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 antiamyloidogenic 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 on amyloid economy.

Significance: Developing new disease-modifying therapeutics remains an urgent need in the treatment of 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 (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 (37–43 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 cases (16–18). Misfolded Aβ monomers can aggregate into higher-order oligomers, eventually forming fibrils that are deposited into the extracellular space to form fibrillar amyloid neuritic plaques. Aβ 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 Aβ 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 Aβ oligomerization and promote Aβ clearance in vitro and in vivo. In addition, the use of cromolyn sodium in addiction models (after
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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 Aβ 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 Aβ 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 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 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 μM, 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 fibrillation assay was performed using synthetic Aβ 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 Aβ40 and Aβ42 peptides were purchased from Peptide Institute, Inc. After resuspension in 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-mm filter, as described previously (31). The stock solution of Aβ40 and Aβ42 peptides was diluted to 5 μM and stored at −80 °C prior to use. Aβ40 or Aβ42 were applied at 50 nM in cell cultures.

In Vitro Aβ Fibrillation, Oligomerization, and Dissociation Assays—An in vitro fibrillation assay was performed using Aβ40 or Aβ42 peptides dissolved in dimethyl sulfoxide at a concentration of 250 μM and sonicated for 1 min. Aβ40 or Aβ42 were diluted to 5 μM in an assay volume of 200 μl with artificial cerebrospinal fluid solution (125 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 1.25 mM Na2HPO4, 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 μM 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 heparin 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.

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 was grown to subconfluence and then treated with PBS or cromolyn sodium at 10 nM, 10 μM, or 1 mM for 12 h at 37 °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 μM, or 1 mM) with conditioned medium from naïve HEK293 cells overexpressing each half of G sansia luciferase fused with Aβ14 for 12 h at 37 °C. The luciferase activity was measured as described previously (32).

Analysis of Aβ42 Fibril Formation by Transmission Electron Microscopy—The antibilirubinogenic properties of cromolyn was confirmed by performing TEM analysis as previously described (33). Briefly, synthetic Aβ42 was dissolved in PBS at a concentration of 0.2 mg/ml for 48 h at 37 °C, with or without addition of Cromolyn Sodium at a concentration of either 5 nM or 500 nM. After incubation for 48 h, 15 μl of the Aβ42 fibril solution was 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 degased ddH2O, air dried, and imaged by transmission electron microscopy (JEOL, JEM-1011) at a magnification of ×150,000.

In Vitro Microglial Uptake Assay—In vitro evaluation of Aβ 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 °C supplied with 5% CO2 for 2 days before treatment with Aβ and cromolyn sodium. After a medium change, microglia cells were incubated with 50 nM Aβ42 with or without cromolyn sodium at 10 nM, 10 μM, or 1 mM for 16 h at 37 °C. After incubation, the medium was collected, the levels of Aβ40 and Aβ42 were measured using a two-site Aβ 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 × 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 Aβ40 and Aβ42 were determined using the commercially available kits BNT77/BA27 for Aβ40 or BNT77/BC05 for Aβ42, 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 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-Aβ antibodies 6E10 (Signet) and 82E1 (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 ×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 Aβ 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, ± 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 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 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 Aβ production in the tissue surrounding the probe. Aβ levels within the ISF were measured for an additional 5 h (38,
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 dimethyl sulfoxide (top panel) or cromolyn sodium (5, 50, and 500 nM, bottom panels). Fibrillization of synthetic Aβ40 (left column) or Aβ42 (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 Aβ40 (top graph) and Aβ42 (bottom graph) fibrillization, which is significantly decreased in presence of cromolyn sodium even though smaller effects were observed on the Aβ42 fibrillation process. C, representative TEM pictures of Aβ42 fibrils formed after 48-h incubation of synthetic Aβ42 peptides (0.2 mg/ml) in the presence of 50 or 500 nM of cromolyn sodium. Scale bar = 100 nm. D, Aβ oligomerization assay using the split-luciferase complementation assay. HEK293 cells, which were engineered to stably express luci-Aβ42 and ferase-Aβ42, were incubated with 0, 10 nM, 10 μM, 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 Aβ were incubated with 0, 10 nM, 10 μM, 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.
Results

Cromolyn Sodium Inhibits Aβ 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 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 Aβ40 and Aβ42 fibrillation using a Thioflavin-T assay. Over 15 h of incubation at 37 °C with increasing concentrations 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 Aβ42 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 Aβ42 fibrils compared with Aβ40. We hypothesized that Aβ42 peptides, being more fibrillogenic, lead to faster formation of stable amyloid plaques.

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 ± S.E.
aggregates compared with Aβ<sub>40</sub>, therefore resulting in a lower antifibrillogenic impact of cromolyn sodium. It is also possible that cromolyn sodium has a higher affinity for Aβ<sub>40</sub> compared with Aβ<sub>42</sub>. 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 Aβ<sub>42</sub> 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 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 on the basis of the presence of Aβ<sub>42</sub> peptides that are more amyloidogenic and aggregate faster than Aβ<sub>40</sub> 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 Aβ 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 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, 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 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 to 2.1 or 3.15 mg/kg cromolyn sodium for only 7 days significantly lowered the content of both TBS-soluble Aβ<sub>40</sub> and Aβ<sub>42</sub> by more than 50% (2.1 mg/kg dose, 39.5% for Aβ<sub>40</sub> and 40.9% for Aβ<sub>42</sub>; 3.15 mg/kg dose, 37.1% for Aβ<sub>40</sub> and 46.2% for Aβ<sub>42</sub>) (Fig. 2A). As reported previously, oligomeric Aβ or Aβ peptides associated with other unidentified molecules may interfere with the interaction with anti-Aβ detection antibodies, therefore altering the outcome of these ELISA systems (37). To circumvent this problem and estimate the exact level of TBS-
soluble Aβ. 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 Aβ and other proteins. As expected, the levels of Aβ after incubation with Gdn-HCl generally increased compared with the native conditions, especially Aβx-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 Aβ in a dose-dependent manner (2.1 mg/kg dose, 50.7% for Aβx-40 and 63.3% for Aβx-42; 3.15 mg/kg dose, 44.6% for Aβx-40 and 76.1% for Aβx-42) (Fig. 2A, 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. 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 4-kDa Aβ band using 6E10 and 82E1

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 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 Aβx-40 (white column) and Aβx-42 (block column) present in the formic acid fraction of brain extracts were measured using Aβ ELISA. n.s., not significant. B, percentages of Aβx-40 and Aβx-42 in each biochemical fraction (TBS, Triton, SDS, and formic acid (FA)) according to the total level of Aβ. 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 μm (bottom panels). D, bar graphs summarizing the amyloid load (top panel) and plaque density (bottom graph) in both group (n = 3–5 mice/group).
Detection antibodies showed that cromolyn sodium decreased the amounts of monomeric Aβx-40, not oligomeric Aβ, 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 Aβx-40 (left panel) and Aβx-42 (right panel) in ISF were measured by ELISA. B, oligomeric Aβ in ISF was quantified using the 82E1/82E1 Aβ oligomer-specific ELISA assay (n = 5 mice/group). *, p < 0.05; n.s., not significant.

FIGURE 5. Acute treatment with cromolyn sodium decreases the levels of monomeric Aβx-40, not oligomeric Aβ, 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 Aβx-40 (left panel) and Aβx-42 (right panel) in ISF were measured by ELISA. B, oligomeric Aβ in ISF was quantified using the 82E1/82E1 Aβ oligomer-specific ELISA assay (n = 5 mice/group). *, p < 0.05; n.s., not significant.

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 compared with the most soluble fractions and because cromolyn sodium only impacts the soluble pool of Aβx-40 and Aβx-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 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.
Taken together, these results indicate that acute intraperitoneal 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 1 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 Aβ₄₀ 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 Aβ, 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 Aβ₄₀ levels by 30% (PBS, 387 pm; cromolyn, 283 pm). A similar trend was observed for the levels of both ISF Aβ₄₂ and Aβ oligomers without reaching statistical significance (Fig. 5, A and B). These results suggest that cromolyn sodium acutely impacts Aβ₄₀ peptides within the ISF, having less effect on the more fibrilligenic Aβ₄₂ 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 observed previously, monomeric Aβ peptides are more efficiently cleared from the brain compared with oligomeric species and higher-order aggregates (19). Because of the antifibrilligenic 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 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 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 Aβ 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 Aβ in cromolyn sodium-treated mice was shorter than that of the control by ~50% (Fig. 6B), therefore 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 kDa compared with previous publications (35 kDa), which allows sampling of oligomeric or apolipoprotein E-associated Aβ species 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 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 Aβ₄₀ nor Aβ₄₂ were changed even at the highest dose of cromolyn sodium (Fig. 7A). These data suggest that the antifibrilligenic effect of cromolyn sodium does not primarily lead to a detectable increase of Aβ 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 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 (3.15 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 Aβ microglial uptake (34). Synthetic Aβ₄₀ and Aβ₄₂ 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 Aβ₄₀ and Aβ₄₂ levels in the 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 the 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 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 Aβ in the plasma but promotes microglial Aβ clearance. A, quantification of the plasmatic levels of Aβ₄₀ and Aβ₄₂, 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 Aβ 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 μm. C, effect of cromolyn sodium on microglial Aβ uptake in vitro. Microglial cells were cultured and incubated with 50 nm of synthetic Aβ₄₀ or Aβ₄₂ and 0, 10 nm, 10 μm, or 1 μm of cromolyn sodium for 16 h. After the incubation, the concentrations of Aβ₄₀ (left panel) and Aβ₄₂ (right panel) in media were measured using Aβ 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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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 also have a beneficial effect on Alzheimer disease.

In this study, we demonstrated that addition of cromolyn sodium to Aβ40 and Aβ42 preparations not only prevents amyloid fibril formation but also inhibits Aβ 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 Aβ 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 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 to activation of microglial cells and an increased efficiency of microglial clearance 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 because 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 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 Aβ42 is less compared with Aβ40, considering the higher potency of Aβ42 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 the content of Aβcromolyn 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 the overall impact 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 contribute to its potential beneficial impact in AD.

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 Aβ fibrillation 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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