

A Food and Drug Administration-approved Asthma Therapeutic Agent Impacts Amyloid β in the Brain in a Transgenic Model of Alzheimer Disease*

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Background: Cromolyn sodium is an FDA-approved drug structurally similar to fisetin, an anti-amyloidogenic molecule.

Results: Cromolyn sodium interferes with amyloid β ($A\beta$) aggregation *in vitro* while rapidly decreasing the levels of soluble $A\beta$ peptides *in vivo* after a week.

Conclusion: Cromolyn sodium may have an impact on amyloid economy.

Significance: Developing new disease-modifying therapeutics remains an urgent need in the treatment of Alzheimer disease.

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

Therapies to prevent Alzheimer disease (AD)² progression remain a highly unmet medical need. Food and Drug Administration-approved drugs such as acetylcholinesterase 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 progression.

The advent and spread of neurotoxic oligomeric aggregates of amyloid β ($A\beta$) 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). $A\beta$ peptides (37–43 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 $A\beta$ generation (12) or increased oligomerization (13) cause AD, whereas a recently reported APP mutation has been shown to decrease $A\beta$ by ~40% and to be protective (14, 15). Aberrant accumulation of $A\beta$ peptides, rather than increased production, is believed to initiate the development of the sporadic form of the disease, which represents the vast majority of cases (16–18). Misfolded $A\beta$ monomers can aggregate into higher-order oligomers, eventually forming fibrils that are deposited into the extracellular space to form fibrillar amyloid neuritic plaques. $A\beta$ oligomers have a longer half-life in the interstitial fluid than monomers so that their formation influences $A\beta$ levels (19). $A\beta$ oligomers rather than monomers have been shown to be neurotoxic for neurons (20), inhibiting long-term potentiation (LTP), leading to neuronal stress, abnormal tau phosphorylation, synapse collapse, and memory impairment (21–26). Therefore, therapeutic agents able to decrease $A\beta$ levels, prevent oligomer formation, or disaggregate soluble oligomers may be of therapeutic interest.

Cromolyn sodium is a Food and Drug Administration-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 has been shown previously to inhibit amyloid aggregation (28), we hypothesized that cromolyn sodium may potentially prevent $A\beta$ oligomerization and promote $A\beta$ clearance *in vitro* and *in vivo*. In addition, the use of cromolyn sodium in addiction models (after

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² The abbreviations used are: AD, Alzheimer disease; $A\beta$, amyloid β ; APP, amyloid precursor protein; ISF, interstitial fluid; Gdn-HCl, guanidine-hydrogen chloride.

subcutaneous infusion (29, 30)) and the physical properties of this molecule (molecular weight: 508.38; polar surface area: 125; and LogP: 1.39) argue for cromolyn sodium as a blood-brain barrier-permeable drug, therefore allowing systemic infusion. In this study, we tested the hypotheses that cromolyn sodium can alter A β aggregation after peripheral delivery. In this study, we demonstrated that cromolyn sodium efficiently prevents the aggregation of monomeric amyloid peptides into higher-order oligomers and fibrils *in vitro*. In addition, *in vivo* intraperitoneal administration of this compound rapidly decreases the amount of soluble monomeric A β in the brain by ~50% after only 1 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 A β was significantly reduced in cromolyn sodium-treated mice compared with PBS-exposed animals, suggesting that preventing A β oligomerization may favor their clearance from the central nervous system. In addition, both *in vivo* and *in vitro* results suggest that A β 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 A β metabolism, with potential impact on toxic oligomers.

EXPERIMENTAL PROCEDURES

Chemicals—Cromolyn sodium USP grade was purchased from Spectrum Chemical Manufacturing Corp. (Gardena, CA) and dissolved in sterile 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 μ M, or 1 mM, whereas a solution of 1.02 mM of the compound was prepared in Dulbecco's PBS before intraperitoneal injection *in vivo* (at three different doses: 1.05, 2.1, or 3.15 mg/kg body weight). An *in vitro* amyloid fibrillization assay was performed using synthetic A β peptides (rPeptide, Bogart, GA) and Thioflavin-T (Sigma-Aldrich), respectively, dissolved in dimethyl sulfoxide and methanol. For the *in vitro* efflux and microglial uptake assay, synthetic A β_{40} and A β_{42} peptides were purchased from Peptide Institute, Inc. After resuspension in 1,1,1,3,3,3-hexafluoro-2-propanol (Kanto Chemical) at a concentration of 1 mg/ml, the peptides were dried, resolubilized in PBS containing 2% (v/v) Me₂SO (Kanto Chemical), and filtered through a 0.2- μ m filter, as described previously (31). The stock solution of A β_{40} and A β_{42} peptides was diluted to 5 μ M and stored at -80°C prior to use. A β_{40} or A β_{42} were applied at 50 nM in cell cultures.

In Vitro A β Fibrillization, Oligomerization, and Dissociation Assays—An *in vitro* fibrillization assay was performed using A β_{40} or A β_{42} peptides dissolved in dimethyl sulfoxide at a concentration of 250 μ M and sonicated for 1 min. A β_{40} or A β_{42} were diluted to 5 μ M in an assay volume of 200 μ l with artificial cerebrospinal fluid 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 a 96-well plate (Corning, Tewksbury, MA). After addition of 10 μ M Thioflavin-T and increasing concentrations of cromolyn sodium (5, 50, and 500 nM), the fibrillization process was initiated by adding 0.5 mg/ml of hep-

arin sulfate (Sigma). Dimethyl sulfoxide 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 Devices, Sunnyvale, CA). The results were normalized for background using fluorescent reading at time 0 by the software provided by the M3 plate reader.

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

Analysis of A β_{42} Fibril Formation by Transmission Electron Microscopy—The antifibrillogenic properties of cromolyn were confirmed by performing TEM analysis as previously described (33). Briefly, synthetic A β_{42} was dissolved in PBS at a concentration of 0.2 mg/ml for 48 h at 37 $^{\circ}\text{C}$, with or without addition of Cromolyn Sodium at a concentration of either 5 nM or 500 nM. After incubation for 48 h, 15 μ l of the A β_{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 ddH₂O, 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 degassed ddH₂O, air dried, and imaged by transmission electron microscopy (JEOL JEM-1011) at a magnification of $\times 150,000$.

In Vitro Microglial Uptake Assay—*In vitro* evaluation of A β uptake was performed as described previously (34). Briefly, human microglial cells (HMG 030, Clonexpress, Inc., Gaithersburg, MD) 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 macrophage-cerebrospinal fluid (AF-300-25, PeproTech Inc., Rocky Hill, NJ). The isolated microglia cells were plated into glass-bottomed well plates (P24G-1.5-13-F, Matrical Bioscience, Spokane, WA) and incubated at 37 $^{\circ}\text{C}$ supplied with 5% CO₂ for 2 days before treatment with A β and cromolyn sodium. After a medium change, microglia cells were incubated with 50 nM A β_{42} with or without cromolyn sodium at 10 nM, 10 μ M, or 1 mM for 16 h at 37 $^{\circ}\text{C}$. After incubation, the medium was collected, the levels of A β_{40} and A β_{42} were measured using a two-site A β ELISA (WAKO Chemicals), microglia cells were fixed in 4% paraformaldehyde, and the number was counted.

Animals and Cromolyn Sodium Treatment—APPswe/PS1dE9 (APP/PS1) mice were purchased from The Jackson Laboratory. These mice express a human mutant amyloid pre-

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cursor protein gene containing the Swedish mutation K594N/M595L as well as the Presenilin 1 gene deleted for 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 this study, 7.5-month-old APP/PS1 males were injected intraperitoneally daily for 1 week with escalating doses of 1.05, 2.1, 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 intraperitoneally 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 CO₂ 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 mixture, Roche) with 25 strokes on a mechanical Dounce homogenizer and centrifuged at 260,000 $\times g$ for 30 min at 4 °C as described previously (36). The TBS-soluble supernatant was collected, and the pellet was then homogenized in 2% Triton X-100/TBSI, 2% SDS/TBSI, and 70% formic acid.

Sandwich ELISA and Immunoblotting—The concentrations of A β ₄₀ and A β ₄₂ were determined using the commercially available kits BNT77/BA27 for A β _{x-40} or BNT77/BC05 for A β _{x-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 A β 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 10–20% Novex Tris-glycine gels (Invitrogen). After transfer to a 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 h. Membranes were then probed with the anti-A β 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 h at room temperature, immunoreactive proteins were developed using an ECL kit (Western Lightning, PerkinElmer Life Sciences) and detected on Hyperfilm ECL (GE Healthcare) (37). A β signal intensity was measured by densitometry using ImageJ software (<http://rsbweb.nih.gov/ij/>).

Immunohistochemistry—Serial paraffin sections were cut at 4 μ m 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 charge-coupled device camera (DP70, Olym-

pus, Tokyo, Japan). Quantitative analyses of amyloid load and plaque density were done using BIOQUANT software (version 6.90.10, MBSR, Nashville, TN) after application of an optical threshold. This software was coordinated with the motorized stage of an upright Leica DMRB microscope equipped with a charge-coupled device camera (DC330, DAGE-MTI, Inc., Michigan City, IN). Immunostained amyloid plaques were thresholded under the $\times 10$ objective after background correction to avoid uneven lighting. For colocalization analysis of A β in microglia, 4- μ m paraffin sections were immunostained with mouse anti-A β antibody 6E10 (Signet) for A β and rabbit anti-Iba1 (WAKO Chemicals) for microglia, followed by Alexa Fluor 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 $\times 63$) between PBS- and cromolyn sodium-treated animals. The percentage of Iba1 colocalizing with amyloid deposits was determined after image analysis using Fiji software (National Institutes of Health). The same thresholds were applied to both 488 and Cy3 channels, and a region of interest was selected corresponding to each plaque. After application of this region of interest on the Cy3 channel (Iba1 staining), an analysis of particles within the region of interest was performed, and the percentage of Iba1 staining overlapping with each amyloid deposit was measured.

In Vivo Microdialysis—*In vivo* microdialysis for ISF A β sampling was performed as reported previously (19). Briefly, the mice were stereotactically implanted with two guide cannulas (PEG-4, Eicom) into both hippocampi (anteroposterior, 3.1 mm; mediolateral, $\pm$ 2.8 mm; dorsoventral, 1.1 mm), under anesthesia with isoflurane (1.5% in O₂). After a recovery time of 3 days, intraperitoneal injections of cromolyn sodium started. ISF sampling was done 1 week after exposure to cromolyn sodium or PBS as a 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 (122 mM NaCl, 1.3 mM CaCl₂, 1.2 mM MgCl₂, 3.0 mM KH₂PO₄, and 25.0 mM NaHCO₃). The outlet and inlet of the probe were then connected to a peristaltic pump (ERP-10, Eicom) and a microsyringe pump (ESP-32, Eicom), respectively, using fluorinated ethylene propylene tubing. The probe was inserted into the mouse hippocampus through the guide cannula. After implantation, artificial cerebrospinal fluid was perfused for 1 h at a flow rate of 10 μ l/min before ISF sampling. ISF samples for the measurement of total A β or oligomeric A β were collected at a flow rate of 0.5 μ l/min or 0.1 μ l/min, respectively, and stored at -80 °C until A β measurement. During *in vivo* microdialysis sampling, mice were awake and moving freely 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 h, 100 nM of the γ -secretase inhibitor compound E (Enzo Life Sciences) diluted in artificial cerebrospinal fluid was perfused into the hippocampus to rapidly inhibit A β production in the tissue surrounding the probe. A β levels within the ISF were measured for an additional 5 h (38,

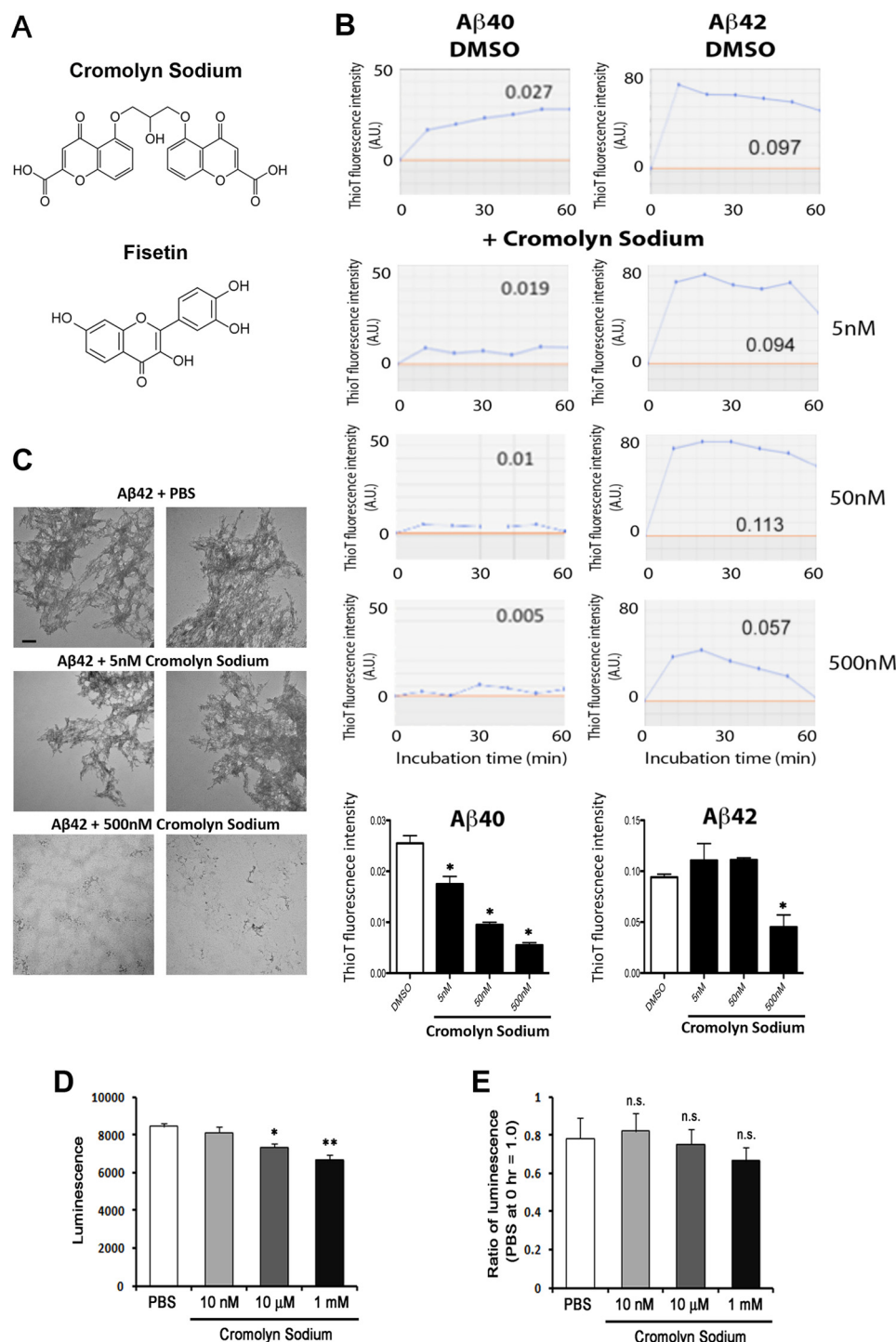


FIGURE 1. Cromolyn sodium inhibits A β polymerization *in vitro* but does not impact pre-existing oligomers. *A*, structures of cromolyn Sodium and fisetin. *B*, left panel, representative curves of Thioflavin-T fluorescence increase upon A β fibrillization after addition of dimethyl sulfoxide (top panel) or cromolyn sodium (5, 50, and 500 nM, bottom panels). Fibrillization of synthetic A β ₄₀ (left column) or A β ₄₂ (right column) peptides was followed over 1 h. The corresponding V_{max} index (milliunits/minute) is indicated on each graph. Right panel, bar graphs summarizing A β ₄₀ (top graph) and A β ₄₂ (bottom graph) fibrillization, which is significantly decreased in presence of cromolyn sodium even though smaller effects were observed on the A β ₄₂ fibrillization process. *C*, representative TEM pictures of A β ₄₂ fibrils formed after 48-h incubation of synthetic A β ₄₂ peptides (0.2 mg/ml) in the presence of 50 or 500 nM of cromolyn sodium. Scale bar = 100 nm. *D*, A β oligomerization assay using the split-luciferase complementation assay. HEK293 cells, which were engineered to stably express luci-A β ₄₂ and ferase-A β ₄₂, were incubated with 0, 10 nM, 10 μ M, and 1 mM of cromolyn sodium for 12 h. 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 A β were incubated with 0, 10 nM, 10 μ M, and 1 mM of cromolyn sodium for 12 h. 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). *, $p < 0.05$; **, $p < 0.01$; n.s., not significant.

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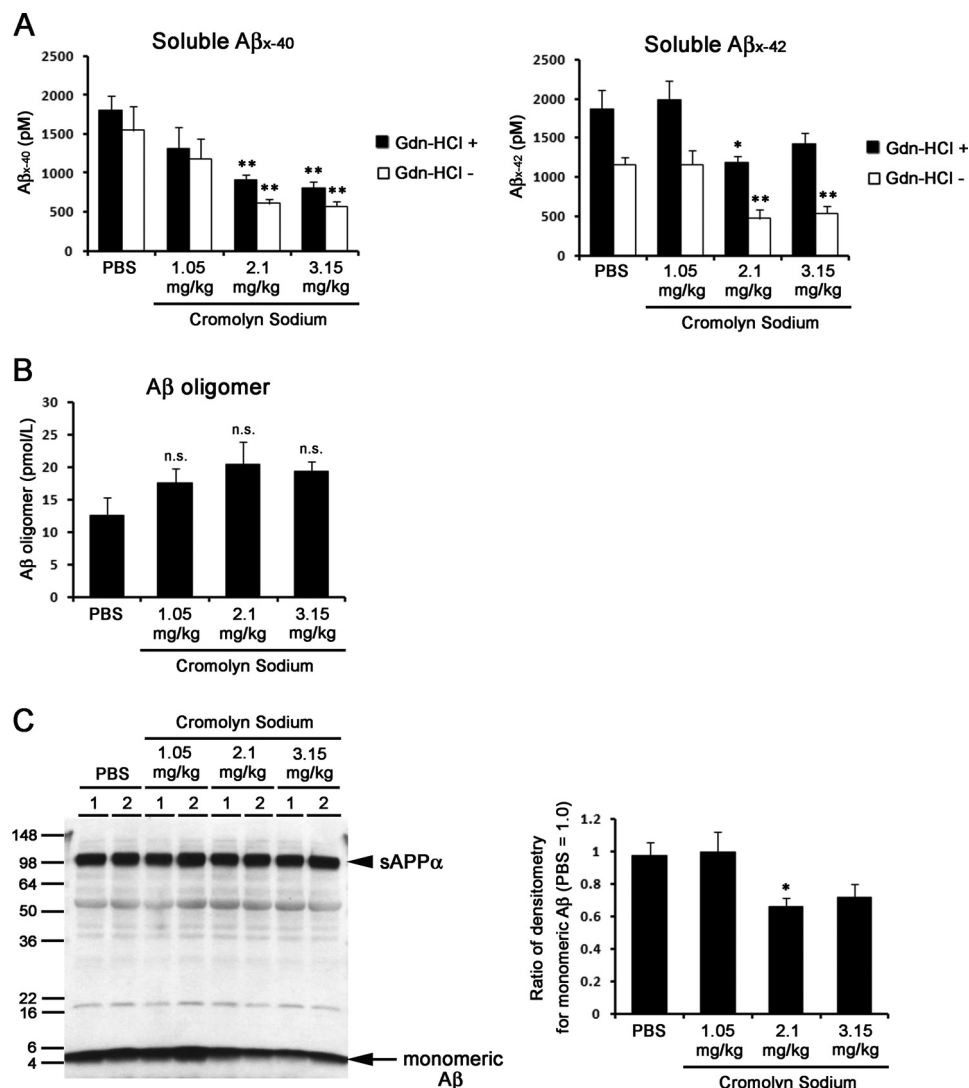


FIGURE 2. Cromolyn sodium reduces the concentration of soluble monomeric A β , not oligomeric A β , in the brain of APP/PS1 mice. A, one week after daily intraperitoneal injection with cromolyn sodium or PBS, TBS-soluble A β_{x-40} (left panel) and A β_{x-42} (right panel) were measured by ELISA with (black column) or without (white column) preincubation with Gdn-HCl. B, oligomeric A β content in TBS-soluble brain extracts was measured using 82E1/82E1 A β oligomer-specific ELISA. C, representative immunoblotting of TBS-soluble brain extracts with anti-A β antibody (6E10 and 82E1, left panel). The densitometry of A β monomers was quantified (right panel) ($n = 3-5$ mice/group). *, $p < 0.05$; **, $p < 0.01$; n.s., not significant.

39). The single logarithmic plot was made from A β levels and extrapolated the half-life of ISF A β .

Statistics Analysis—Statistical analyses were performed using GraphPad 5 Prism software. *In vitro*, each experiment 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 analysis of variance followed by Bonferroni post hoc test. *In vivo* data were averaged per mouse and analyzed using a non-parametric Kruskal-Wallis test, followed by Dunn multiple comparison test. For the quantification of amyloid plaques, data were analyzed using a non-parametric Mann-Whitney test. $p < 0.05$ was considered significant. Data in all figures are expressed as mean $\pm$ S.E.

RESULTS

Cromolyn Sodium Inhibits A β Polymerization *In Vitro* but Does Not Impact Preexisting Oligomers—Cromolyn sodium (sodium cromoglycate) is a Food and Drug Administration-

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 A β polymerization (27, 28), we hypothesized that cromolyn sodium may also impact A β aggregation processes and modulate A β metabolism *in vitro* and *in vivo*. To test this hypothesis, we first evaluated the effect of cromolyn sodium on A β_{40} and A β_{42} fibrillization using a Thioflavin-T assay. Over 1 h of incubation at 37 °C with increasing concentrations of cromolyn sodium (5, 50, and 500 nM), we observed an inhibition of A β fibril formation *in vitro* at a nanomolar concentration (Fig. 1B). Using TEM, we confirmed that the formation of A β_{42} fibrils was inhibited after incubation with 500 nM cromolyn sodium (Fig. 1C), whereas no effect was detected at a lower concentration (50 nM). Importantly, this effect was significantly less on the formation of A β_{42} fibrils compared with A β_{40} . We hypothesized that A β_{42} peptides, more fibrillogenic, lead to faster formation of stable amyloid

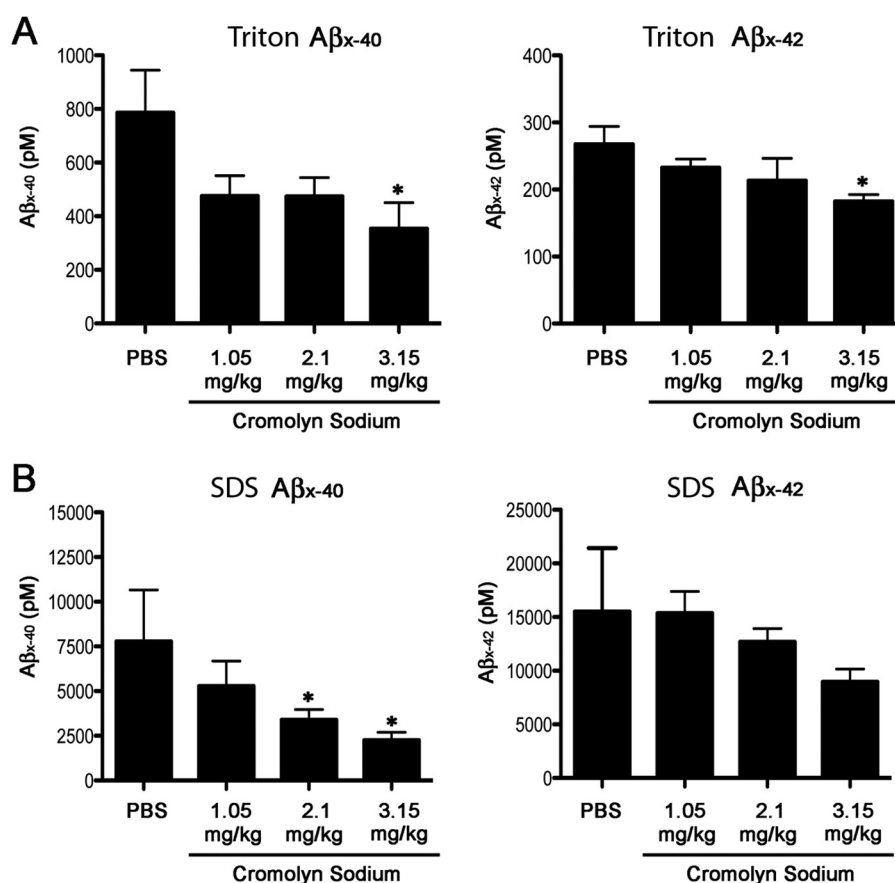


FIGURE 3. **Cromolyn sodium lowers the levels of A β _{x-40} and A β _{x-42} in both Triton-X and SDS fractions of APP/PS1 brain extracts.** As mentioned previously, AD transgenic mice were treated with PBS or escalating concentrations of cromolyn sodium (1.05, 2.1, and 3.15 mg/kg) for 1 week before euthanasia. *A*, bar graph representing the concentrations of A β _{x-40} (left panel) and A β _{x-42} (right panel) measured by ELISA in the Triton X extracts. *B*, bar graph representing the concentrations of A β _{x-40} (left panel) and A β _{x-42} (right panel) measured by ELISA in the SDS extracts ($n = 3$ –5 mice/group). *, $p < 0.05$.

aggregates compared with A β ₄₀, therefore resulting in a lower antifibrillogenic impact of cromolyn sodium. It is also possible that cromolyn sodium has a higher affinity for A β ₄₀ compared with A β ₄₂.

Because prefibrillar oligomeric amyloid aggregates are known to be highly neurotoxic *in vitro* and *in vivo* (21–26), we also asked whether cromolyn sodium could prevent the formation of these particularly harmful species. Using a split-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 β ₄₂ with cromolyn sodium significantly decreased the luminescence signal in a dose-dependent manner, therefore 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 μ M. 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 on the basis of the presence of A β ₄₂ peptides that are more amyloidogenic and aggregate faster than A β ₄₀ peptides. By contrast, addition of cromolyn sodium to conditioned medium that already contains pre-existing oligomers failed to impact the luminescence signal (Fig. 1E). These data indicate that cromolyn sodium efficiently prevents A β polymerization

into higher-ordered oligomers or fibrils but cannot dissociate pre-existing aggregates.

One-week Exposure to Cromolyn Sodium of APP/PS1 Mice Significantly Lowers the Content of Soluble A β *In Vivo* but Does Not Affect Amyloid Deposition or Highly Fibrillar A β Species—Our preliminary results confirmed that cromolyn sodium may interfere with A β 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, 2.1, or 3.15 mg/kg body weight) or PBS intraperitoneally to APP/PS1 mice daily for 1 week.

We first evaluated the levels of the most soluble A β 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 to 2.1 or 3.15 mg/kg cromolyn sodium for only 7 days significantly lowered the content of both TBS-soluble A β _{x-40} and A β _{x-42} by more than 50% (2.1 mg/kg dose, 39.5% for A β _{x-40} and 40.9% for A β _{x-42}; 3.15 mg/kg dose, 37.1% for A β _{x-40} and 46.2% for A β _{x-42}) (Fig. 2A, *Gdn-HCl*–). As reported previously, oligomeric A β or A β peptides associated with other unidentified molecules may interfere with the interaction with anti-A β detection antibodies, therefore altering the outcome of these ELISA systems (37). To circumvent this problem and estimate the exact level of TBS-

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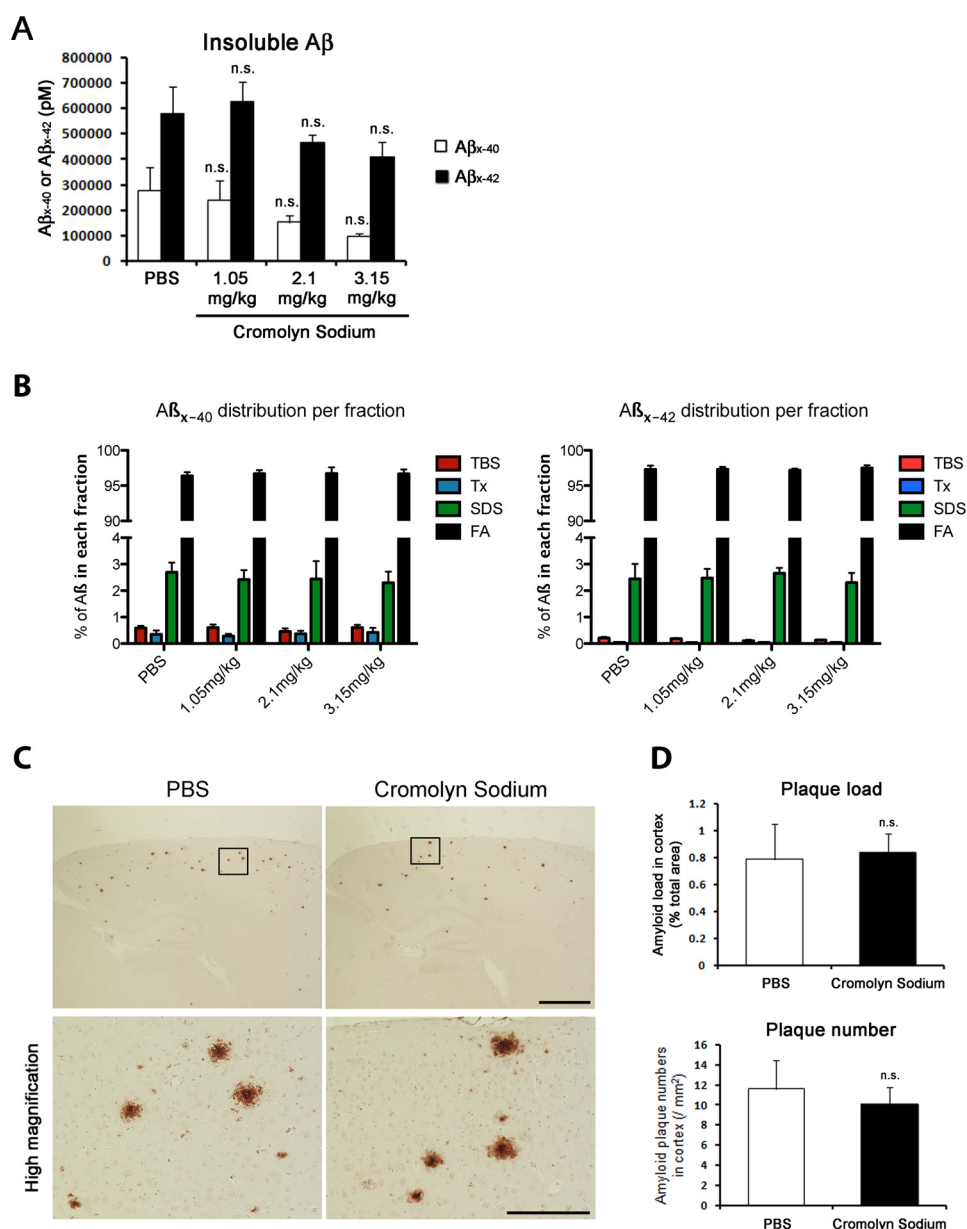


FIGURE 4. Cromolyn sodium does not alter the levels of insoluble A β peptides and amyloid deposition in APP/PS1 mice after a week of treatment. A, cromolyn sodium was intraperitoneal injected into 7.5-month-old APP/PS1 mice daily for 1 week with three different doses (1.05, 2.1, and 3.15 mg/kg). One week after daily injection, insoluble A β_{x-40} (white column) and A β_{x-42} (black column) present in the formic acid fraction of brain extracts were measured using A β ELISA. *n.s.*, not significant. B, percentages of A β_{x-40} and A β_{x-42} in each biochemical fraction (TBS, Triton, SDS, and formic acid (FA)) according to the total level of A β . Distributions were compared between control transgenic mice and animals treated with 1.05, 2.1, 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 1 week (using the rabbit anti-human amyloid β (N) antibody from Immuno-Biological Laboratories). Scale bars = 2 mm (top panels) and 200 μ m (bottom panels). D, bar graphs summarizing the amyloid load (top panel) and plaque density (bottom graph) in both group ($n = 3-5$ mice/group).

soluble A β , TBS-soluble fractions were incubated with 0.5 M guanidine (Gdn-HCl) at 37 °C for 30 min to dissociate oligomers or other complexes formed between A β and other proteins. As expected, the levels of A β after incubation with Gdn-HCl generally increased compared with the native conditions, especially A β_{x-42} , which is more prone to aggregation. Using this complementary assay, we confirmed that treatment with cromolyn sodium lowered the total level of TBS-soluble A β in a dose-dependent manner (2.1 mg/kg dose, 50.7% for A β_{x-40} and 63.3% for A β_{x-42} ; 3.15 mg/kg dose, 44.6% for A β_{x-40} and 76.1% for A β_{x-42}) (Fig. 2A, Gdn-HCl+).

When the amounts of oligomeric A β (or bound A β) were estimated according to the difference between the A β levels with and without incubation of Gdn-HCl, we found that cromolyn sodium did not significantly alter the content of higher-order amyloid species. To further examine this result, the concentrations of A β 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 4-kDa A β band using 6E10 and 82E1

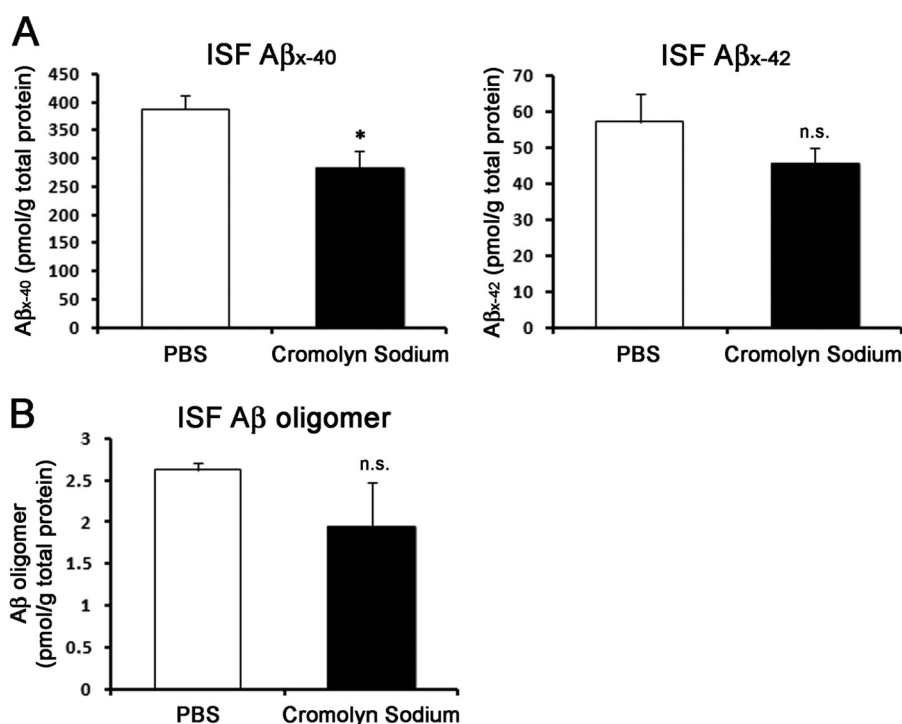


FIGURE 5. Acute treatment with cromolyn sodium decreases the levels of monomeric A β_{x-40} , not oligomeric A β , in the ISF of APP/PS1 mice. A, cromolyn sodium was injected intraperitoneally into 9-month-old APP/PS1 mice daily for 1 week at the 3.15 mg/kg dose. One week after daily injection, ISF sample were collected by *in vivo* microdialysis. The concentrations of A β_{x-40} (left panel) and A β_{x-42} (right panel) in ISF were measured by ELISA. B, oligomeric A β in ISF was quantified using the 82E1/82E1 A β oligomer-specific ELISA assay ($n = 5$ mice/group). *, $p < 0.05$; n.s., not significant.

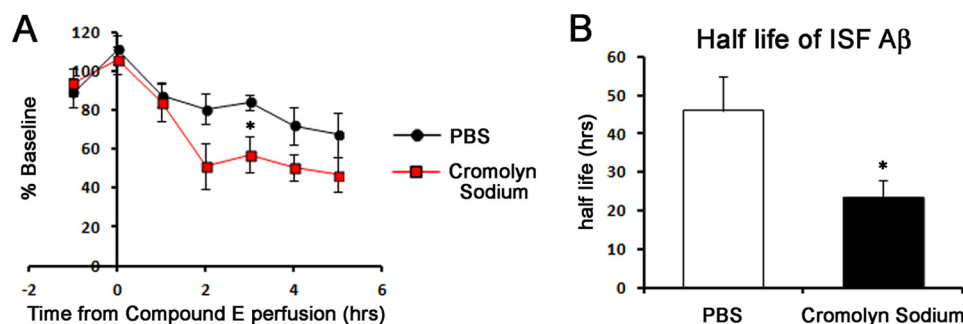


FIGURE 6. Cromolyn sodium decreases the half-life of A β in ISF. A, representative decay A β_{x-42} curves in mice treated with PBS and cromolyn sodium (3.15 mg/kg) after infusion of 100 nm of γ -secretase inhibitor (compound E) by reverse microdialysis. ISF samples were collected every hour before and after γ -secretase inhibitor perfusion. The concentration of ISF A β_{x-42} was measured by ELISA after preincubation with Gdn-HCl. B, bar graph summarizing the extrapolated A β_{x-42} half-life 1 week after intraperitoneal injection with cromolyn sodium or PBS ($n = 5$ mice/group). *, $p < 0.05$.

detection antibodies showed that cromolyn sodium decreased the amounts of monomeric A β (Fig. 2C), confirming our initial ELISA data. Because of the low proportion of soluble A β oligomers compared with total levels of A β , we did not detect those specific aggregates by Western blotting.

We then quantified the concentrations of A β 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 β_{x-40} and A β_{x-42} compared with PBS controls. Of note, and consistent with our *in vitro* results, cromolyn sodium appeared to have a bigger impact on decreasing A β_{x-40} than A β_{x-42} for all the fractions considered (TBS, Triton, and SDS).

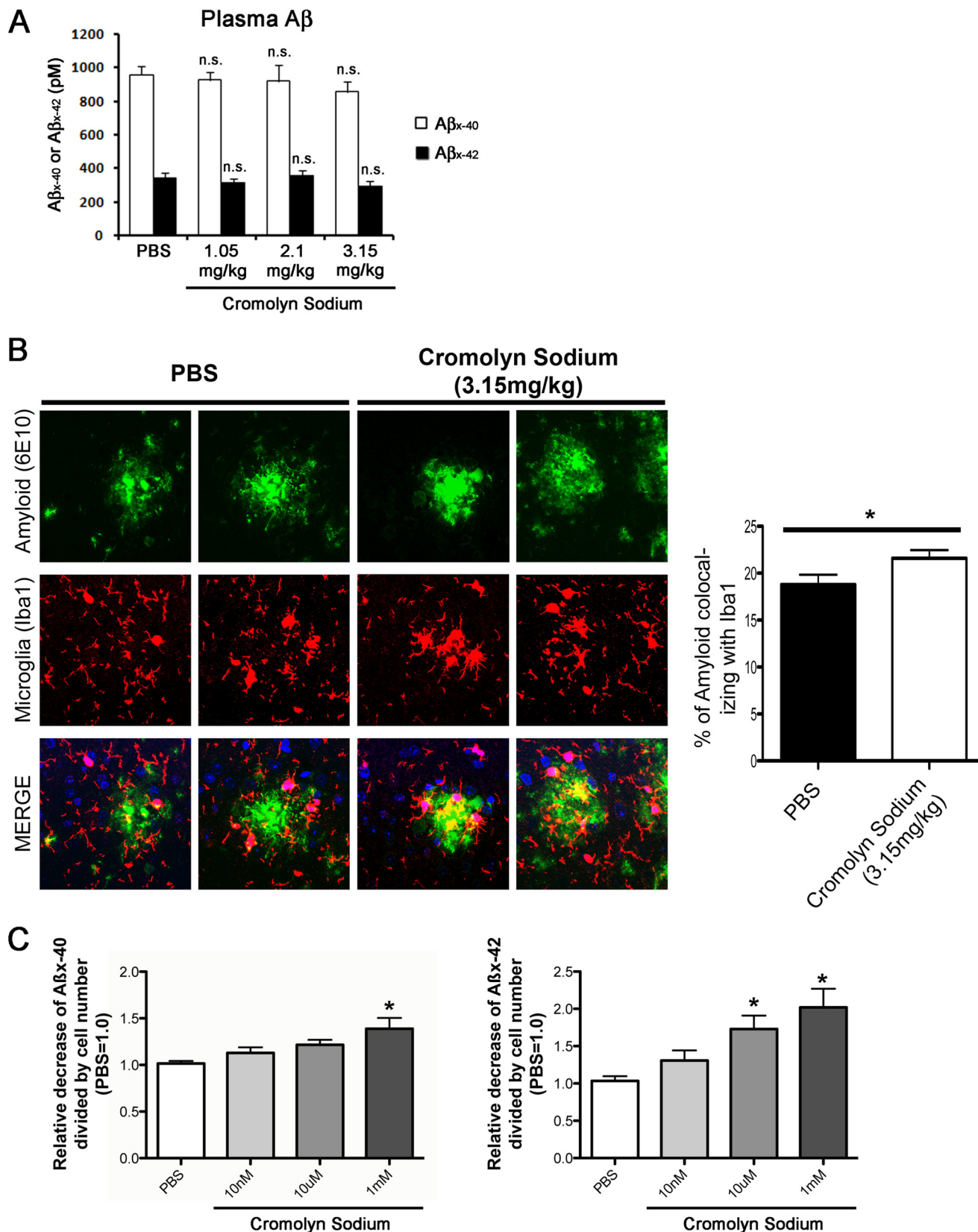
Finally, we evaluated the impact of cromolyn sodium on the most insoluble fraction of A β peptides (formic acid extracts) and

on the density of amyloid deposits. We observed that insoluble A β levels were not affected by acute cromolyn sodium administration (Fig. 4A). Because the levels of insoluble A β peptides are much higher compared with the most soluble fractions and because cromolyn sodium only impacts the soluble pool of A β_{x-40} and A β_{x-42} in TBS, Triton, and SDS extracts, it did not overall alter the distribution of A β 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-A β antibody, confirmed that the amount of extracellularly deposited aggregates of amyloid peptides remained unaffected after 1 week of cromolyn sodium treatment (Fig. 4, C and D). These data indicate 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.

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Taken together, these results indicate that acute intraperitoneal administration of cromolyn sodium rapidly decreases the amount of TBS-, Triton-, and SDS-soluble monomeric A β *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 1 week of treatment, which correlates



with a predominant effect of cromolyn sodium in preventing A β aggregation rather than dissociating previously existing aggregates.

Cromolyn Sodium Decreases the Concentration of A β_{40} in the Interstitial Fluid of APP/PS1 Mice—Our preliminary results indicate that acute exposure to 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 A β , we used a technique of *in vivo* microdialysis that allows the sampling of interstitial fluid closely in contact with neuronal cells.

APP/PS1 mice were injected intraperitoneally with PBS or cromolyn sodium at the highest dose (3.15 mg/kg body weight) daily for 1 week. Acute administration of cromolyn sodium dramatically decreased ISF A β_{x-40} levels by 30% (PBS, 387 pM; cromolyn, 283 pM). A similar trend was observed for the levels of both ISF A β_{x-42} and A β oligomers without reaching statistical significance (Fig. 5, A and B). These results suggest that cromolyn sodium acutely impacts A β_{x-40} peptides within the ISF, having less effect on the more fibrillogenic A β_{x-42} peptides.

Cromolyn Sodium Reduces the Half-life of A β within ISF, a Process Potentially Related to Microglial Uptake Rather Than Egress of A β through the Blood-Brain Barrier—As observed previously, monomeric A β peptides are more efficiently cleared from the brain compared with oligomeric species and higher-order aggregates (19). Because of the antifibrillogenic effect of cromolyn sodium observed *in vitro*, we hypothesized that exposure of AD transgenic mice to this compound may prevent A β aggregation and, therefore, promote its clearance from the brain. To test this hypothesis, we estimated the half-life of A β in ISF using reverse microdialysis with the γ -secretase inhibitor compound E to acutely block A β 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 8 days with PBS or cromolyn sodium at the highest dose (3.15 mg/kg body weight). In mice injected with cromolyn sodium, the ISF A β level started decreasing only 2 h 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 A β in cromolyn sodium-treated mice was shorter than that of the control by ~50% (Fig. 6B), therefore indicating that ISF A β was more rapidly cleared after treatment with this compound. Of note, the half-life of A β 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 kDa compared with previous publications (35 kDa), which allows sampling of oligomeric or apolipoprotein E-associated A β spe-

cies that have an increased half-life in the ISF compared with monomeric amyloid peptides (19).

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

We then tested whether Cromolyn sodium may impact the interaction of microglial cells with amyloid deposits and eventually affect A β clearance by microglia. We first performed a double immunostaining between A β and the microglial marker Iba1 in brain sections of mice treated with PBS or the highest dose of cromolyn sodium (3.15 mg/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 modestly 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 A β microglial uptake (34). Synthetic A β_{40} and A β_{42} peptides were applied to microglia in culture in the presence or absence of cromolyn sodium. After 16 h of incubation, we observed a dose-dependent decrease of A β_{40} and A β_{42} levels in the presence of cromolyn sodium, indicating that the impact of cromolyn sodium on A β aggregation mechanisms may promote A β clearance by microglial uptake (Fig. 7C). The combination of the *in vivo* and *in vitro* results may suggest that, in addition to inhibiting A β fibrillization, cromolyn sodium may also affect microglial activation and A β clearance.

DISCUSSION

Amyloid-reducing agents remain potentially promising therapeutic drugs in the treatment of Alzheimer disease as the only molecules currently available that 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 Food and Drug Administration since the 1970s for the treatment of asthma (40, 41). For asthma treatment, cromolyn sodium powder is 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

FIGURE 7. Cromolyn sodium does not affect the levels of A β in the plasma but promotes microglial A β clearance. A, quantification of the plasmatic levels of A β_{x-40} and A β_{x-42} 1 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 (3.15 mg/kg) or PBS daily for 7 days. The percentage of amyloid occupied by Iba1-positive processes was calculated for each deposit and showed an increased overlap between A β and Iba1 after treatment with cromolyn sodium ($n = 3$ mice for PBS and $n = 5$ mice for cromolyn sodium; approximately 20 plaques were evaluated for each animal). Scale bar = 10 μ m. C, effect of cromolyn sodium on microglial A β uptake *in vitro*. Microglial cells were cultured and incubated with 50 nM of synthetic A β_{40} or A β_{42} and 0, 10 nM, 10 μ M, or 1 mM of cromolyn sodium for 16 h. After the incubation, the concentrations of A β_{x-40} (left panel) and A β_{x-42} (right panel) in media were measured using A β ELISA and normalized with microglia cell number and according to the PBS control condition ($n = 3$ experiments). *, $p < 0.05$, **, $p < 0.01$; n.s., not significant.

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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 also have a beneficial effect on Alzheimer disease.

In this study, we demonstrated that addition of cromolyn sodium to A β_{40} and A β_{42} preparations not only prevents amyloid fibril formation but also inhibits A β oligomerization *in vitro*, therefore 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 A β economy *in vivo*, an effect detectable after only 1 week of treatment. We observed that intraperitoneal administration of cromolyn sodium reduces the levels of TBS-, Triton X-, and SDS-soluble A β 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 A β 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 A β but may rather be related to activation of microglial cells and an increased efficiency of microglial clearance of intracerebral A β peptides.

The specific effect of cromolyn sodium on A β 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 because we anticipated that the mobilization of one pool of amyloid would have induced a concomitant decrease in small soluble aggregates. However, oligomeric A β species have been shown previously to have a longer ISF half-life (19), therefore suggesting that a longer chronic exposure time to 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 β_{42} is less compared with A β_{40} , considering the higher potency of A β_{42} to aggregate. Our results illustrate the complex relationships between the kinetics of monomeric and oligomeric A β in the interstitial fluid *in vivo*. Nonetheless, the marked change in soluble monomeric A β levels after only a week of treatment with cromolyn sodium is remarkable, with about 50% reduction in the content of A β 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 A β , if sustained chronically, may have important effects in AD.

Finally, numerous synthetic or naturally occurring compounds (such as curcuminoids or flavonoids (45)), small peptides (eventually derived from A β itself (46)), endogenously expressed chaperones (such as DNAJB6 (47)), or even synthetic nucleotides (48) have been reported previously to prevent A β aggregation into higher-order species or fibrils. Despite their

potency to inhibit or destabilize the formation of amyloid fibrils *in vitro*, so far only a few of these compounds have been associated with an impact on the neuropathological hallmarks of the disease *in vivo*. Among them, curcuminoids and flavonoids have been associated with decreased A β deposition, A β -dependent neurotoxicity, and 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 A β aggregation *in vitro*, those particular compounds also modulate other processes (such as inflammation, oxidative stress, or endoplasmic reticulum stress) that concomitantly counteract the buildup of amyloid within the brain but also alleviate A β -related deleterious effects. It is also difficult to draw a clear conclusion regarding the potential therapeutic impact of cromolyn sodium compared with other antifibrillogenic molecules, considering that the duration of exposure, the route of administration (intravenous, intracerebroventricular infusion, intraperitoneal injection, etc.), and the cellular or animal models are highly variable between studies.

In conclusion, additional investigations will be necessary to determine whether 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 A β 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 contribute to its potential beneficial impact in AD.

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