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J. Biol. Chem. published online December 2, 2014

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JBC Papers in Press. Published on December 2, 2014 as Manuscript M114.586602
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M114.586602

FDA approved asthma therapeutic agent impacts amyloid β in the brain in a transgenic model of Alzheimer's disease

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* Running title: *Cromolyn Sodium prevents Aβ aggregation and lowers cerebral Aβ*

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Keywords: Alzheimer's disease, amyloid-beta (Aβ), aggregation, asthma, Cromolyn Sodium, *in vivo* microdialysis

Background: Cromolyn Sodium is a FDA approved drug structurally similar to fisetin, an anti-amyloidogenic molecule.
Results: Cromolyn Sodium interferes with Amyloid β (Aβ) aggregation *in vitro*, while rapidly decreasing the levels of soluble Aβ peptides *in vivo* after a week.
Conclusion: Cromolyn Sodium may have an impact amyloid economy.
Significance: Developing new disease-modifying therapeutics remains an urgent need in the treatment of Alzheimer's disease.

ABSTRACT

Interfering with the assembly of Amyloid β (Aβ) peptides from monomer to oligomeric species and fibrils, or promoting their clearance from brain, are targets of anti-Aβ directed therapies in Alzheimer's disease (AD). Here, we demonstrate that Cromolyn Sodium (disodium cromoglycate), an FDA-approved drug already in use for the treatment of asthma, efficiently inhibits the aggregation of Aβ monomers into higher order oligomers and fibrils *in vitro*, without affecting Aβ production. *In vivo*, the levels of soluble Aβ are decreased by over 50% after only 1 week of daily intraperitoneal administered Cromolyn Sodium. Additional *in vivo* microdialysis studies also show that this compound decreases the half-life of soluble Aβ in the brain. These data suggest a clear effect of a peripherally administered, FDA approved medication on Aβ economy, supporting further investigation of the potential long term efficacy of Cromolyn Sodium in AD.

INTRODUCTION

Therapies to prevent Alzheimer's disease (AD) progression remain a high-unmet medical need. FDA-approved drugs, such as acetylcholinesterase (AChE) inhibitors (donepezil, rivastigmine and galantamine), are indicated for symptomatic relief in patients with mild to moderate AD (1-3). However, none of the molecules currently available efficiently target the underlying causative pathophysiological processes of the disease. The development of successful disease-modifying treatments, in contrast, would have a long-term beneficial outcome on the course of AD

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progression.

The advent and spread of neurotoxic oligomeric aggregates of amyloid β (Aβ) is widely regarded as a key trigger in AD pathogenesis (4), leading to neuronal damage, oxidative stress, mitochondrial dysfunction, synaptic collapse and finally to cognitive deficits (5-10). Aβ peptides (37 to 43 amino acids in length) are formed by sequential cleavage of the native transmembrane amyloid precursor protein (APP) successively by β- and γ-secretases (11). Autosomal dominantly inherited mutations in APP that lead to enhanced Aβ generation (12), or increased oligomerization (13), cause AD, whereas a recently reported APP mutation has been shown to decrease Aβ by ~40% and to be protective (14,15). Aberrant accumulation of Aβ peptides, rather than increased production, is believed to initiate the development of the sporadic form of the disease, which represents the vast majority of the cases (16-18). Misfolded Aβ monomers can aggregate into higher order oligomers, eventually forming fibrils that get deposited into the extracellular space to form fibrillar amyloid neuritic plaques. Aβ oligomers have a longer half-life in the interstitial fluid than monomers, so that their formation influences Aβ levels (19). Aβ oligomers rather than monomers have been shown to be neurotoxic for neurons (20), inhibiting LTP, leading to neuronal stress, abnormal tau phosphorylation, synapse collapse and memory impairment (21-26). Therefore, therapeutic agents able to decrease Aβ levels, prevent oligomer formation, or disaggregate soluble oligomers, may be of therapeutic interest.

Cromolyn Sodium is an FDA-approved drug used in the treatment of asthma. Because of its close structural homology with fisetin (3,3',4',7-tetrahydroxyflavone) (27), a neurotrophic molecule that was previously shown to inhibit amyloid aggregation (28), we hypothesized that Cromolyn Sodium may potentially prevent Aβ oligomerization and promote Aβ clearance *in vitro* and *in vivo*. In addition, the use of Cromolyn Sodium in addiction models (after sub-cutaneous infusion (29,30)), as well as the physical properties of this molecule (MW - 508.38; PSA – 125; and LogP -1.39), argues for Cromolyn Sodium as a blood-brain barrier permeable drug, thus allowing systemic infusion. In the present study, we tested the hypotheses that Cromolyn Sodium can alter Aβ aggregation ¯ter peripheral delivery. We first demonstrated that Cromolyn Sodium efficiently prevents the aggregation of monomeric Aβ peptides into higher order oligomers and fibrils *in vitro*. Importantly, *in vivo* intraperitoneal administration of this compound rapidly decreases the amount of soluble monomeric Aβ in the brain by ~50% after only one week of treatment, and more specifically within the interstitial fluid that contains neurotoxic molecules directly in contact with the neuropil. Using *in vivo* microdialysis, we showed that the half-life of Aβ was significantly reduced in Cromolyn Sodium treated mice compared with PBS exposed animals, suggesting that preventing Aβ oligomerization may favor their clearance from the central nervous system. In addition, both *in vivo* and *in vitro* results suggest that Aβ uptake by microglial cells may be exacerbated in presence of Cromolyn Sodium. Overall, these results suggest that Cromolyn Sodium may be of interest in changing cerebral Aβ metabolism, with potential impact on toxic oligomers.

EXPERIMENTAL PROCEDURES

Chemicals - Cromolyn Sodium U.S.P. grade was purchased from Spectrum Chemical Mfg. Corp. (Gardena, CA.) and dissolved in sterile phosphate buffered saline (PBS). A stock solution of 100 mM was used for *in vitro* experiments, and of 10.2 mM for *in vivo* administration. *In vitro*, Cromolyn Sodium stock solution was directly diluted in the cell culture media at final concentrations of 10 nM, 10 μM or 1 mM, while a solution of 1.02 mM of the compound was prepared in Dulbecco's Phosphate Buffer saline (DPBS) before intraperitoneal injection *in vivo* (at three different doses: 1.05 mg/kg, 2.1 mg/kg or 3.15 mg/kg body weight). *In vitro* amyloid fibrillization assay was performed using synthetic Aβ peptides (rPeptide, Bogart, GA) as well as Thioflavin-T (Sigma-Aldrich), respectively dissolved in DMSO and in methanol. For the *in vitro* efflux and microglial uptake assay, synthetic $Aβ_{40}$ and $Aβ_{42}$ peptides were purchased from Peptide Institute, Inc. After resuspension in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Kanto Chemical) at a concentration of 1 mg/ml, the peptides were dried, resolubilized in PBS containing 2% (V/V) Me_2SO (Kanto

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Chemical) and filtered through a 0.2 mm filter, as previously described (31). The stock solution of $A\beta_{40}$ and $A\beta_{42}$ peptides were diluted to 5 μM and stored at -80°C prior to use. $A\beta_{40}$ or $A\beta_{42}$ were applied at 50 nM in cell cultures.

In vitro Aβ fibrillization, oligomerization and dissociation assays - *In vitro* fibrillization assay was performed using $A\beta_{40}$ or $A\beta_{42}$ peptides dissolved in DMSO at a concentration of 250 μM and sonicated for 1 min. $A\beta_{40}$ or $A\beta_{42}$ were diluted to 5 μM in an assay volume of 200 μl with artificial CSF solution (125 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1.25 mM NaH2PO4, 2 mM CaCl2, 25 mM NaHCO3, and 25 mM Glucose, pH 7.3) in 96 well plate (Corning, Tewksbury, MA). After addition of 10 μM Thioflavin-T and increasing concentrations of Cromolyn Sodium (5 nM, 50 nM and 500 nM), the fibrillization process was initiated by adding 0.5 mg/ml of Heparin sulfate (Sigma, St. Louis MO). DMSO was used as control. The progression of fibrillization was followed every 10 min for 60 min at room temperature by measuring the fluorescence intensity at excitation and emission wavelengths of 450 nm and 480 nm, respectively, using an M3 microplate reader (Molecular Devises, Sunnyvale, CA). The results were normalized for background using fluorescent reading at time 0 by the software provided by the M3 plate reader.

Aβ oligomerization and oligomer dissociation assays were performed *in vitro* using an Aβ split-luciferase complementation assay, as described previously (32). To evaluate the effect of Cromolyn Sodium on the formation of Aβ oligomers, a HEK293 cell line designed to stably overexpress the N- and C-terminal fragments of *Gaussia* luciferase (Gluc) conjugated to $A\beta_{42}$ was incubated without or with Cromolyn Sodium at 10 nM, 10 μM or 1 mM for 12 hours at 37°C. The conditioned media from these cells was collected, 10 mM of coelenterazine (NanoLight technology) was added and the luciferase activity was measured using a Wallac 1420 (PerkinElmer). The oligomer dissociation assay was performed by incubating PBS or Cromolyn Sodium (10 nM, 10 μM or 1 mM) with conditioned media from naïve HEK293 cells overexpressing each half of Gluc fused with $A\beta_{42}$, 12 hours at 37°C. The luciferase activity was measured as previously described (32).

Analysis of $A\beta_{42}$ fibril formation by transmission electron microscopy – The anti-fibrillogenic properties of Cromolyn was confirmed by performing TEM analysis as previously described (33). Briefly, synthetic $A\beta_{42}$ was dissolved in PBS at a concentration of 0.2 mg/ml for 48 hours at 37°C, with or without addition of Cromolyn Sodium at a concentration of either 5nM or 500nM. After incubation for 48 hours, 15μl of the $A\beta_{42}$ fibril solution were adsorbed on carbon-coated EM grids (Electron Microscopy Sciences, Hatfield, PA) for 20 min at room temperature. After 3 washes in sterile PBS and ddH_2O, the grids were allowed to dry before negative staining with 2% (w/v) uranyl-acetate in water, two times for 8 min. Each grid was then briefly washed in degased ddH_2O, air dried, and imaged by TEM (JEOL JEM-1011) at a magnification of 150,000x.

In vitro microglial uptake assay – *In vitro* evaluation of Aβ uptake was performed as previously described (34). Briefly, human microglial cells (HMG 030, Clonexpress, Inc., Gaithersburg, MD, USA) were isolated from fetal brain tissue samples and suspended in a culture medium (50:50 of DMEM: F-12) supplemented with 5% FBS, 1% Penicillin/Streptomycin, and 10 ng/mL of M-CSF (AF-300-25, PeproTech Inc., Rocky Hill, NJ, USA). The isolated microglia cells were plated into glass-bottomed well plates (P24G-1.5-13-F, Matrical Bioscience, Spokane, WA, USA) and incubated at 37 °C supplied with 5% CO_2 for two days before treatment with Aβ and Cromolyn Sodium. After a medium change, microglia cells were incubated with 50 nM $A\beta_{42}$ with or without Cromolyn Sodium at 10 nM, 10 μM or 1 mM for 16 hours at 37°C. After incubation, the medium was collected and the levels of $A\beta_{40}$ and $A\beta_{42}$ were measured using a two-site Aβ ELISA (WAKO Chemicals) and microglia cells were fixed in 4% paraformaldehyde and the number counted.

Animals and Cromolyn Sodium treatment - APPswe/PS1dE9 (APP/PS1) were purchased from the Jackson laboratory. These mice express a human mutant amyloid precursor protein gene containing the Swedish mutation K594N/M595L as well as the Presenilin 1 gene deleted for the exon 9, both under the control of the prion promoter (35). This AD mouse model presents a severe phenotype with amyloid deposition beginning at 6 months of age. In

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the present study, 7.5-month-old APP/PS1 males were injected intraperitoneally (i.p.) daily for one week with escalating doses of 1.05 mg/kg, 2.1 mg/kg or 3.15 mg/kg body weight of Cromolyn Sodium or PBS. For interstitial fluid (ISF) sampling, 9-month-old male APP/PS1 mice were i.p. injected daily with the highest dose of Cromolyn Sodium (3.15 mg/kg body weight) or PBS for 7 days, just before ISF sampling. One day after the last injection or ISF collection, the mice were euthanized by CO_2 inhalation. Plasma was then collected via cardiac puncture. After transcardiac PBS perfusion, the brain was dissected and one brain hemisphere was fixed in 4% paraformaldehyde for immunohistochemistry whereas the contralateral hemisphere was snap-frozen in liquid nitrogen for biochemical assays. All experiments were performed under the agreement of the Massachusetts General Hospital Subcommittee for Research Animal Care and the Institutional Animal Care and Use Committee at Harvard Medical School.

Biochemical sample preparation - Brain tissue samples were homogenized in 10 volumes of TBSI (Tris-buffered saline with Protease inhibitor Cocktail; Roche) with 25 strokes on a mechanical Dounce homogenizer and centrifuged at 260,000*g* for 30 min at 4°C, as previously described (36). The TBS soluble supernatant was collected and the pellet was then successively homogenized in 2% tritonX-100/TBSI, 2% SDS/TBSI and 70% formic acid.

Sandwich ELISA and immunoblotting - The concentrations of $A\beta_{40}$ and $A\beta_{42}$ were determined using the commercially available kits BNT77/BA27 for $A\beta_{x\text{-}40}$ or BNT77/BC05 for $A\beta_{x\text{-}42}$ respectively (WAKO Chemicals). For guanidine (Gdn-HCl) treatment, samples were incubated with 0.5 M Gdn-HCl at 37°C for 30 min. Oligomeric Aβ species were quantified using the 82E1/82E1 ELISA kit (Immuno-Biological laboratories), in which both capture and detection antibodies are identical. For immunoblotting, TBS-soluble fractions were electrophoresed on a 10-20% Novex Tris-Glycine gels (Invitrogen). After transfer on nitrocellulose membrane (GE Healthcare), the blots were blocked in 5% nonfat skim milk/TBST (Tris-buffer saline with 0.1% Tween 20) buffer for 1 hour. Membranes were then probed with the anti-Aβ antibodies 6E10 (Signet) and 82E1 (Immuno-Biological laboratories) overnight at 4°C. Following incubation with horseradish peroxidase-conjugated secondary antibody Mouse True Blot (eBioscience) for 1 hour at room temperature, immunoreactive proteins were developed using an ECL kit (Western Lightning, PerkinElmer) and detected on Hyperfilm ECL (GE Healthcare) (37). Aβ signal intensity was measured by densitometry using Image J software (http://rsbweb.nih.gov/ij/).

Immunohistochemistry - Serial paraffin sections were cut at 4-μm and immunostained with a rabbit anti-Human Amyloid β (N) antibody (Immuno-Biological Laboratories) for amyloid plaques, followed by biotinylated goat anti-rabbit secondary antibody (Vector Laboratories) and developed using the ABC Elite and DAB kits (Vector Laboratories). Images were taken using an Olympus BX51 epifluorescence upright microscope equipped with a CCD camera model DP70 (Olympus, Tokyo, Japan). Quantitative analyses of amyloid load and plaque density were done using the BIOQUANT software (version 6.90.10, MBSR, Nashville, TN) after application of an optical threshold. This software is coordinated with the motorized stage of an upright Leica DMRB microscope equipped with a CCD camera (model DC330, DAGE-MTI, Inc., Michigan City, IN). Immunostained amyloid plaques were thresholded under the 10x objective after background correction to avoid uneven lighting. For colocalization analysis of Aβ in microglia, 4-μm paraffin sections were immunostained with mouse anti-Aβ antibody 6E10 (Signet) for Aβ and rabbit anti-Iba1 (WAKO Chemicals) for microglia followed by Alexa 488- or Cy3- conjugated secondary antibodies. Images were acquired on a Zeiss LSM 510 META confocal microscope, using the same pinhole settings and gain for taking all the pictures (objective x63) between PBS and Cromolyn Sodium treated animals. The percentage of Iba1 colocalizing with amyloid deposits was determined after image analysis using the Fiji software (National Institutes of Health). The exact same thresholds were applied to both 488 and Cy3 channels and an ROI was selected corresponding to each plaque. After application of this ROI on the Cy3 channel (Iba1 staining), an analysis of particles within the ROI was performed and the %

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of Iba1 staining overlapping with each amyloid deposit was measured.

In vivo microdialysis - *In vivo* microdialysis for ISF Aβ sampling was performed as previously reported (19). Briefly, the mice were stereotactically implanted with two guide cannulas (PEG-4, Eicom) into both hippocampi (AP -3.1 mm, L +/-2.8 mm, DV -1.1 mm), under anesthesia with isoflurane (1.5% in O_2). After a recovery time of three days, i.p. injections of Cromolyn Sodium started. ISF sampling was done one week after exposure with Cromolyn Sodium or PBS as control. For ISF sampling, a 1000 kDa molecular probe was used (PEP-4-03, Eicom). Before use, the probe was washed with artificial cerebrospinal fluid (aCSF: in mM: 122 NaCl, 1.3 CaCl2, 1.2 MgCl2, 3.0 KH2PO4, 25.0 NaHCO3). The probe's outlet and inlet were then connected to a peristaltic pump (ERP-10, Eicom) and a microsyringe pump (ESP-32, Eicom), respectively, using fluorinated ethylene propylene (FEP) tubing. The probe was inserted into mice hippocampus through the guide cannula. After implantation, aCSF was perfused for 1 hour at a flow rate of 10 µl/min before ISF sampling. ISF samples for the measurement of total Aβ or oligomeric Aβ were collected at a flow rate of 0.5 µl/min or 0.1 µl/min, respectively, and stored at -80°C until Aβ measurement. During *in vivo* microdialysis sampling, mice were awake and freely moving in the microdialysis cage designed to allow unrestricted movement without applying pressure on the probe assembly (AtmosLM microdialysis system, Eicom).

Compound E treatment using reverse microdialysis - The contralateral hippocampus was used for this experiment. After baseline sampling for 4 hours, 100 nM of γ-secretase inhibitor Compound E (Enzo Life Sciences) diluted in aCSF was perfused into the hippocampus to rapidly inhibit Aβ production in the tissue surrounding the probe. Aβ levels within the ISF were measured for an additional 5 hours (38,39). The single logarithmic plot was made from Aβ levels, and extrapolated the half-life of ISF Aβ.

Statistics analysis – Statistical analyses were performed using Graph Pad 5 Prism software. *In vitro*, each experiments was performed at least three times independently and normality was verified. Comparison of means among three or more groups was analyzed using a one-way ANOVA followed by a Bonferroni's *post hoc* test. *In vivo* data were averaged per mouse and analyzed using a non-parametric Kruskal-Wallis test, followed by a Dunn's Multiple Comparison Test. For the quantification of amyloid plaques, data were analyzed using a non-parametric Mann-Whitney test. *P* values less than 0.05 were considered significant. Data in all the figures are expressed as mean ±S.E.M.

RESULTS

Cromolyn Sodium inhibits Aβ polymerization in vitro, but does not impact pre-existing oligomers - Cromolyn Sodium (Sodium-cromoglycate) is an FDA-approved drug already in use for the treatment of asthma. Because of its structural similarities with fisetin (3,3',4',7-tetrahydroxyflavone, Fig. 1A), a flavonoid compound that has been previously shown to inhibit Aβ polymerization (27,28), we hypothesized that Cromolyn Sodium may also impact Aβ aggregation processes and modulate Aβ metabolism *in vitro* and *in vivo*. To test this hypothesis, we first evaluated the effect of Cromolyn Sodium on $Aβ_{40}$ and $Aβ_{42}$ fibrillization using a Thioflavin T assay. Over one hour of incubation at 37°C with increasing concentration of Cromolyn Sodium (5, 50, and 500 nM), we observed an inhibition of Aβ fibril formation *in vitro* at a nanomolar concentration (Fig. 1B). Using TEM, we confirmed that the formation of $Aβ_{42}$ fibrils was inhibited after incubation with 500nM of Cromolyn Sodium (Fig. 1C), whereas no effect was detected at a lower concentration (50nM). Importantly, this effect was significantly dimmer on the formation of $Aβ_{42}$ fibrils compared with $Aβ_{40}$. We hypothesized that $Aß_{42}$ peptides, more fibrillogenic, lead to faster formation of stable amyloid aggregates as compared with $Aß_{40}$, therefore resulting in lower anti-fibrillogenic impact of Cromolyn Sodium. It is also possible that Cromolyn Sodium has higher affinity for $Aß_{40}$ compared with $Aß_{42}$.

Because pre-fibrillar oligomeric amyloid aggregates are known to be highly neurotoxic *in vitro* and *in vivo* (21-26), we also asked if Cromolyn Sodium could prevent the formation of these particularly harmful species. Using a split-

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luciferase complementation method to specifically monitor oligomer formation (32), we observed that treatment of HEK293 cells overexpressing both N- or C-terminal of luciferase conjugated $A\beta_{42}$ with Cromolyn Sodium significantly decreased the luminescence signal in a dose-dependent manner, thus demonstrating the efficacy of this compound to inhibit oligomer formation *in vitro* (Fig. 1D). However, this effect could only be detected with concentrations of Cromolyn Sodium above 10 μM. This discrepancy with the Thioflavin-T assay may be due to the fact that our split-luciferase complementation method is performed in a cellular environment. In addition, this oligomerization assay is based on the presence of $A\beta_{42}$ peptides that are more amyloidogenic and aggregate faster than $A\beta_{40}$ peptides. By contrast, addition of Cromolyn Sodium to conditioned media that already contains pre-existing oligomers failed to impact the luminescence signal (Fig. 1E). These data indicated that Cromolyn Sodium efficiently prevents Aβ polymerization into higher ordered oligomers or fibrils, but cannot dissociate pre-existing aggregates.

One week exposure with cromolyn sodium in APP/PS1 mice significantly lowers the content of soluble Aβ in vivo but does not affect amyloid deposition or highly fibrillar Aβ species - Our preliminary results confirmed that Cromolyn Sodium may interfere with Aβ aggregation processes *in vitro,* and therefore may be classified as an anti-amyloidogenic compound. To further examine the effect of Cromolyn Sodium *in vivo*, we administered escalating doses of Cromolyn Sodium (1.05 mg/kg, 2.1 mg/kg or 3.15 mg/kg body weight) or PBS intraperitoneally (i.p.) to APP/PS1 mice daily for one week.

We first evaluated the levels of the most soluble Aβ species, hypothesizing that they may be preferentially impacted by the treatment over a short period of time *in vivo*. We observed that acute exposure of AD transgenic mice with 2.1 mg/kg or 3.15 mg/kg Cromolyn Sodium for only seven days significantly lowered the content of both TBS-soluble $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$ by more than 50% (2.1 mg/kg dose: 39.5% for $A\beta_{x\text{-}40}$, 40.9% for $A\beta_{x\text{-}42}$; 3.15 mg/kg dose: 37.1% for $A\beta_{x\text{-}40}$, 46.2% for $A\beta_{x\text{-}42}$, respectively) (Fig. 2A, shown as Gdn-HCl -). As reported previously, oligomeric Aβ or Aβ peptides associated with other unidentified molecules may interfere with the interaction with anti-Aβ detection antibodies, thus altering the outcome of these ELISA systems (37). To circumvent this problem and estimate the exact level of TBS soluble Aβ, TBS soluble fraction were incubated with 0.5M guanidine (Gdn-HCl) at 37°C for 30 min to dissociate oligomers or other complexes formed between Aβ and other proteins. As expected, the levels of Aβ after incubation with Gdn-HCl generally increased compared with the native conditions, especially $A\beta_{x\text{-}42}$ that is more prone to aggregation. Using this complementary assay, we confirmed that treatment with Cromolyn Sodium lowered the total level of TBS soluble Aβ in a dose-dependent manner (2.1 mg/kg dose: 50.7% for $A\beta_{x\text{-}40}$, 63.3% for $A\beta_{x\text{-}42}$; 3.15 mg/kg dose: 44.6% for $A\beta_{x\text{-}40}$, 76.1% for $A\beta_{x\text{-}42}$, respectively) (Fig.2A, shown as Gdn-HCl +).

When the amounts of oligomeric Aβ (or bound Aβ) were estimated according to the difference between the Aβ levels with and without incubation of Gdn-HCl, we found that Cromolyn Sodium did not significantly alter the content of higher-order amyloid species. In order to further examine this result, the concentrations of Aβ oligomers were also specifically measured using the 82E1/82E1 ELISA assay that uses the same capture and detection antibody. Again, no changes in the levels of oligomeric aggregates could be detected (Fig. 2B). TBS soluble extracts were also subjected to SDS-PAGE. Quantification of the 4kDa Aβ band using 6E10 and 82E1 detection antibodies showed that Cromolyn Sodium decreased the amounts of monomeric Aβ (Fig. 2C), confirming our initial ELISA data. Because of the low proportion of soluble Aβ oligomers as compared with total levels of Aβ, we did not detect those specific aggregates by western blotting.

We then quantified the concentrations of Aβ detergent resistant species sequentially extracted in 2% Triton (Fig. 3A) and 2% SDS (Fig. 3B) buffers. Interestingly, we observed that treatment with the highest dose of Cromolyn Sodium (3.15 mg/kg) was associated with a significant decrease in the amounts of $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$ as compared with PBS controls. Of note, and consistently with our *in vitro* results, Cromolyn Sodium appeared to have a bigger impact in decreasing $A\beta_{x\text{-}40}$ than

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$A\beta_{x-42}$ for all the fractions considered (TBS, Triton and SDS).

Finally, we evaluated the impact of Cromolyn Sodium on the most insoluble fraction of Aβ peptides (formic acid extracts) and on the density of amyloid deposits. We observed that insoluble Aβ levels were not affected by acute Cromolyn Sodium administration (Fig. 4A). Because the levels of insoluble Aβ peptides are much higher as compared with the most soluble fractions, and because Cromolyn Sodium only impacts the soluble pool of $A\beta_{x-40}$ and $A\beta_{x-42}$ in TBS, Triton and SDS extracts, it did not overall alter the distribution of Aβ peptides within each biochemical fraction (TBS, Triton, SDS and formic acid, Fig. 4B). Additional quantification of the amyloid burden and the density of amyloid deposits, assessed immunohistochemically with an anti-Aβ antibody, confirmed that the amount of extracellular deposited aggregates of amyloid peptides remained unaffected after one week of Cromolyn Sodium treatment (Fig. 4C and 4D). These data indicated that Cromolyn Sodium does not primarily affect the most fibrillar forms of amyloid when administered in AD transgenic mice for a short period of time.

Taken together, these results indicate that acute i.p. administration of Cromolyn Sodium rapidly decreases the amount of TBS, Triton and SDS soluble monomeric Aβ *in vivo*, which may constitute the most exchangeable pool of amyloid within the brain. However, this compound had no detectable impact on the amount of oligomers already present at the time of treatment or on the steady state levels of fibrillar amyloid after one week of treatment, which correlates with a predominant effect of Cromolyn Sodium in preventing Aβ aggregation rather than dissociating previously existing aggregates.

Cromolyn Sodium decreases the concentration of $A\beta_{40}$ in the interstitial fluid of APP/PS1mice - Our preliminary results indicate that acute exposure with Cromolyn Sodium primarily decreases the amount of soluble monomeric amyloid peptides rather than aggregated species *in vivo*. To further investigate the effects of Cromolyn Sodium on the most diffusible pool of Aβ, we next used a technique of *in vivo* microdialysis that allows the sampling of interstitial fluid closely in contact with neuronal cells.

APP/PS1 mice were i.p. injected with PBS or Cromolyn Sodium at the highest dose (3.15 mg/kg body weight) daily for one week. Acute administration of Cromolyn Sodium dramatically decreased ISF $A\beta_{x-40}$ level by 30% (PBS: 387pM, Cromolyn: 283pM). A similar trend was observed for the levels of both ISF $A\beta_{x-42}$ and Aβ oligomers, without reaching statistical significance (Fig. 5A and 5B). These results suggest that Cromolyn Sodium accutely impacts $A\beta_{x-40}$ peptides within the ISF, having less effect on the more fibrillogenic $A\beta_{x-42}$ peptides.

Cromolyn Sodium reduces the half-life of Aβ within ISF, a process potentially related to microglial uptake rather than egress of Aβ through the blood brain barrier - As previously observed, monomeric Aβ peptides are more efficiently cleared from the brain compared with oligomeric species and higher-order aggregates (19). Because of the anti-fibrillogenic effect of Cromolyn Sodium observed *in vitro*, we hypothesized that exposure of AD transgenic mice to this compound may prevent Aβ aggregation and therefore promote its clearance from the brain. To test this hypothesis, we estimated the half-life of Aβ in ISF using reverse microdialysis with the γ-secretase inhibitor Compound E, in order to acutely block Aβ production within the local environment of the microdialysis probe while following its decay over time (19,39). ISF sampling was done before and after delivery of Compound E in APP/PS1 mice that were treated daily for eight days with PBS or Cromolyn Sodium at the highest dose (3.15 mg/kg body weight). In mice injected with Cromolyn Sodium, ISF Aβ level started decreasing only 2 hours after the administration of Compound E, significantly faster than in PBS treated mice (Fig. 6A). When extrapolated from Fig. 6A, we calculated that the half life of ISF Aβ in Cromolyn Sodium treated mice was shorter than control by ~50% (Fig. 6B), thus indicating that ISF Aβ was more rapidly cleared after treatment with this compound. Of note, the half-life of Aβ calculated in our study was significantly longer than initially reported in other works (39). This discrepancy may be explained by the use of a larger pore size of 1000

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kDa as compared with previous publications (35kDa), which allows sampling of oligomeric or APOE-associated Aβ species that have an increased half-life in the ISF than monomeric amyloid peptides (19).

We then tried to identify the underlying mechanism leading to the decreased Aβ half-life upon treatment with Cromolyn Sodium. We first hypothesized that monomeric Aβ peptides may be efficiently cleared through the blood brain barrier or via perivascular drainage. However, the plasma levels of Aβ in mice treated with PBS or Cromolyn Sodium remained comparable, and neither $Aβ_{x-40}$ nor $Aβ_{x-42}$ were changed even at the highest dose of Cromolyn Sodium (Fig. 7A). These data suggest that the anti-fibrillogenic effect of Cromolyn Sodium does not primarily leads to a detectable increase of Aβ efflux from the central nervous system to the periphery.

We then tested if Cromolyn sodium may impact the interaction of microglial cells with amyloid deposits and eventually affects Aβ clearance by microglia. We first performed a double immunostaining between Aβ and the microglial marker Iba1 in brain sections of mice treated with PBS or the highest dose of Cromolyn sodium (3.15mg/kg). A systematic analysis of the overlap between both stainings revealed that animals that received Cromolyn Sodium showed a higher percentage of Iba1 immunoreactivity overlapping with amyloid (Fig. 7B), which may indicate a modest increased recruitment of microglia around plaques induced by the compound. To go further in our understanding of these mechanisms, and considering that evaluating change in microglial function is challenging *in vivo*, we then used an additional *in vitro* system of Aβ microglial uptake (34). Synthetic $Aβ_{40}$ and $Aβ_{42}$ peptides were applied to microglia in culture in the presence or absence of Cromolyn Sodium. After 16 hours of incubation, we observed a dose-dependent decrease of $Aβ_{40}$ and $Aβ_{42}$ levels in presence of Cromolyn Sodium, indicating that the impact of Cromolyn Sodium on Aβ aggregation mechanisms may promote Aβ clearance by microglial uptake (Fig. 7C). The combination of those *in vivo* and *in vitro* results may suggest that, in addition to inhibiting Aβ fibrillization, Cromolyn Sodium may also affect microglial activation and Aβ clearance.

DISCUSSION

Amyloid reducing agents remain potentially promising therapeutic drugs in the treatment of Alzheimer's disease, as the only molecules currently available mainly target associated symptoms rather than the neuropathological causes of the disease. Cromolyn Sodium is a synthetic chromone derivative that has been approved for use by the FDA since the 1970s for the treatment of asthma (40,41). For asthma treatment, Cromolyn Sodium powder was micronized for inhalation to the lungs via dry powder inhaler, whereas liquid intranasal and ophthalmic formulations have also been developed for the treatment of rhinitis and conjunctivitis (42). The broad use of Cromolyn Sodium in the treatment of several diseases, its capacity to cross the blood brain barrier after peripheral injection in rodents (29,30) and its structural similarity with fisetin, an anti-amyloid compound that has been shown to inhibit amyloid fibril formation (27) and has neuroprotective functions *in vitro* and *in vivo* (43,44), raise the possibility that Cromolyn Sodium may have a beneficial effect on Alzheimer's disease as well.

In the present study, we demonstrated that addition of Cromolyn Sodium to $Aβ_{40}$ and $Aβ_{42}$ preparations not only prevents amyloid fibril formation but also inhibits Aβ oligomerization *in vitro*, thus potentially preventing the formation of the most neurotoxic species of amyloid (20-26). Importantly, short-term exposure with peripherally administered Cromolyn Sodium also efficiently impacts Aβ economy *in vivo*, an effect detectable after only one week of treatment. We observed that intraperitoneal administration of Cromolyn Sodium reduces the levels of TBS-, TritonX- and SDS-soluble Aβ monomers in the brain within days in a dose dependent fashion. However, the concentrations of oligomers and fibrillar amyloid remain unchanged during the same period of time. Microdialysis experiments also show that the half-life of Aβ is shorter in the presence of Cromolyn Sodium, leading to lower total amounts in the brain. Interestingly, these significant modifications detected in the central nervous system did not impact the plasma concentrations of Aβ, but may rather be related with activation of microglial cells and an increased efficiency of microglial clearance

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of intracerebral Aβ peptides.

The specific effect of Cromolyn Sodium on Aβ monomers versus oligomers (assessed with two different assays – the addition of Guanidine hydrochloride to break apart oligomeric structures, and the use of an established same site ELISA assay that specifically differentiates monomeric from oligomeric species) may be surprising, as we anticipated that the mobilization of one pool of amyloid would have induced a concomitant decrease in small soluble aggregates. However, oligomeric Aβ species have been previously shown to have a longer ISF half-life (19), therefore suggesting that longer chronic exposure time with Cromolyn Sodium may be needed to detect a significant decrease in the levels of oligomers. It also explains why the overall impact of Cromolyn Sodium on $A\beta_{42}$ is dimmer as compared with $A\beta_{40}$, considering the higher potency of $A\beta_{42}$ to aggregate. Our results illustrate the complex relationships between the kinetics of monomeric and oligomeric Aβ in the interstitial fluid *in vivo*. Nonetheless, the marked change in soluble monomeric Aβ levels after only a week of treatment with Cromolyn Sodium is remarkable, with about 50% reduction in the content of Aβ after a few days, an effect of the same order of magnitude as the decrease seen in patients who have inherited a protective mutation in APP (14), suggesting that this magnitude of reduction in Aβ, if sustained chronically, may have important effects in AD.

Finally, numerous synthetic or naturally occurring compounds (such as cucurminoids or flavonoids (45)), small peptides (eventually derived from Aβ itself (46)), endogenously expressed chaperones (such as DNAJB6, (47)) or even synthetic nucleotides (48) have been previously reported to prevent Aβ aggregation into higher order species or fibrils. Despite their potency to inhibit or destabilize the formation of amyloid fibrils *in vitro*, so far only few of these compounds have been associated with an impact on the neuropathological hallmarks of the disease *in vivo*. Among them, curcuminoids as well as flavoinoids have been associated with decreased Aβ deposition, Aβ-dependent neurotoxicity as well as improved cognitive functions in AD mouse models, an effect reported after weeks or months of treatments (49-51). However, besides their evident inhibitory effect on Aβ aggregation *in vitro*, those particular compounds also modulate other processes (such as inflammation, oxidative stress or endoplasmic reticulum stress) that concomitantly counteract the build-up of amyloid within the brain but also alleviate Aβ-related deleterious effects. It is also difficult to draw a clear conclusion on the potential therapeutic impact of Cromolyn Sodium as compared with other anti-fibrillogenic molecules, considering that the duration of exposure, the route of administration (intravenous, intracerebro-ventricular infusion, intraperitoneal injection, etc…), the cellular or animal models are highly variable between studies.

In conclusion, additional investigations will be necessary to determine if appropriate levels of Cromolyn Sodium can reach the central nervous system in patients and further studies will be needed to understand the mechanisms of action of Cromolyn Sodium on Aβ fibrillization and clearance. In particular, the anti-inflammatory effect of this molecule has been well reported in the treatment of asthma, a property that may also contributes to its potential beneficial impact in AD.

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FOOTNOTES

* This study was supported by AZTherapies Inc., a postdoctoral fellowship from the Uehara Memorial Foundation (Y.H.) and the postdoctoral fellowship for research abroad from Japan Society for the Promotion of Science (JSPS) (S.T.), and the Dreyfoos fund Against Alzheimer's Disease.

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[5]The abbreviations used are: FDA, Food and Drug Administration; Aβ, amyloid β; AD, Alzheimer's disease; ISF, interstitial fluid; Gdn-HCl, guanidine-hydrogen chloride; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay.

FIGURE LEGENDS

FIGURE 1. Cromolyn sodium inhibits Aβ polymerization *in vitro* but does not impact pre-existing oligomers. A. Structures of Cromolyn Sodium and fisetin. B. Left panel: representative curves of Thioflavin T fluorescence increase upon Aβ fibrillization after addition of DMSO (upper panel) or Cromolyn Sodium (5nM, 50nM and 500nM, lower panels). Fibrillization of synthetic $A\beta_{40}$ (left column) or $A\beta_{42}$ (right column) peptides was followed over 1 hour. The corresponding Vmax index (milli-units/minute) is indicated on each graph. Right panel: Bar graphs summarizing $A\beta_{40}$ (upper graph) and $A\beta_{42}$ (lower graph) fibrillization, which is significantly decreased in presence of Cromolyn Sodium, even though smaller effects were observed on $A\beta_{42}$ fibrillization process. C. Representative TEM pictures of $A\beta_{42}$ fibrils formed after 48 hours incubation of synthetic $A\beta_{42}$ peptides (0.2 mg/ml), in presence of 50nM or 500nM of Cromolyn Sodium. Scale bar = 100nm. D. Aβ oligomerization assay using the split-luciferase complementation assay. HEK 293 cells, which were engineered to stably expressing luci-$A\beta_{42}$ and ferase-$A\beta_{42}$, were incubated with 0, 10 nM, 10 μM, 1 mM of Cromolyn Sodium for 12 hours. The amounts of oligomers were measured using luciferase activity. E. Oligomer dissociation assay using the split-luciferase complementation assay. Pre-existing oligomers composed of split-luciferase conjugated Aβ were incubated with 0, 10 nM, 10 μM, 1 mM of Cromolyn Sodium for 12 hours. The amounts of oligomers were measured using luciferase activity. The ratio of luminescence after incubation with Cromolyn Sodium compared with PBS was calculated. (n=3 experiments; *, $P<0.05$, **, $P<0.01$)

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FIGURE 2. Cromolyn Sodium reduces the concentration of soluble monomeric Aβ, not oligomeric Aβ, in the brain of APP/PS1 mice. A. One week after daily i.p. injection with Cromolyn Sodium or PBS, TBS soluble $A\beta_{x-40}$ (left graph) and $A\beta_{x-42}$ (right graph) were measured by ELISA, with (black bar) or without (white bar) pre-incubation with Gdn-HCl. B. Oligomeric Aβ content in TBS soluble brain extracts was measured using 82E1/82E1 Aβ oligomer specific ELISA. C. Representative immunoblotting of TBS soluble brain extracts with anti-Aβ antibody (6E10 and 82E1) (left panel). The densitometry of Aβ monomers was quantified (right bar-graph). (n=3-5 mice/group; *, $P<0.05$, **, $P<0.01$)

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FIGURE 3. Cromolyn Sodium lowers the levels of $A\beta_{x-40}$ and $A\beta_{x-42}$ in both Triton-X and SDS fractions of APP/PS1 brain extracts. As previously mentioned, AD transgenic mice were treated with PBS or escalating concentrations of Cromolyn Sodium (1.05mg/kg, 2.1mg/kg and 3.15mg/kg) for one week before euthanasia. A. Bar graph representing the concentrations of $A\beta_{x-40}$ (left panel) and $A\beta_{x-42}$ (right panel) measured by ELISA in the Triton-X extracts. B. Bar graph representing the concentrations of $A\beta_{x-40}$ (left panel) and $A\beta_{x-42}$ (right panel) measured by ELISA in the SDS extracts. (n=3-5 mice /group; *, $P<0.05$)

FIGURE 4. Cromolyn Sodium does not alter the levels of insoluble Aβ peptides and amyloid deposition in APP/PS1 mice after a week of treatment. A. Cromolyn Sodium was i.p. injected to 7.5 months old APP/PS1 mice daily for one week with three different doses (1.05 mg/kg; 2.1 mg/kg and 3.15 mg/kg). One week after daily injection, insoluble $A\beta_{x-40}$ (white bar) and $A\beta_{x-42}$ (black bar) present in the formic acid fraction of brain extracts were measured using Aβ ELISA. B. Percentages of $A\beta_{x-40}$ and $A\beta_{x-42}$ in each biochemical fraction (TBS, Triton, SDS and formic acid) accordingly to the total level of Aβ. Distributions were compared between control transgenic mice and animals treated with 1.05mg/kg, 2.1mg/kg and 3.15 mg/kg of Cromolyn Sodium for a week. No significant changes could be detected. C. Representative pictures of the amount of amyloid deposits in mice treated with PBS or Cromolyn Sodium (3.15 mg/kg) for one week (using the rabbit anti-Human Amyloid β (N) antibody from Immuno-Biological laboratories). Scale bar=2 mm for upper panels or 200 μm for lower panels. D. Bar graphs summarizing the amyloid load (upper graph) and plaque density (lower graph) in both group. (n=3-5 mice/group)

FIGURE 5. Acute treatment with Cromolyn Sodium decreases the levels of monomeric $A\beta_{x-40}$, not oligomeric Aβ, in the ISF of APP/PS1 mice. A. Cromolyn Sodium was i.p. injected to 9 months old APP/PS1 mice daily for one week at 3.15 mg/kg dose. One week after daily injection, ISF sample were collected by *in vivo* microdialysis. The concentrations of $A\beta_{x-40}$ (left graph) and $A\beta_{x-42}$ (right graph) in ISF were measured by ELISA. B. Oligomeric Aβ in ISF were quantified using the 82E1/82E1 Aβ oligomer specific ELISA assay. (n=5 mice/group; *, $P<0.05$)

FIGURE 6. Cromolyn Sodium decreases the half-life of Aβ in ISF. A. Representative decay $A\beta_{x-42}$ curves in PBS and Cromolyn Sodium (3.15 mg/kg) treated mice after infusion of 100 nM of γ-secretase inhibitor (Compound E) by reverse microdiaysis. ISF samples were collected every hour before and after γ-secretase inhibitor perfusion. The concentration of ISF $A\beta_{x-42}$ was measured by ELISA after pre-incubation with Gdn-HCl. B. Bar-graph summarizing the extrapolated $A\beta_{x-42}$ half-life one week after i.p. injection with Cromolyn Sodium or PBS. (n=5 mice/group; *, $P<0.05$)

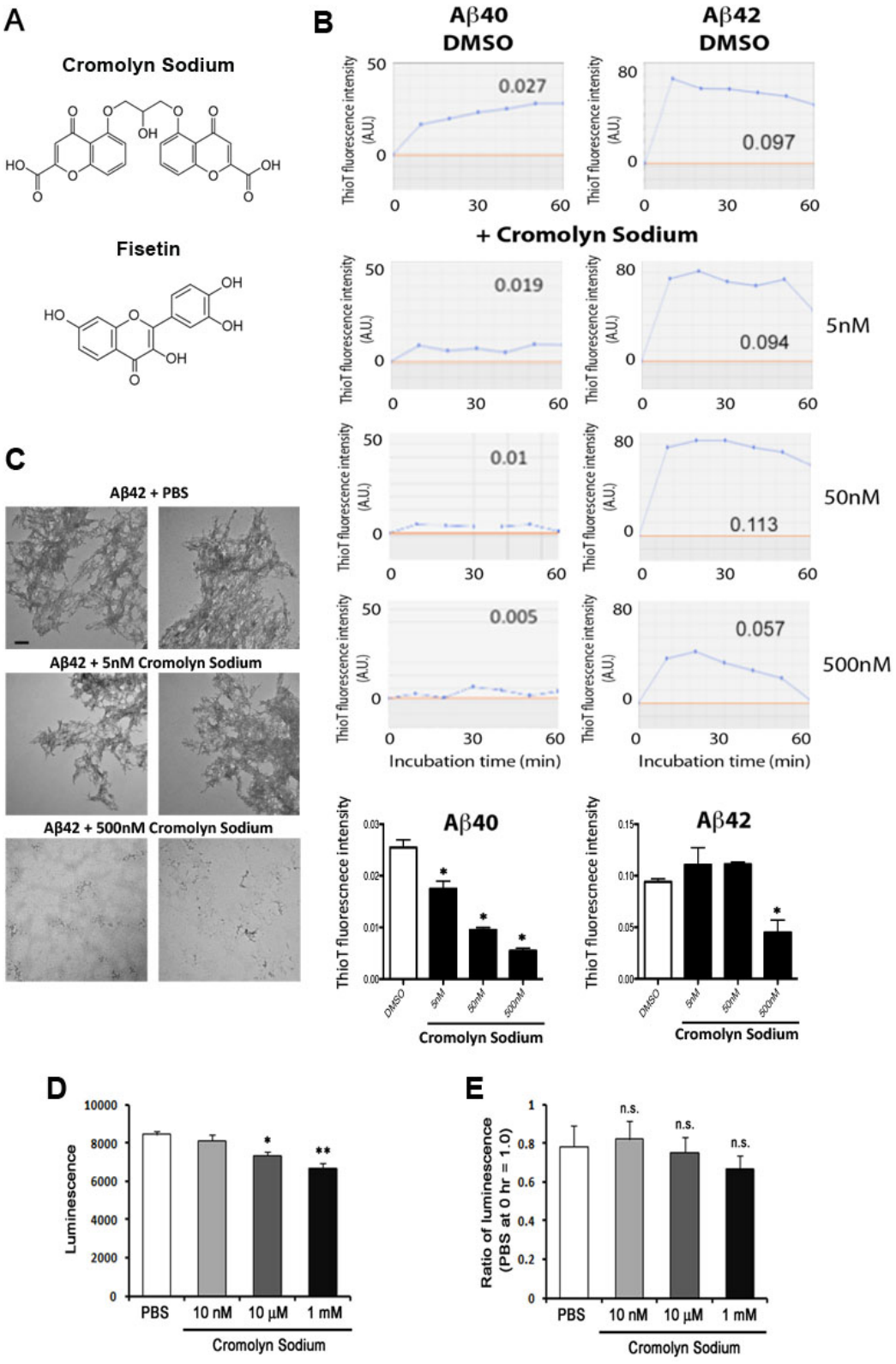
FIGURE 7. Cromolyn Sodium does not affect the levels of Aβ in the plasma but promotes microglial Aβ clearance. A. Quantification of the plasmatic levels of $A\beta_{x-40}$ and $A\beta_{x-42}$ one week after treatment with PBS or escalating doses of Cromolyn Sodium (n=3-5 mice/group). B. Representative images of localization of amyloid deposits (6E10, green) and microglia (Iba1, red) in mice treated with Cromolyn Sodium (3.15mg/kg) or PBS daily for seven days. The percentage of amyloid occupied by Iba1 positive processes was calculated for each deposit and showed an increased overlap between Aβ and Iba1 after treatment with Cromolyn Sodium (n=3 mice for PBS and n=5mice for Cromolyn Sodium. Between 20 to 20 plaques were evaluated for each animal). Scale bar=10 μm. C. Effect of Cromolyn Sodium on microglial Aβ uptake *in vitro*. Microglial cells were cultured and incubated with 50 nM of synthetic $A\beta_{40}$ or $A\beta_{42}$ and 0, 10 nM, 10 μM or 1 mM of Cromolyn Sodium for 16 hours. After the incubation, the concentrations of $A\beta_{x-40}$ (left panel) $A\beta_{x-42}$ (right panel) in media were measured using Aβ ELISA and

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normalized with microglia cell number and according to the PBS control condition. (n=3 experiments; *, $P<0.05$, **, $P<0.01$)

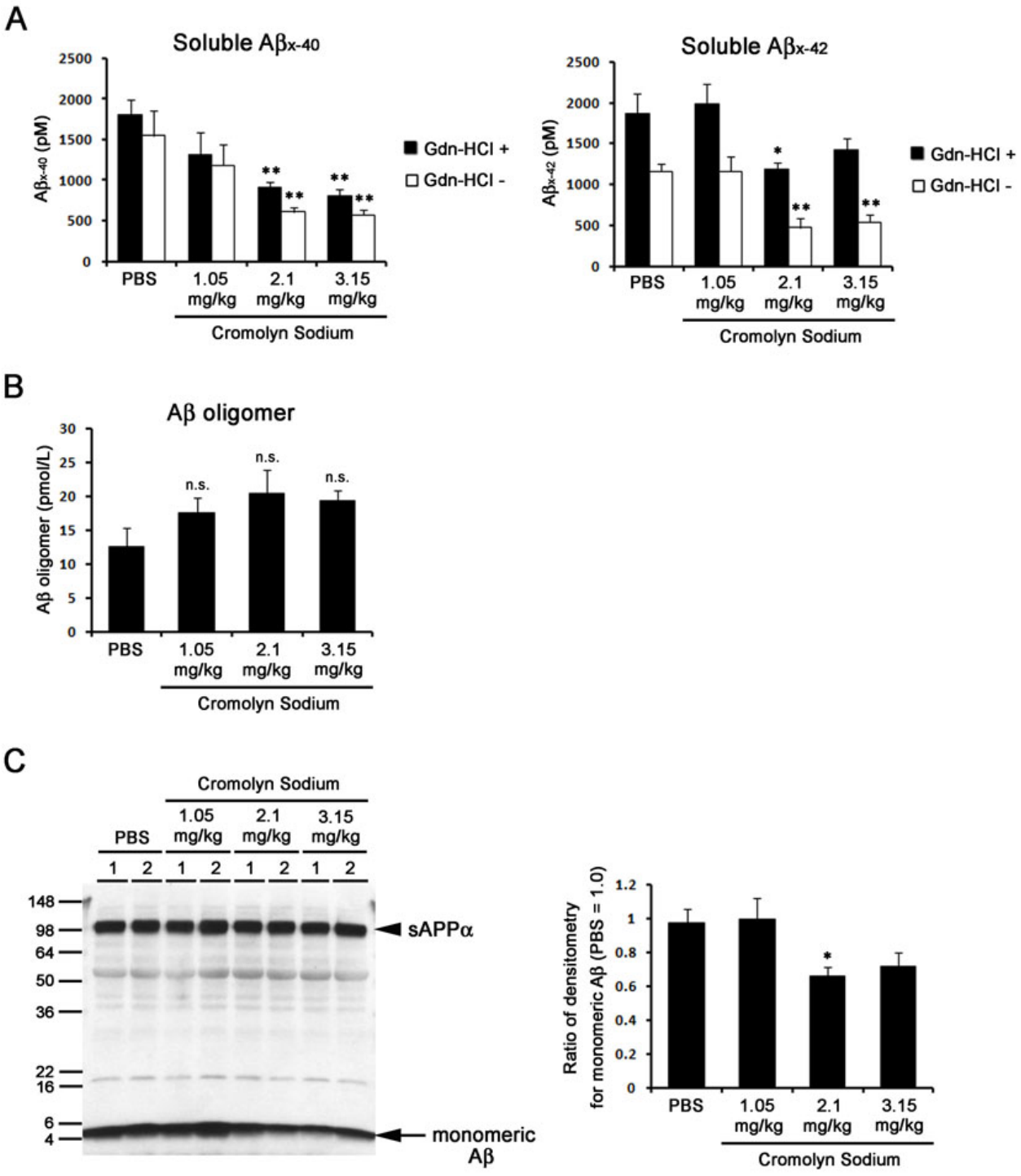
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FIGURE 1



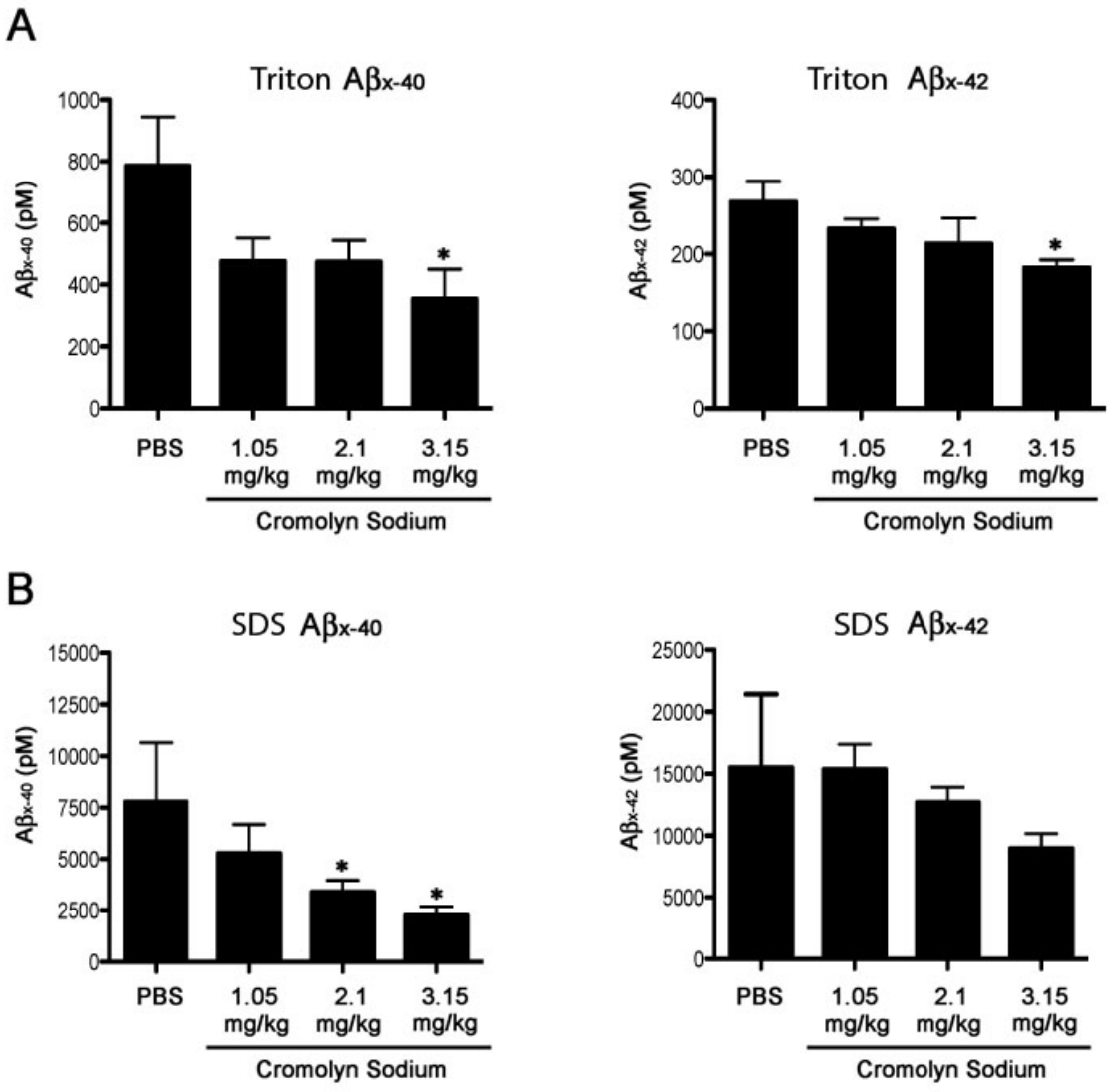
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FIGURE 2



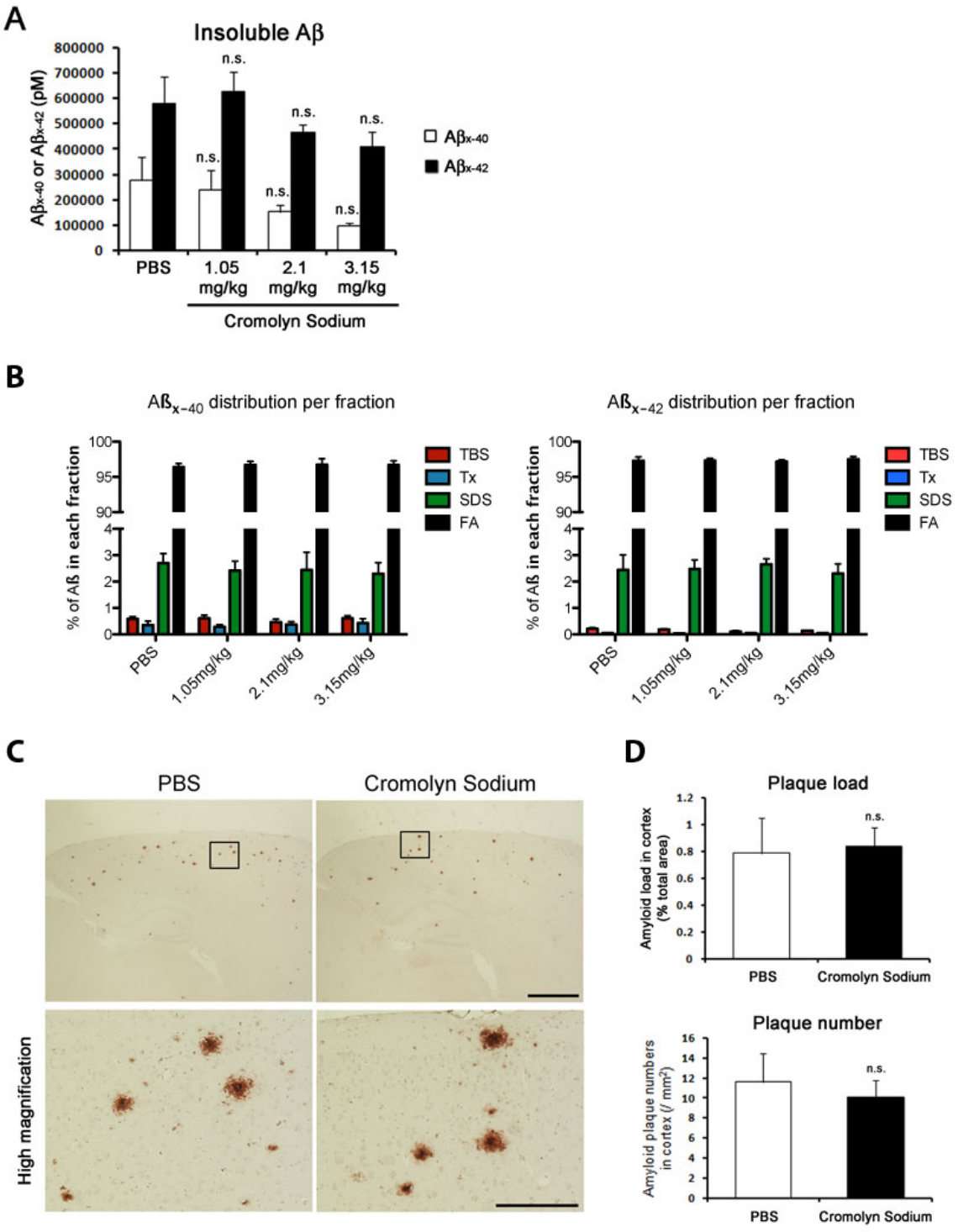
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FIGURE 3



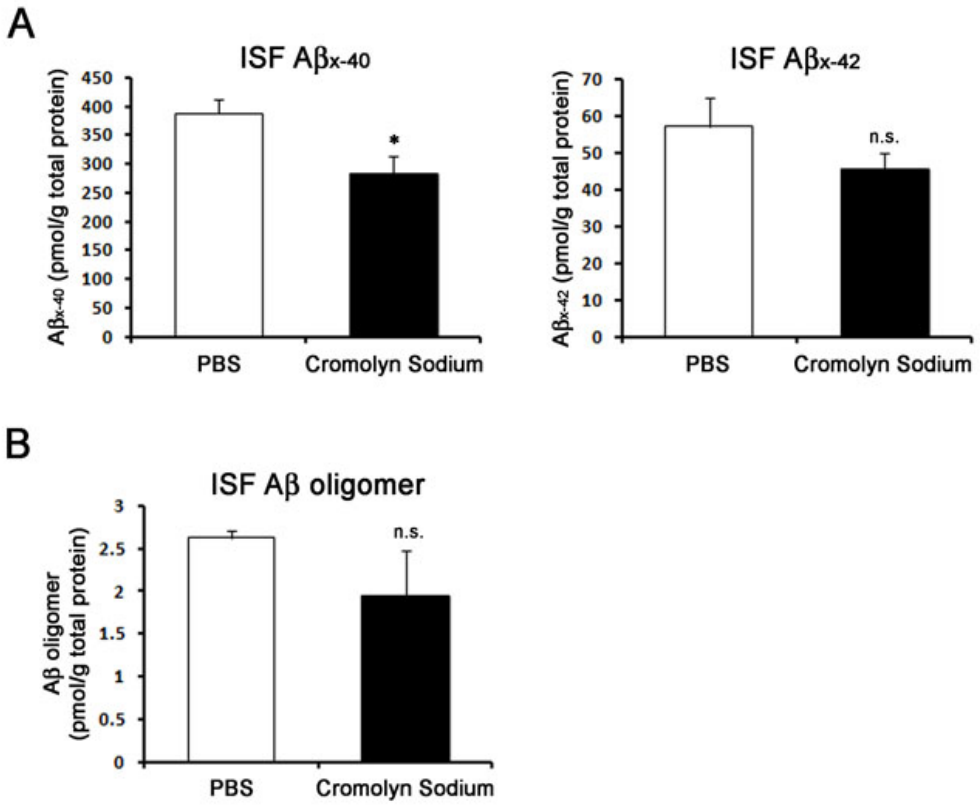
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FIGURE 4



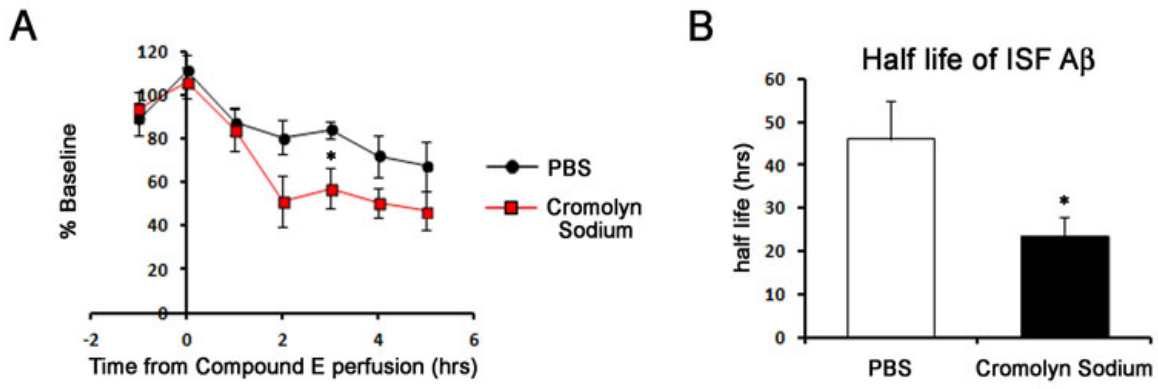
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FIGURE 5



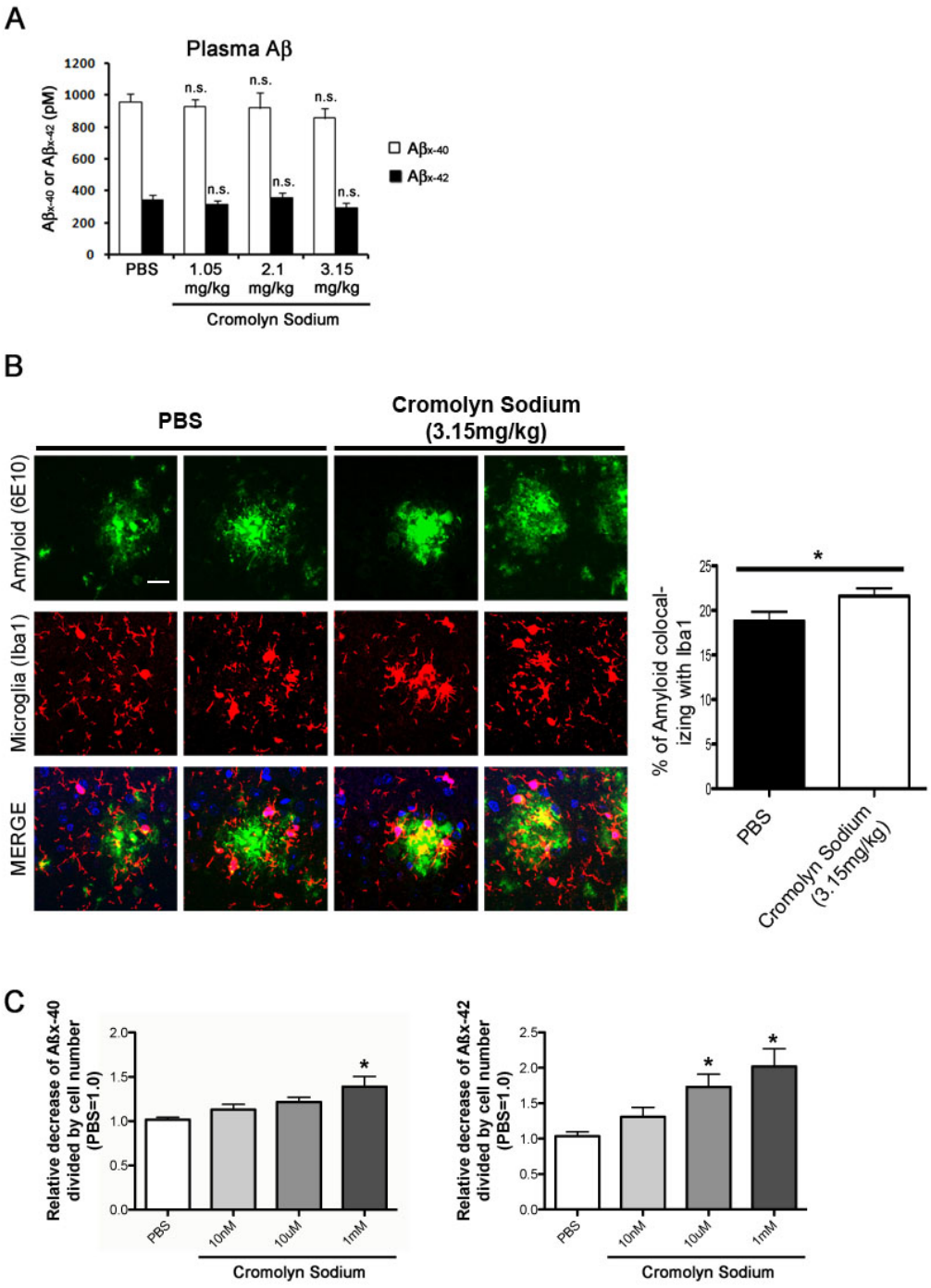
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FIGURE 6



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FIGURE 7



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