Supporting Information

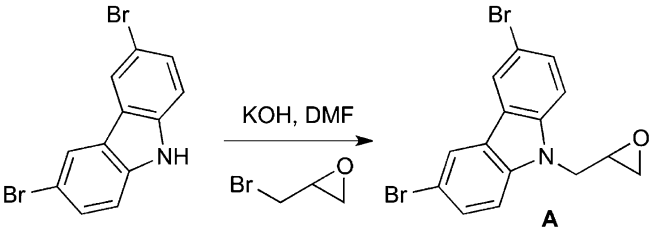
De Jesús-Cortés et al. 10.1073/pnas.1213956109

SI Materials and Methods

Thirty-Day Survival Assay of Newborn Hippocampal Neurons. Because both social activity and voluntary exercise enhance hippocampal neurogenesis, mice were individually housed without access to running wheels throughout the entire procedure, beginning 1 wk before bromodeoxyuridine (BrdU; Sigma-Aldrich) labeling of newborn cells. Throughout the study, mice had ad libitum access to food and water. BrdU was injected intraperitoneally at 150 mg/kg i.p., and 24 h later administration of test compounds or vehicle was initiated. P7C3 and P7C3A20 were dissolved in 5% dextrose (pH 7.0) with 2.5% DMSO and 10% Cremaphor EL (Sigma; C5135). Dimebon was dissolved in normal saline. Compounds were compared with their respective controls and were tested at 2.5, 5, 10, and 20 mg/kg twice daily (i.p.) for 30 d. The injection site was alternated between right and left sides. Each group consisted of six 12-wk-old adult male C57Bl/6 mice. Animals were monitored daily for general health and weight loss. Cage changes were performed per routine scheduling. After 30 d of compound administration, mice were killed by transcardial perfusion with 4% paraformaldehyde at pH 7.4, and their brains were processed for immunohistochemical detection of incorporated BrdU in the dentate gyrus. Dissected brains were immersed in 4% paraformaldehyde overnight at 4 °C and then cryoprotected in sucrose before being sectioned with a Leica SM2000R sliding microtome coronally into 40-μM-thick free-floating sections. Unmasking of BrdU antigen was achieved through incubating tissue sections for 2 h in 50% formamide/2× SSC at 65 °C, followed by a 5-min wash in 2× SSC and subsequent incubation for 30 min in 2 M HCl at 37 °C. Sections were processed for immunohistochemical staining with mouse monoclonal anti-BrdU (1:100; Roche). Diaminobenzidine was used as a chromagen, and tissue was counterstained with hematoxylin to aid in visualization of the neuroanatomy. Images were analyzed with a Nikon Eclipse 90i motorized research microscope with Plan Apo lenses coupled with Metamorph Image Acquisition software (Nikon). Quantification of all staining was done blind to treatment group. The number of $BrdU^+$ cells in the entire dentate gyrus was quantified by counting $BrdU^+$ cells within the dentate gyrus in every fifth section throughout the entire hippocampus and then normalizing for dentate gyrus volume.

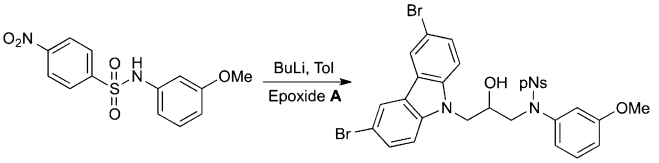
Assessment of MPTP-Mediated Neurotoxicity to Murine SNc Neurons. Fifteen adult male C57BL/6 mice were individually housed for 1 wk and then injected daily for 5 d with 30 $mg \cdot kg^{-1} \cdot d^{-1}$ (i.p.) free base 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Sigma). On day 6, 24 h after receiving the fifth and final dose of MPTP, daily treatment with P7C3, P7C3A20, Dimebon, or vehicle was initiated. Mice were housed in disposable caging, and protective gear and precautions were implemented for handling MPTP in accordance with University of Texas Southwestern Medical Center policy. Dose–response studies were conducted in which mice received twice daily doses of each compound (or vehicle) by i.p. injection for the following 21 d, after which mice were killed by transcardial perfusion with 4% paraformaldehyde. Brains were dissected, fixed overnight in 4% paraformaldehyde, and cryoprotected in sucrose for freezing by standard procedures. Frozen brains were sectioned through the striatum and substantia nigra pars compacta (SNc) at 30-μm intervals, and every fourth section (spaced 120 μm apart) was stained with antibodies directed against tyrosine hydroxylase (TH) (Abcam; rabbit anti-TH, 1:2,500). Diaminobenzidine was used as a chromagen, and tissue was counterstained with hematoxylin to aid in visualization of the neuroanatomy. Images were analyzed with a Nikon Eclipse 90i motorized research microscope with Plan Apo lenses coupled with Metamorph Image Acquisition software (Nikon). TH^+ neurons were counted with Image J software (National Institutes of Health) in every section by two blinded investigators and results were averaged and multiplied by the sectioning interval to determine the total number of TH^+ neurons per SNc.

Synthesis of P7C3-A20. Scheme S1. 3,6-Dibromo-9-(oxiran-2-ylmethyl)-9H-carbazole.



P7C3-A20 was synthesized using a modification of a procedure described previously (1). Thus, following a literature procedure (1), powdered KOH (0.103 g, 1.85 mmol) was added to a solution of 3,6-dibromocarbazole (0.500 g, 1.54 mmol) in DMF (1.5 mL) at ambient temperature and stirred for 30 min until dissolved. Epibromohydrin (0.32 mL, 3.8 mmol) was added via syringe and the reaction was stirred at room temperature overnight. Upon completion, the solution was partitioned between EtOAc and H_2O. The aqueous layer was washed three times in EtOAc, and the combined organics were washed with saturated aqueous NaCl, dried over Na_2SO_4, filtered, and concentrated in vacuo. The crude residue was recrystallized from EtOAc/Hexane to afford epoxide **A**, 3,6-dibromo-9-(oxiran-2-ylmethyl)-9H-carbazole (80%). ^1H NMR ($CDCl_3$, 500 MHz) δ 8.10 (d, 2H, J = 2.0 Hz), 7.54 (dd, 2H, J = 2.0, 8.5 Hz), 7.31 (d, 2H, J = 8.5 Hz), 4.62 (dd, 1H, J = 2.5, 16.0 Hz), 4.25 (dd, 1H, J = 5.5, 16.0 Hz), 3.29 (m, 1H), 2.79 (dd, 1H, J = 4.0, 4.5 Hz), 2.46 (dd, 1H, J = 2.5, 5.0 Hz).

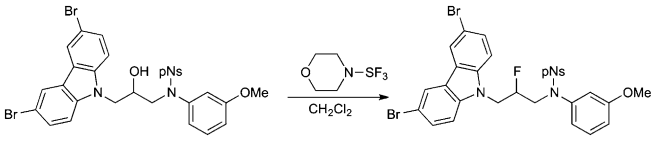
Scheme S2. *N*-(3-(3,6-dibromo-9H-carbazol-9-yl)-2-hydroxypropyl)-*N*-(3-methoxyphenyl)-4-nitrobenzenesulfonamide.



A heterogeneous mixture of *N*-(4-methoxyphenyl)-4-nitrobenzenesulfonamide (100.2 mg, 0.32 mmol) in toluene (2.5 mL, 0.13 M) under a N_2 atmosphere was cooled in a dry ice/acetone bath before dropwise addition of *n*-butyllithium (200 μL of 1.78 M in hexanes, 0.36 mmol). The reaction was stirred at –78 °C for 10 min before addition of epoxide **A**. The heterogeneous mixture was stirred at room temperature for 5 min before heating at 100 °C for 48 h. The cooled reaction was diluted with EtOAc and washed three times in 5% acetic acid solution, followed by a brine wash. The organic layer was dried over Na_2SO_4, filtered, and condensed. The crude mixture was purified by chromatography (SiO_2, 100% CH_2Cl_2) to afford the product (88%). ^1H NMR ($CDCl_3$, 400 MHz) δ 8.23 (d, 2H, J = 8.5 Hz), 8.06 (d, 2H, J = 1.9 Hz), 7.65 (d, 2H, J = 8.5 Hz), 7.46, (dd, 2H, J = 1.9, 8.6 Hz), 7.22 (d, 2H, J = 8.8 Hz), 6.94 (d, 2H, J = 8.8 Hz), 6.83

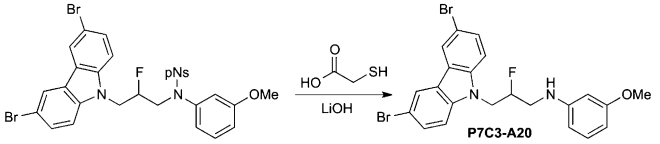
(d, 2H, J = 9.1 Hz), 4.44 (dd, 1H, J = 3.6,14.9 Hz), 4.26–4.34 (m, 1H), 4.17–4.24 (br s, 1H), 3.81 (s, 3H), 3.62–3.75 (m, 2H). ESI m/z: 732.0 ($[M+HCOO]^-$, $C_{29}H_{24}Br_2N_3O_8S$ requires 732.0).

Scheme S3. *N*-(3-(3,6-dibromo-9H-carbazol-9-yl)-2-fluoropropyl)-*N*-(3-methoxyphenyl)-4-nitrobenzenesulfonamide.



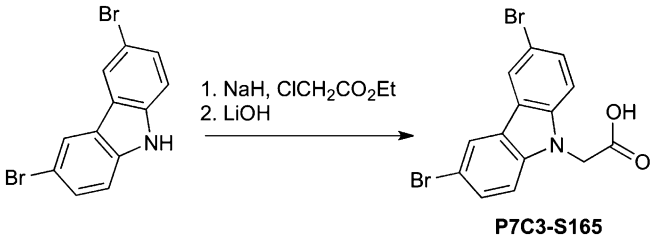
The product from the previous step was fluorinated as follows: An oven-dried 20-mL scintillation vial containing Ns-A (18.3 mg, 0.027 mmol) was purged with N2 and charged with anhydrous CH_2Cl_2 (1.5 mL, 0.018 M). The sealed vial was cooled in a dry ice acetone bath before the dropwise addition of morpholinosulfur trifluoride (morpho-DAST, 0.053 mmol). The reaction temperature was maintained at –78 °C for 1 h and then slowly warmed to room temperature and stirred overnight. The reaction was quenched with 2.0 mL of saturated $NaHCO_3$ solution and diluted with 6 mL CH_2Cl_2 and extracted three times. The combined organics were dried over Na_2SO_4, filtered, and condensed. The crude product was carried forward without further purification. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.28 (d, 2H, J = 8.0 Hz), 8.13 (s, 2H), 7.72 (d, 2H, J = 8.7 Hz), 7.54, (d, 2H, J = 8.0 Hz), 7.21 (d, 3H, J = 8.1 Hz), 6.89 (dd, 1H, J = 2.4, 8.3 Hz), 6.67 (t, 1H, J = 2.0 Hz), 6.55 (d, 1H, J = 8.0 Hz), 4.93 (m, 1H), 4.43–4.68 (m, 2H), 4.20 (t, 1H, J = 6.2 Hz), 3.81–3.99 (m, 2H), 3.75 (s, 3H). ESI m/z: 733.9 ([M+HCOO]–, $C_{29}H_{23}Br_2FN_3O_7S$ requires 734.0).

Scheme S4. *N*-(3-(3,6-dibromo-9H-carbazol-9-yl)-2-fluoropropyl)-3-methoxyaniline (P7C3-A20).



Lithium hydroxide (3.2 mg, 0.134 mmol), DMF (0.5 mL, 0.06 M), and mercaptoacetic acid (4.2 μL, 0.060 mmol) were added to a vial containing nosyl-protected P7C3-A20 (21.0 mg, 0.030 mmol). After stirring at room temperature for 1 h the reaction mixture was diluted with EtOAc and washed sequentially with H_2O, saturated aqueous $NaHCO_3$, H_2O (three times), and brine. The organic layer was dried over Na_2SO_4, filtered, and condensed. The crude reaction mixture was purified (SiO_2, 30% EtOAc/Hexanes + 0.2% Et_3N), to afford **P7C3-A20** (13.6 mg, 88%). ^{1}H NMR ($CDCl_3$, 500 MHz) δ 8.16 (d, 2H, J = 2.0 Hz), 7.56 (dd, 2H, J = 1.9, 8.7 Hz), 7.31 (d, 2H, J = 8.6 Hz), 7.11 (t, 1H, J = 8.1 Hz), 6.36 (dd, 1H, J = 2.2, 8.1 Hz), 6.23 (dd, 1H, J = 2.0, 8.0 Hz), 6.15 (t, 1H, J = 2.3 Hz), 5.11 (dddd, 1H, J = 4.6, 5.8, 10.4, 47.7 Hz), 4.60 (m, 2H), 4.39 (dm, 2H), 3.95 (t, 1H, J = 6.3 Hz), 3.75 (s, 3H). ESI m/z: 504.9 ($[M+H]^+$, $C_{22}H_{20}Br_2FN_2O$ calculated 505.0).

Synthesis of P7C3-S165. Scheme S5. Ethyl [2-(3,6-dibromo-9H-carbazol-9-yl]acetate.



Sodium hydride was added to a stirred solution of 3,6-dibromocarbazole (250 mg, 0.77 mmol) in DMF (4 mL). The solution was stirred for 30 min before the dropwise addition of ethyl chloroacetate. After 12 h water was added and a fine white precipitate formed, which was filtered and rinsed with water and hexanes to afford the desired ethyl ester in 93% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15 (s, 2H), 7.56 (d, J = 8.6 Hz, 2H), 7.21 (d, J = 8.5 Hz, 2H), 4.94 (s, 2H), 4.20 (q, J = 6.3 Hz, 2H), 1.26–1.18 (m, 3H). ESI m/z: 409.7 ([M+H]+, $C_{16}H_{13}Br_2NO_2$ requires 409.9).

Scheme S6. 2-(3,6-Dibromo-9H-carbazol-9-yl)acetic acid (P7C3-S165).

Methanol (0.2 mL), water (0.2 mL), and lithium hydroxide (14.5 mg, 0.6 mmol) were added to a stirred solution of the ethyl ester (50 mg, 0.12 mmol) in 0.6 mL of THF. After 1 h all starting material had been consumed. The pH was adjusted to about 4 with 1 N HCl, and a white precipitate was collected and rinsed with fresh water to afford the desired acid **P7C3-S165** in 95% yield. ^{1}H NMR (500 MHz, acetone) δ 8.41 (s, 2H), 7.62 (dt, J = 8.6, 1.7 Hz, 2H), 7.58 (dd, J = 8.7, 1.5 Hz, 2H), 5.31 (d, J = 1.6 Hz, 2H). ^{13}C NMR (acetone-d_6, 126 MHz) δ 173.8, 141.0 (2C), 129.7 (2C), 124.4 (2C), 124.0 (2C), 113.0 (2C), 112.5 (2C), 76.3. ESI m/z: 381.7 ([M+H]+, $C_{14}H_9Br_2NO_2$ requires 381.9).

P7C3-S7, -S8, -S40, -S41, and -S54 were synthesized as previously described (1). P7C3-S184 was synthesized as previously described by Asso et al. (2).

Pharmacokinetic Analysis. C57BL/6 mice treated with MPTP and then dosed i.p. with compound for 21 d were used for pharmacokinetic (PK) analysis of total P7C3, P7C3A20, and Dimebon levels in plasma and brain. In a separate set of experiments designed to test the ability of unique P7C3 analogs to cross the blood–brain barrier, C57BL/6 mice were dosed i.p. a single time with compounds at 10 mg/kg. P7C3, P7C3A20, and Dimebon were formulated for administration as described above. Analogs were formulated in 5% Dextrose, pH 7.4, containing 5% DMSO and 10% Cremophor EL with the exception of P7C3-S8, which required 10% DMSO and 20% Cremophor EL dissolved in 5% Dextrose for delivery. Six hours after the final compound dose, animals were given an inhalation overdose of CO_2 and whole blood and brain were collected. Plasma was prepared from blood and was stored along with the brain tissue at −80 °C until analysis. Brain homogenates were prepared by homogenizing the tissues in a threefold volume of PBS. Total brain homogenate volume was estimated as volume of PBS added plus volume of brain in milliliters. One hundred milliliters of either plasma or brain homogenate was processed by addition of a two- or fourfold excess of methanol or acetonitrile containing formic acid and an internal standard (IS), *N*-benzylbenzamide (Sigma-Aldrich, lot 02914LH) to precipitate plasma or tissue protein and release bound drug. The final formic acid concentration was 0.1%, and the final IS concentration was 25 ng/mL. Extraction conditions were optimized before PK analysis for efficient and reproducible recovery over a 3-log range of concentrations. The samples were vortexed 15 s, incubated at room temperature for 10 min, and spun two times at 16,100 × g in a standard refrigerated microcentrifuge. The supernatant was then analyzed by liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). Standard curves were prepared by addition of the appropriate compound to plasma or brain homogenate. A value three times above the signal obtained in the blank plasma or brain homogenate was designated the limit of detection (LOD). The limit of quantitation (LOQ) was defined as the lowest concentration at which back calculation yielded a concentration within 20% of the theoretical value and above the LOD signal. LOQ values for plasma and brain ranged from 0.5 to 500 ng/mL but were well below the concentrations measured at 6 h for all of the compounds. Compound levels were monitored by LC/MS/MS, using an AB/Sciex 3200 Qtrap mass spectrometer coupled to a Shimadzu Prominence LC. The compounds were detected

with the mass spectrometer in multiple-reaction monitoring (MRM) mode by following the precursor to fragment ion transition 474.9 → 337.8 for P7C3 [positive (pos.) mode; $M+H^+$], 507.0 → 204.1 for P7C3A20 (pos. mode; $M+H^+$), 320.3 → 277.3 for Dimebon (pos. mode; $M+H^+$), 381.9 → 80.7 for P7C3-S165 [negative (neg.) mode; $M-H^+$], 519.0 → 338.0 for P7C3-S54 (pos. mode; $M+H^+$), 536.0 → 536.0 (redundant MRM) for P7C3-S7 (neg mode; $M+HCOO^-$), 520.0 → 520.0 for P7C3-S41 (neg mode; $M+HCOO^-$); 520.1 → 520.1 for P7C3-S40 (neg mode; $M+HCOO^-$), 477.1 → 138.2 for P7C3-S8 (pos. mode; $M+H^+$), 478.0 → 153.2 for P7C3-S25 (pos. mode; $M+H^+$), and 435.2 → 248.2 for P7C3-S184 (pos. mode; $M+H^+$). The IS, *N*-benzylbenzamide, was monitored using a 212.1 → 91.1 transition (pos. mode; $M+H^+$). An Agilent XDB C18 column (50 × 4.6 mm, 5-μm packing) was used for chromatography with the following conditions: buffer A, dH_20 + 0.1% formic acid; buffer B, methanol + 0.1% formic acid, 0–1.5 min 0% B, 1.5–2.5 min gradient to 100% B, 2.5–3.5 min 100% B, 3.5–3.6 min gradient to 0% B, 3.6–4.5 min 0% B. Chromatography conditions were identical for all compounds, except the initial and final concentrations of buffer B, which were set to 0% for P7C3 and P7C3A20 and 3% for Dimebon and all of the other P7C3 analogs.

Maintenance of *Caenorhabditis elegans*. *C. elegans* were grown at 20 °C on nematode growth medium (NGM) agar in 60-mm Petri plates according to standard protocols (3). Worms were fed the *Escherichia coli* nutrient-rich strain HB101. All experiments were performed using BZ555 [Pdat-1::GFP], obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota. BZ555 is an integrated transgenic strain (chromosome IV) that expresses GFP under the control of the dopamine neuron-specific promoter *dat-1* (4). To obtain first-stage synchronous larvae (L1s), gravid adults were treated with alkaline hypochlorite solution, rinsed three times in M9 buffer, suspended in 6 mL of M9, and shaken for 12–14 h at room temperature, according to standard protocols (5, 6). Compound tests were performed in a 500-μL solution of PBS, compounds, and a bacterial density of HB101 at OD_{600} of 2 at 20 °C in 12-well plates (BD Falcon; Thermo Fisher Scientific).

Assessment of MPP^+ Dopaminergic Neuron Toxicity in *C. elegans*. Synchronized L1 larvae were plated into each well of a 12-well plate (~400 larvae per well) containing PBS, vehicle, or compounds, with or without 5 mM MPP^+ iodide (Sigma) freshly diluted in PBS. DMSO was used as vehicle (VEH) and the concentration in treatment groups was maintained below 1%. The assay solution (500 mL) was incubated for 40 h at 20 °C. The worms were then washed in dH_2O and supernatant was aspirated. To examine dopaminergic neuron toxicity, worms were anesthetized (0.1% tricaine, 0.01% tetramizole) for 5 min and then transferred to microscope slides and coverslipped. Pictures were taken at 40× magnification (AMG; Evos fl microscope). Each experiment was conducted in triplicate with 10–20 worms counted per condition. For quantification, investigators were blind to treatment condition. Quantification was done by observing all four cephalic sensilla (CEP) dendrites, per standard protocol (7). Briefly, GFP fluorescence was visualized from the nerve ring to the tip of the nose, and if any portion of a dendrite was absent, then it was counted as being degenerated.

Locomotion Analysis of *C. elegans*. A video-based assay was used to assess the swim speed, distance traveled, and length of worms. After exposure to MPP^+ for 32 h, worms were washed, resuspended in M9 buffer (500 μL), and transferred to microscope slides. A 10-s movie was recorded at 4× magnification, using a Nikon Eclipse 80i microscope. Each movie consisted of 160 frames and the distance traveled by the head of each worm was manually tracked in each frame, using Imera software. This software was also used to measure the length of the worm body. The ratio of movement distance to body length was used as a movement index and defined as locomotion per standard protocols (8).

BACE1 Activity Assay. The kinetic BACE1 FRET assay was performed with the BACE1 (β-secretase) FRET Assay kit (Invitrogen; P2985). The standard enzyme reaction contains 250 nM FRET substrate (Rh-EVNLDAEFK-Quencher) and 10 milliunits BACE1. Rhodamine fluorescence was measured every 10 min at room temperature for nine cycles on an EnVison multimode plate reader (Perkin-Elmer).

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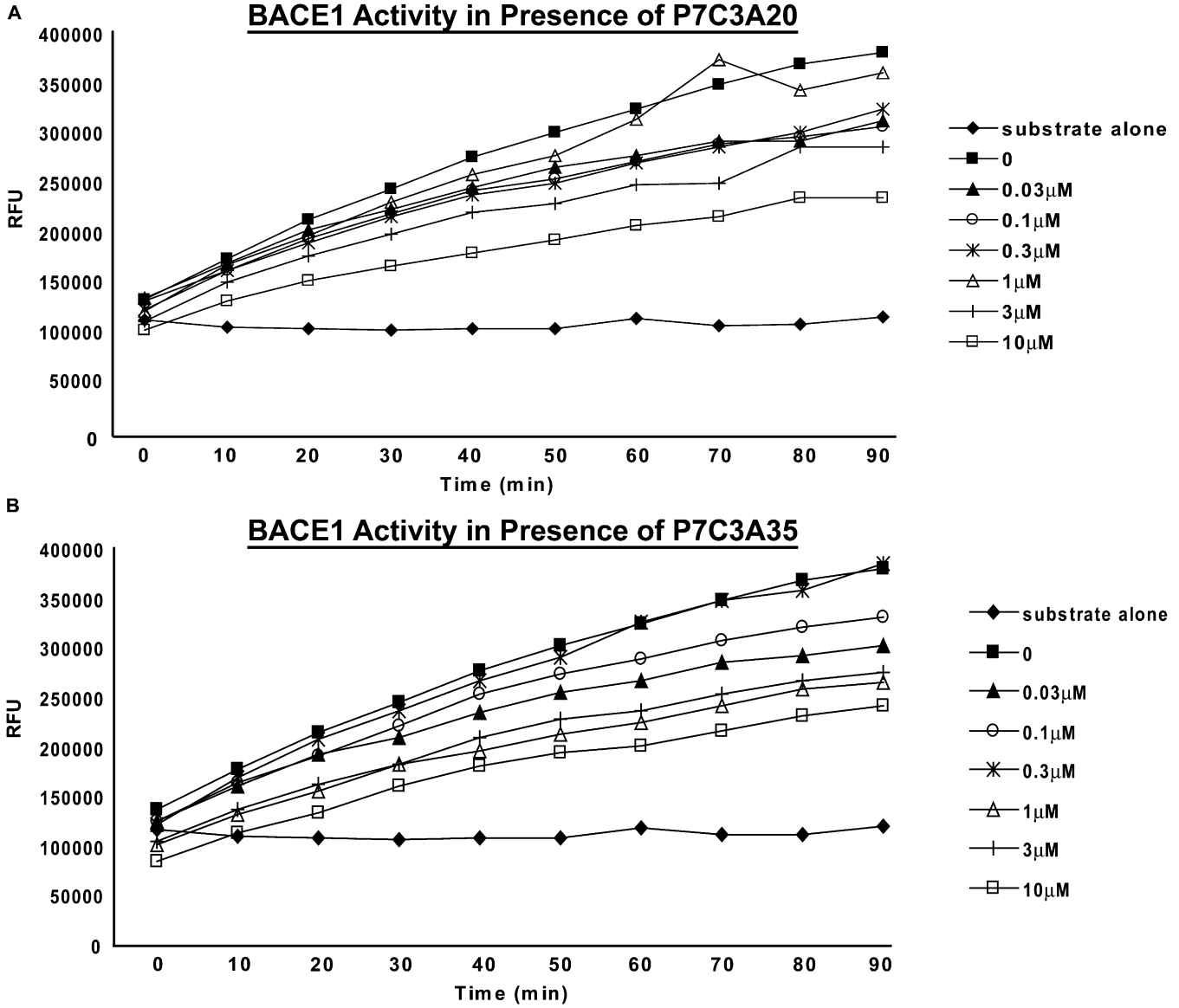
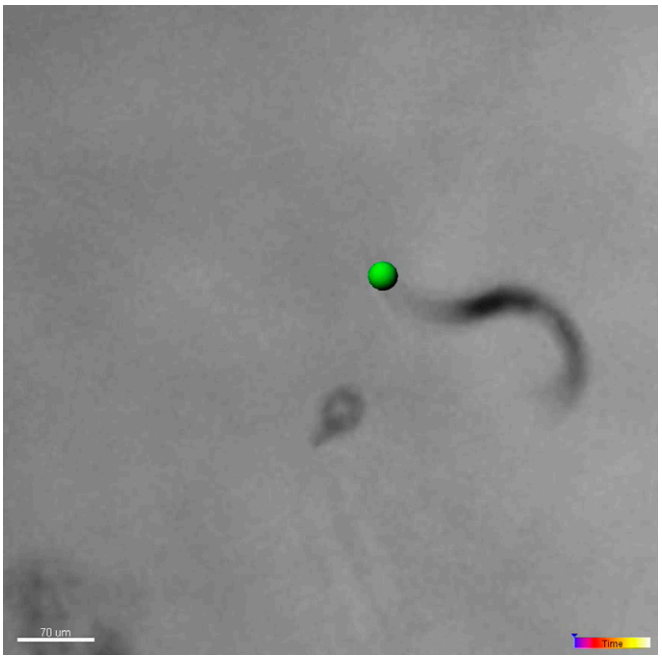
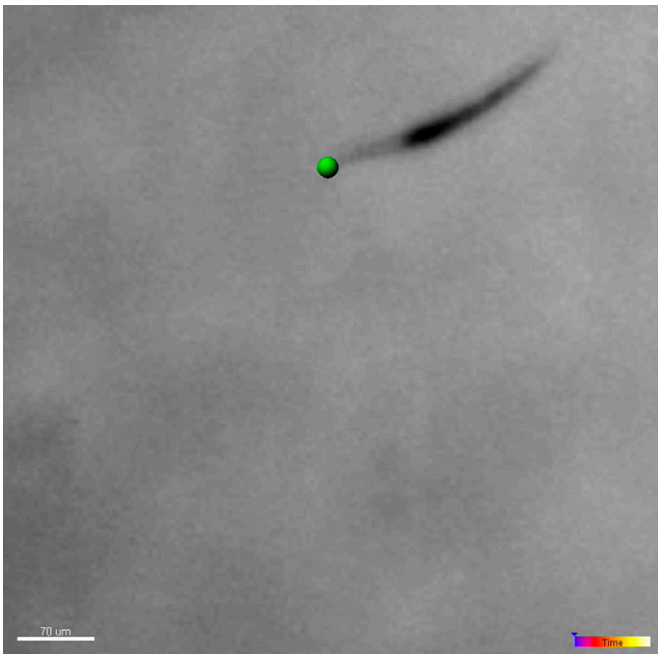


Fig. S1. BACE1 inhibition by P7C3 derivatives. The highly active proneurogenic analog P7C3A20 (*A*) and inactive analog P7C3A35 (*B*) were serially diluted and incubated with the BACE1 enzyme reaction for 90 min. BACE1 activity was measured every 10 min. Both compounds showed similar modest inhibition of BACE1 at the highest concentration (10 μM), indicating no correlation between neuroprotective efficacy and inhibition of BACE1. P7C3A20 did not inhibit BACE1 at the nanomolar concentrations shown to achieve neuroprotective efficacy.



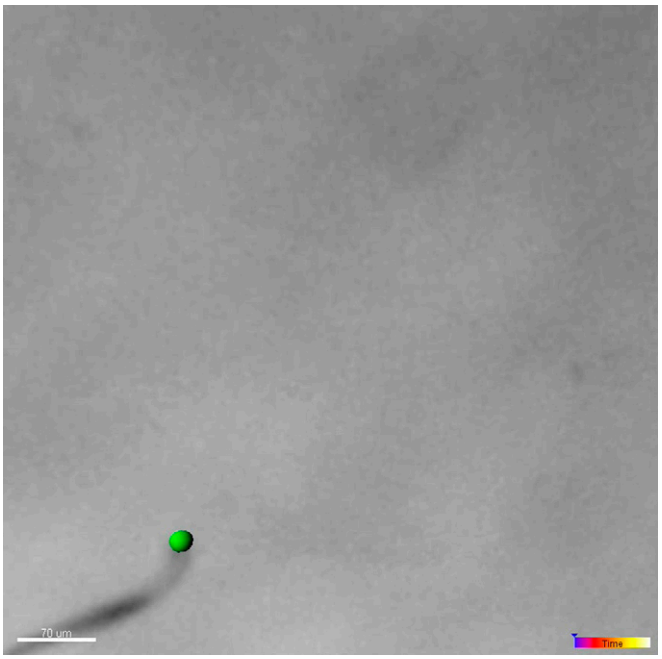
Movie S1. Worm mobility following exposure to vehicle. This is a 10-s movie of worm motion (160 frames) recorded at 4× magnification after 32 h of vehicle exposure. The green sphere represents the head of the worm, which was manually tracked in each frame to quantify movement.

Movie S1



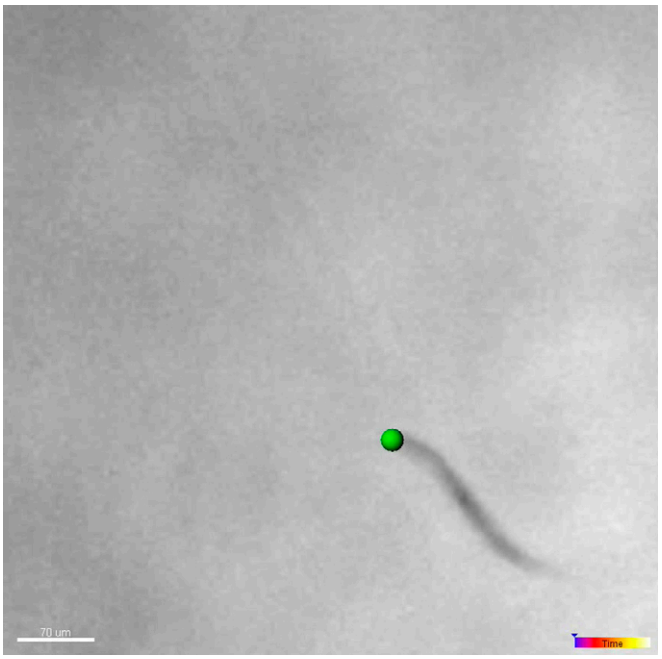
Movie S2. Worm mobility is reduced following exposure to MPP^+. This is a 10-s movie of worm motion (160 frames) recorded at 4× magnification after 32 h of MPP^+ exposure, showing that mobility is reduced. Worms were also pretreated for 30 min with the vehicle used to dissolve P7C3 and P7C3A20 in the subsequent studies. The green sphere represents the head of the worm, which was manually tracked in each frame to quantify movement.

Movie S2



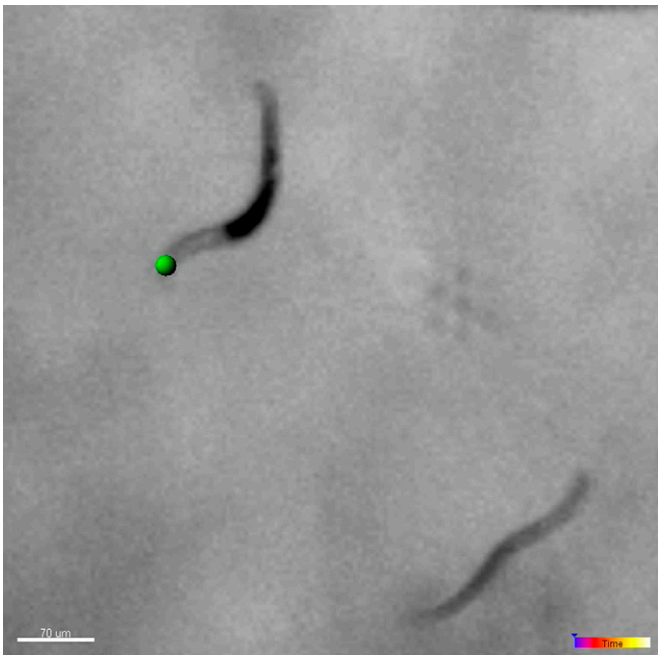
Movie S3. Treatment with P7C3A20 helps preserve worm mobility after exposure to MPP^+. This is a 10-s movie of worm motion (160 frames) recorded at 4× magnification after 32 h of MPP^+ exposure to worms that were pretreated for 30 min with 10 μM P7C3A20. The green sphere represents the head of the worm, which was manually tracked in each frame to quantify movement.

Movie S3



Movie S4. Treatment with P7C3 helps preserve worm mobility after exposure to MPP^+. This is a 10-s movie of worm motion (160 frames) recorded at 4× magnification after 32 h of MPP^+ exposure to worms that were pretreated for 30 min with 10 μM P7C3. The green sphere represents the head of the worm, which was manually tracked in each frame to quantify movement.

Movie S4



Movie S5. Treatment with Dimebon does not help preserve worm mobility after exposure to MPP^+. This is a 10-s movie of worm motion (160 frames) recorded at 4× magnification after 32 h of MPP^+ exposure to worms that were pretreated for 30 min with 10 μM Dimebon. The green sphere represents the head of the worm, which was manually tracked in each frame to quantify movement.

Movie S5