LPS (10 nM). ACN compounds 018 and 030 displayed the highest activity. Lanes: 1: vehicle (DMSO), 2: Dexamethasone+LPS, 3: vehicle+LPS, 4: ACN015+LPS, 5: ACN016+LPS, 6: ACN017+LPS, 7: ACN018+LPS, 8: Vehicle, 9: Dexamethasone+LPS, 10: Vehicle+LPS, 11: ACN026+LPS, 12: ACN029+LPS, 13: ACN030+LPS. All compounds were tested at 10  $\mu$ M. C) Neurons, but not astrocytes, die after challenged with A $\beta$  (2  $\mu$ M). Thus, activated caspase 3 staining (red) was found only in neuronal cultures ( $\beta$ 3-tubulin+ cells in green) but not in astrocytes (GFAP+, in green). D) Neurons (Neu) in culture do not show association of calcineurin with FOXO3 after LPS or A $\beta$  challenge. Cultured astrocytes (As) that show association of FOXO3 with calcineurin were used as positive controls. E) Compounds ACN 018 and 030 showed the greatest inhibitory activity on A $\beta$ -induced neuronal death. Neurons were cultured with astrocytes and challenged with A $\beta$  (2  $\mu$ M). Twenty-four hours later activated caspase levels were measured by western blot in culture lysates. Bars in the right show quantification of blots. Lanes: 1: Control, 2,3: A $\beta$ , 4,5: ACN018+A $\beta$ , 6,7: ACN030+A, 8: ACN030. (\*\*p < 0.01 versus A $\beta$  and \*\*\*p < 0.001 versus control, n = 3). All compounds were tested at 10  $\mu$ M. When given alone, neither compound affected neuronal viability (not shown).

Figure 2. Interfering calcineurin/FOXO3 interactions reduces levels of A $\beta$ PP mRNA and its amyloidogenic processing. A) ACN018 and 030 reduce levels of A $\beta$ PP mRNA in wild type astrocyte-neuronal co-cultures exposed to A, and in astrocyte-neuronal co-cultures from APP/PS1 mice, B) Similarly, ACN018 and 030 reduce levels of A $\beta$  in wild type astrocyte-neuronal co-cultures exposed to A $\beta$  and in astrocyte-neuronal co-cultures from APP/PS1 mice. Representative blots using in combination anti-A $\beta$  antibodies 6E10 and 82E1. Monomer and dimer forms of A $\beta$  are shown. Quantitation histograms are shown below. (\*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001 versus control, n = 5).

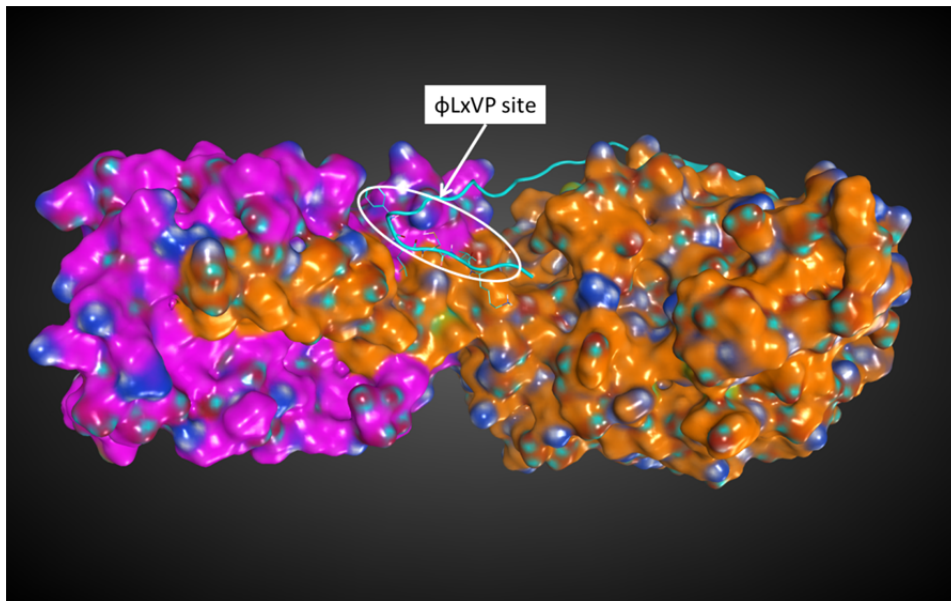
Figure 3. ACN018 and 030 stimulate the release of neurotrophic factors in astrocytes exposed to inflammatory challenge. A, B) In the presence of LPS (A) or A $\beta$  (B), ACN018 and 030 stimulate the secretion of IGF-I, IGF-II, FGF-8, and sA $\beta$ PP $\alpha$ . C) Astrocytes derived from APP/PS1 mice show enhanced secretion of IGF-I, IGF-II, FGF-8, and

sA $\beta$ PP $\alpha$  when cultured in the presence of ACN018 or 030. Normalization of protein load was performed with bovine serum albumin (BSA) from culture supernatants.

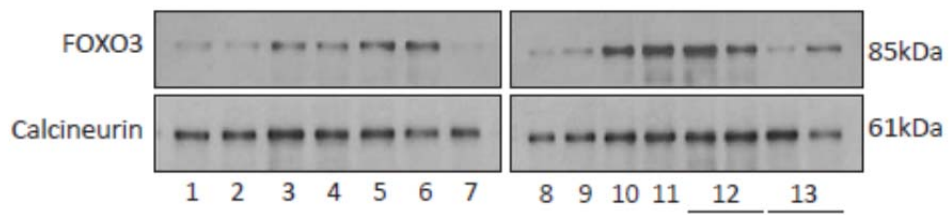
Figure 4. Compounds 018 and 030 decrease mRNA levels of pro-inflammatory cytokines TNF $\alpha$  (left chart) and IL-6 (right) that are increased after exposure of wild type astrocytes to A $\beta$  or in APP/PS1 astrocytes (\*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001 versus control, n = 5).

FIGURE 1

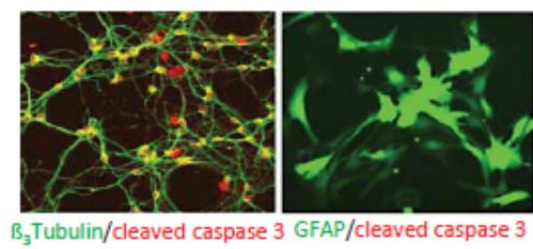
a)



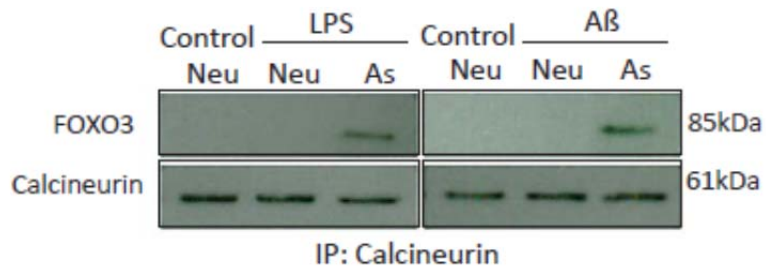
b)



c)



d)



e)

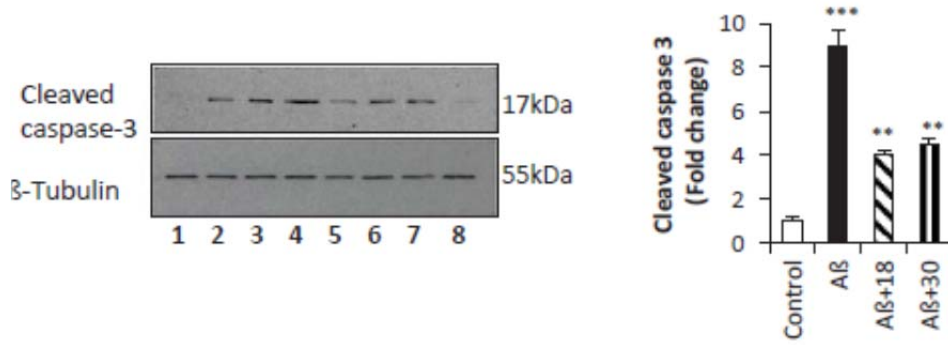




FIGURE 2

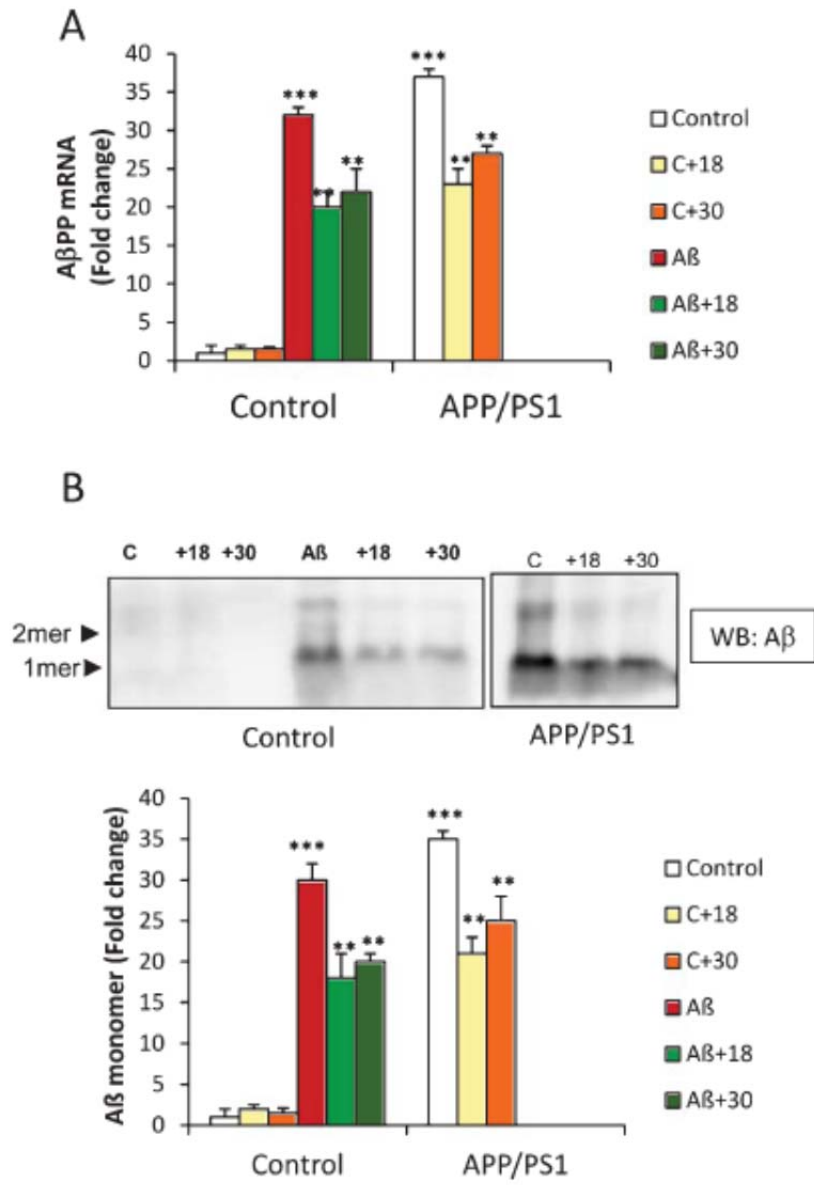


FIGURE 3

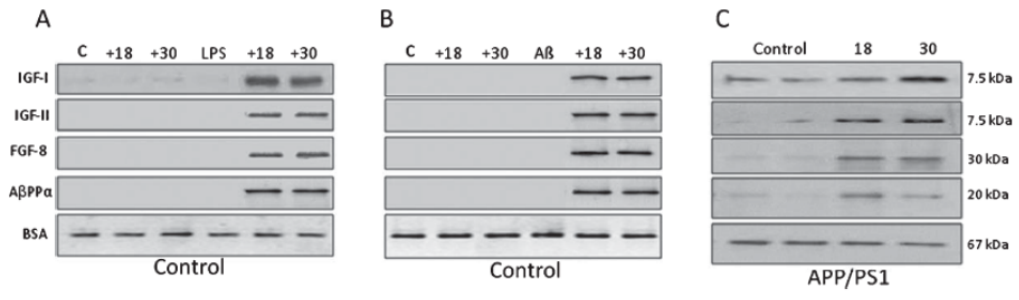


FIGURE 4

