Inventory of Supplemental Material

1. Supplemental experimental procedures
2. Supplemental Figure Legends
3. Supplemental References
4. Supplemental Figures

Supplemental Experimental Procedures:

Selection of 1000 representative small molecules from the UTSWMC chemical compound collection: 1,000 compounds of optimal diversity were selected for screening from the UT Southwestern Medical Center chemical compound library by means of filtering software deployed from the CheD and SARNavigator programs that implemented the following selection criteria: 1) molecular weight 350-650; 2) minimum of two hydrogen bond donors; 3) minimum of three hydrogen bond acceptors; 4) more than five degree of unsaturation; 5) net charge not to exceed +2 or -2; 6) chirality with 1-3 asymmetric centers. This selection strategy was thus biased towards chemical complexity and enrichment in functional groups capable of forming specific hydrogen bonds and electrostatic contacts with potential receptors, as well as the presence of chiral centers. We further endeavored to eliminate compounds with alkylating functionality or other properties that might be anticipated to lead to covalent modification of proteins, chelation of metals, or obvious signs of metabolic liability.

***In vivo* hippocampal neurogenic assay:** Pools of 10 compounds each from the UTSWMC chemical compound library were dissolved at 10 mM concentration each (100 mM total solute concentration) in artificial cerebrospinal fluid (aCSF: 128 mM NaCl/2.5 mM KCl/0.95 mM CaCl2/1.9 mM MgCl2). Compounds were infused intracerebroventricularly (i.c.v.) into the left lateral ventricle of two adult (12 week old) wild type C57BL/J6 mice by means of surgically implanted Alzet osmotic minipumps that delivered solution into animals at a constant rate of 0.5 μL / hour for 7 days. After seven days of infusion at a constant rate of 0.5μL/ hour, a total of 84μL of volume will have left the pump (0.00084μMoles) and entered the cerebrospinal fluid. The average volume of a brain from a 12 week old male, C57Bl/ J6 mouse in our study is $500mm^3$. We thus estimated the maximal amount of drug that could potentially be present in the brain, taking the extreme and unlikely scenario of 100% absorbance of the drug into brain tissue and 0% clearance throughout the seven day infusion period. Under these conditions, at the end of one week of infusion each compound would be present at 1.7μMolar concentration. Since the actual amount of chemical compound in the brain is likely to be only a fraction of this predicted level, it is reasonable to estimate that compounds were administered at low-micromolar to mid-nanomolar concentrations.

Surgical implantation of Alzet osmotic minipumps (Alzet 1007D; Cupertino, CA) was conducted as follows. Animals were anesthetized with intraperitoneally-administered ketamine:xylazine, and the back of the neck and upper back area were then shaved and swabbed with 70% ethanol and betadine. A small midline incision was made in the skin between the scapulae to allow use of a hemostat to create a small pocket by spreading the subcutaneous connective tissue apart. The pump was inserted into the

pocket with the flow moderator pointing away from the incision, and the incision was closed with surgical thread. Surgery to implant the cannula into the lateral ventricle followed immediately, while animals were still anesthetized. The top portion of the head was shaved and the animal was then placed in a stereotactic holder. The head was swabbed with 70% alcohol followed by betadine, and an incision was made in the scalp to expose the surface of the skull in the area of bregma. The skull was cleaned of fascial tissue and a burr hole was made in the skull such that the guide cannula could be lowered into the burr hole to a depth 3 mm deep to the pial surface, -0.3 mm anteroposterior relative to bregma, and 1.3 mm lateral to midline. The guide cannula was secured to the skull with dental cement and the scalp incision was closed by suture thread. Animals were then placed in a clean warm cage on a heating pad until mobile.

Because both social activity and voluntary exercise stimulate hippocampal neurogenesis, mice were individually housed without access to running wheels throughout the entire procedure, beginning one week prior to pump implantation. Mice had *ad libitum* access to food and water. Bromodeoxyuridine (BrdU, Sigma-Aldrich) was injected intraperitoneally at 50 mg/kg/day for six days during pump infusion in order to detect agents that either promote proliferation or augment survival of proliferating neural stem cells in the SGZ. The injection site was alternated between right and left sides. Animals were monitored daily for general health and weighed every 3 days. Accumulated weight loss > 20% relative to pre-surgical weight was considered a sign of sickness and any such animals were euthanized for humane treatment. Cage changes were performed per routine scheduling. All bedding was dumped into a biohazard bag, in a fume hood, and then incinerated. All carcasses were incinerated, and cages were washed with 70% ethanol in a fume hood at the conclusion of the experiment in order to extract any compounds excreted in animal waste that may have accumulated on the cage. Material used to wipe the cages was also packaged into a biohazard bag and incinerated.

Twenty-four hours after the final BrdU administration, mice were sacrificed by transcardial perfusion with 4% paraformaldehyde at pH 7.4, and their brains were processed for immunohistochemical detection of incorporated BrdU in the SGZ. Dissected brains were immersed in 4% paraformaldehyde overnight at 4 degrees Celsius, 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 two hours in 50% formamide / 2X SSC at 65 degrees Celsius, followed by five minute wash in 2X SSC and subsequent incubation for thirty minutes in 2M HCl at 37 degrees Celsius. Sections were processed for immunohistochemical staining with mouse monoclonal anti-BrdU (1:100, Roche). Adjacent sections were used as described in the text to stain for

doublecortin and cleaved caspase 3, and quantified in similar manner. This same staining procedure was used to analyze aged rat brain tissue and *npas3*$^{-/-}$ brain tissue as well. Quantification of all staining was done blind to treatment group and animal genotype. Diaminobenzidine was used as a chromagen, and tissue was counter-stained 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). The number of BrdU+ cells in the entire dentate gyrus SGZ in the contralateral hemisphere (opposite side of surgically implanted pump) was quantified by counting BrdU+ cells within the SGZ and dentate gyrus in every fifth section throughout the entire hippocampus and then normalizing for dentate gyrus volume. The SGZ was defined as a two-cell-body-wide zone of the hilus along the base of the granular layer of the dentate gyrus. By quantifying only those cells in the contralateral hemisphere we avoided any artifact that could result from nonspecific incorporation of BrdU in the regions surrounding the site of mechanical trauma from surgical cannula implantation into the left lateral ventricle. The results are compared to that seen with infusion of either vehicle (aCSF) or commercially available recombinant FGF (0.04 mg/kg/day, Sigma-Aldrich) over the same period of time.

Any positive pools were subsequently repeated in additional animals to ensure statistical significance. Confirmed positive pools were broken down into their individual components in order to isolate any single chemical species with pro-neurogenic effect. Isolated pro-neurogenic chemicals were then re-supplied, their purity verified by HPLC, and re-assessed for pro-neurogenic effect in 4 additional animals to ensure that the original pro-neurogenic effect was not an artificat of storage conditions used for compounds in the UTSWMC chemical compound library.

Immunohistochemistry for Astrocytes, Oligodendrocytes, Neurons

Sections were removed from antifreeze and washed three times in 1X TBS for 15 minutes each and transferred to 12-well plates. Sections were blocked in 0.5% normal donkey serum (NDS) and 0.5% Triton X-100 in TBS for 30 minutes and incubated in primary antibodies diluted in blocking solution overnight. Primary antibodies used include: rabbit Prox1 (1:1000, Chemicon, Temecula, CA), mouse NeuN (1:1000, Millipore, Temecula, CA), chicken GFAP (1:2000, Millipore, Temecula, CA), mouse S100β (1:2000 Sigma, St. Louis, MO), mouse GSTπ (1:3000, BD Biosciences, San Jose, CA), and rat PDGFα (1:500, BD Pharmingen, San Jose, CA). For doublestaining, antibodies were applied at the same time and incubated in the same solution. After primary antibody administration, sections were washed with TBS and incubated in appropriate secondary antibodies conjugated to fluorophores (1:1000 in TBS, Jackson Immunoresearch, West Grove, PA) for 3-6 hours. Sections were then washed and

mounted on charged slides, costained with DAPI, and coverslipped with PVA/DABCO. For those sections stained with BrdU, prior to DAPI staining, the mounted sections were treated for 10 minutes with trypsin (0.1% trypsin, 0.1M TrisHCl pH 7.5, and 0.1% $CaCl_2$ in water), washed in TBS, and denatured in 2N HCl for 30 minutes. Blocking, primary antibody (rat BrdU, 1:1000, Accurate Chemical, Westbury, NY), and secondary antibody incubation was repeated as before except on mounted slides. Sections were costained with DAPI and coverslipped with PVA/DABCO.

Microscopy

Imaging of GFAP/S100β and PDGFα/GSTπ stained sections was done using a Nikon TE2000-U inverted microscope (Nikon Inc) at 400X. Post-processing was done using Adobe Photoshop CS2 (Adobe Systems). Colocalization of BrdU with NeuN and Prox1 was carried out using a Leica confocal microscope (emission wavelengths 488, 543, and 633nm) at 400X. Total counts of cells positive for all three markers or double positive for BrdU and Prox1 were normalized to total BrdU+ cells.

Pharmacokinetic analysis of P7C3: P7C3 was prepared for dosing by dissolving a recrystallized stock in DMSO at 50 mg/ml. The compound was diluted to a final formulation of 3% DMSO/10% cremophor EL (Sigma, St. Louis, MO)/87.5% D5W (5% dextrose in water, pH 7.2). Adult mice were dosed IV, IP or via oral gavage in a total volume of 0.2 ml.

Compound P7C3 levels were monitored by LC/MS/MS using an AB/Sciex (Applied Biosystems, Foster City, CA) 3200 Qtrap mass spectrometer coupled to a Shimadzu Prominence LC. The compound was detected with the mass spectrometer in MRM (multiple reaction monitoring) mode by following the precursor to fragment ion transition 474.9 → 337.8. Instrument settings were as follows: Dwell time of 150 ms, DP (declustering potential) 41.0 volts, EP (entrance potential) 7.5 volts, CEP (collision cell entrance potential) 20 V, CE (collision energy) 35 volts, CXP (collision cell exit potential) 4 volts, CUR (curtain gas) 35, CAD (collision gas) high, IS (ion spray voltage) 4000 V, TEM (turbo heater temperature) 650°C, GS1 (nebulizing gas) 70 psi, GS2 (auxiliary gas) 70 psi. A Phenomenex (Torrance, CA) Synergi 4 u Fusion-RP column (75 X 2.00 mm) was used for chromatography with the following conditions: Buffer A: dH20 + 0.1% formic acid, Buffer B: acetonitrile + 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 to 3.6 min gradient to 0% B, 3.6 to 4.5 min 0% B.

Whole blood was collected with an ACD solution (sodium citrate) coated syringe and needle. The blood was subsequently centrifuged at 9300 x g for 10’ to isolate plasma. Plasma was stored at -80°C until analysis. Brains were isolated from mice immediately

after sacrifice, rinsed three times with PBS and blotted gently to remove any surface adhering blood, weighed, and snap frozen in liquid nitrogen. Lysates were prepared by homogenizing the brain tissue in a 3-fold volume of PBS (weight of brain in g X = volume of PBS in ml added). Total lysate volume was estimated as volume of PBS added + volume of brain in ml. One hundred μl of either plasma or brain was processed by addition of 200 μl of acetonitrile to precipitate plasma or tissue protein and release bound drug. In some experiments, this mixture was centrifuged at 16,100 X g for 5 min and the supernatant analyzed directly by LC/MS/MS while in other experiments the sample was additionally processed by passage over a solid phase extraction column. An additional 700 μl of PBS was added to the plasma or lysate acetonitrile mixture and the sample centrifuged at 16,100 x g for 5 min. Nine hundred μl of supernatant was mixed with 1 ml of PBS and passed over a Waters (Milford, MA) OASIS HLB solid phase extraction column primed by addition of 2 ml acetonitrile followed by 2 ml of water. The column was washed twice with 2 ml of 5% acetonitrile in H20 and compound was eluted by addition of 2ml of acetonitrile. 500 μl of the eluant was added to 500 μl of H20 containing 0.2% formic acid (final formic acid 0.1%) and analyzed by LC/MS/MS as described above. Standard curves were prepared by addition of P7C3 to blank plasma or blank brain lysate. A value of 3X above the signal obtained from blank plasma or brain lysate 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 theoretical. The LOQ for both plasma and brain was 5 ng/ml. In general back calculation of points on both curves yielded values within 25% of theoretical over 4 orders of magnitude (10000 to 5 ng/ml). Pharmacokinetic parameters were calculated using the noncompartmental analysis tool of WinNonLin (Pharsight). Bioavailability was calculated as AUC_{oral}/AUC_{iv} x $Dose_{iv}/Dose_{oral}$ x 100 (AUC is area under the concentration time curve). The Brain:Blood ratio was calculated using AUC values.

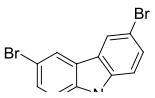
Synthesis and Preparation of P7C3 analogs:

3,6-Dibromo-9-(oxiran-2-ylmethyl)-9H-carbazole (I)

Following established procedures (Asso et al., 2008), 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 3× with 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 the desired product (389 mg, 66%).
^{1}H 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).

1-(3,6-dibromo-9H-carbazol-9-yl)-3-(3-methoxyphenylamino)propan-2-ol (II, P7C3-OMe)



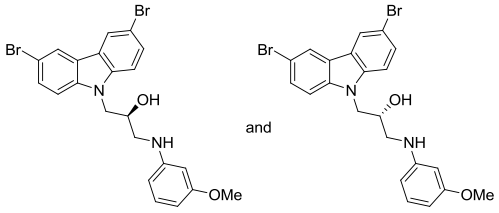
Following established procedures (Asso et al., 2008), *m*-anisidine (1.0 mL, 8.95 mmol) was added to a suspension of epoxide **I** (3.02 g, 7.92 mmol) in cyclohexane (73 mL). $BiCl_3$ (0.657 g, 2.08 mmol) was added and the mixture was heated at reflux overnight. Upon completion, the reaction was partitioned between EtOAc and H_2O. The aqueous layer was washed 3× with 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 purified by chromatography (SiO_2, 0–50% EtOAc/Hexane) to afford the desired alcohol as an opaque yellow solid (998 mg, 25%).
^{1}H NMR ($CDCl_3$, 400 MHz) δ = 8.12 (d, 2H, *J* = 1.6 Hz), 7.52 (dd, 2H, *J* = 2.0, 8.8 Hz), 7.32 (d, 2H, *J* = 8.8 Hz), 7.07 (dd, 1H, *J* = 8.0 Hz), 6.31 (dd, 1H, *J* = 2.4, 8.0 Hz), 6.21 (dd, 1H, *J* = 2.0, 8.0 Hz), 6.12 (dd, 1H, *J* = 2.0, 2.4 Hz), 4.34–4.39 (m, 3H), 4.00 (br s, 1H), 3.71 (s, 3H), 3.30 (dd, 1H, *J* = 3.6, 13.2 Hz), 3.16 (dd, 1H, *J* = 6.4, 13.2 Hz), 2.16 (br s, 1H).
^{13}C NMR ($CDCl_3$, 100 MHz) δ = 161.0, 149.2, 139.9 (2C), 130.4 (2C), 129.5 (2C), 123.8 (2C), 123.5 (2C), 112.8, 111.0 (2C), 106.7, 103.8, 99.8, 69.5, 55.3, 48.0, 47.4.
ESI *m*/*z* 502.9 ($[M+H]^+$, $C_{22}H_{21}Br_2N_2O_2$ requires 503.0).

1-(3,6-Dibromo-9H-carbazol-9-yl)-3-(3-methoxyphenylamino)propan-2-yl 3,3,3-trifluoro-2-methoxy-2-phenylpropanoate (III)

Alcohol **II** (0.150 g, 0.298 mmol) was dissolved in anhydrous dichloromethane (6 mL) and cooled to 0 °C. Pyridine (0.053 mL, 0.655 mmol) was added, followed by *S*-(+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (*S*-Mosher's acid chloride, 0.083 mL, 0.446 mmol) and dimethylaminopyridine (0.004 g, 0.030 mmol). The reaction was allowed to warm to room temperature over 4 hours, after which it was quenched by addition of saturated aqueous $NaHCO_3$. The mixture was extracted 3× with 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 purified by chromatography (SiO_2, 0–50% EtOAc/Hexane) to afford a mixture of both possible esters and both possible amides (~5:1 ester:amide ratio by ^{1}H NMR, 132 mg, 64%). Separation of the mixture was achieved using HPLC

(Phenomenex SiO_2 Luna, 21×250 mm, 15% EtOAc/Hexane, 16 mL/min; HPLC Retention time: 25.6 min (ester 1) and 41.2 min (ester 2)).
Ester 1: ^1H NMR ($CDCl_3$, 500 MHz) δ = 8.11 (d, 2H, *J* = 2.0 Hz), 7.45 (dd, 2H, *J* = 8.5 Hz), 7.24 (m, 2H), 7.22 (m, 4H), 7.05 (t, 1H, *J* = 8.0 Hz), 6.32 (dd, 1H, *J* = 2.0, 8.0 Hz), 6.12 (dd, 1H, *J* = 2.0, 8.0 Hz), 6.05 (dd, 1H, *J* = 2.0, 2.5 Hz), 5.59 (m, 1H), 4.54 (d, 2H, *J* = 6.5 Hz), 3.71 (br s, 1H), 3.69 (s, 3H), 3.43 (m, 1H), 3.29 (ddd, 1H, *J* = 5.5, 13.5 Hz), 3.19 (s, 3H).
Ester 2: ^1H NMR ($CDCl_3$, 500 MHz) δ = 8.08 (d, 2H, *J* = 2.0 Hz), 7.42 (dd, 2H, *J* = 2.0, 9.0 Hz), 7.28 (m, 2H), 7.24 (m, 4H), 7.04 (t, 1H, *J* = 8.0 Hz), 6.31 (dd, 1H, *J* = 2.0, 8.5 Hz), 6.11 (dd, 1H, *J* = 2.0, 8.0 Hz), 6.01 (dd, 1H, *J* = 2.0, 2.5 Hz), 5.63 (m, 1H), 4.49 (d, 2H, *J* = 6.5 Hz), 3.82 (dd, 1H, *J* = 5.5, 6.0 Hz), 3.66 (s, 3H), 3.42 (s, 3H), 3.39 (m, 1H), 3.28 (dd, 1H, *J* = 5.0, 13.5 Hz).

_R_- or _S_-1-(3,6-Dibromo-9H-carbazol-9-yl)-3-(3-methoxyphenylamino)propan-2-ol ((+) and (-)-P7C3-OMe)



Following established procedures (Abad et al., 1996) ester **III** (0.011 g, 0.015 mmol) was dissolved in degassed Et_2O (0.150 mL) and cooled to 0 °C. Lithium aluminum hydride (1M in THF, 0.018 mL, 0.018 mmol) was added via syringe and the reaction was stirred for 20 min. Upon completion by TLC the reaction was quenched by the addition of MeOH and stirred for 45 min. The mixture was partitioned between EtOAc and H_2O. The aqueous layer was extracted 3× with 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 purified by chromatography (SiO_2, 0–30% EtOAc/Hexane) to afford the desired alcohol (4.7 mg, 64%). Optical purity was determined by HPLC (ChiralCel OD-H; 20% iPrOH/Hexane; 1ml/min.
(R)-(+) configuration ret. time = 87 min. (S)-(-) configuration ret. time = 46 min

(*R*)-(+)-**P7C3-A17** (From Ester 1): $[\alpha]_D$ = +10° (*c* = 0.1, CH_2Cl_2); >95% ee
(*S*)-(–)-**P7C3-A17** (From Ester 2): $[\alpha]_D$ = –14° (*c* = 0.1, CH_2Cl_2); >95% ee

N-(3-(3,6-dibromo-9H-carbazol-9-yl)-2-fluoropropyl)-3-methoxyaniline (P7C3A20)

DAST [(Et_2NSF_3) 0.12 ml, 0.916 mmol] was added dropwise to a solution of alcohol **II** (0.102 g, 0.203 mmol) in 6.0 ml of anhydrous DCM at -78 °C. The reaction was stirred at –78 °C for one hour before being slowly warmed to 0 °C over 5 hours. The reaction was quenched by addition of phosphate buffer (pH=8) and extracted with CH_2Cl_2. The aqueous phase was extracted twice with 10 ml CH_2Cl_2. The combined organics were dried over Na_2SO_4, filtered and concentrated. The crude reaction material

was purified by flash chromatography on SiO_2 (20% EtOAc/hexanes/0.2%TEA). Fractions containing the desired fluorinated product were further purified with 40% EtOAc/hexanes (+ 0.1%TEA) to provide 5.7 mg desired product.

^{1}H NMR ($CDCl_3$, 500 MHz) δ = 8.16 (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),.

MS (ESI), *m/z*: 504.9 $[M+1]^+$. ($[M+1]^+$ for $C_{22}H_{19}Br_2FN_2O$ calculated 505.0).

3,6-dibromo-9-(2-(oxiran-2-yl)ethyl)-9H-carbazole (VI)

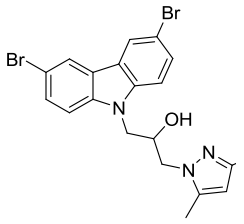
Crushed KOH (0.0054 g, 0.0954 mmol, 1.2 equiv) was added to 3,6-dibromocarbazole (0.0258 g, 0.0795 mmol, 1 equiv) in 0.5 mL DMF, and the mixture was stirred for 30 min. 1-Bromo-3,4-epoxybutane (0.0300 g, 0.199 mmol) in 0.5 mL DMF was added dropwise into the mixture, and it was stirred at room temperature overnight. The crude reaction was diluted with 20 mL EtOAc and washed with water 5 x 10 mL. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to afford 31.2 mg white solid as product, yield 97.9%.
^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.65 - 1.81 (m, 1H), 2.13 - 2.27 (m, 1H), 2.34 (dd, *J* = 4.88, 2.64 Hz, 1H), 2.64 (dd, *J* = 4.78, 4.05 Hz, 1H), 2.69 - 2.80 (m, 1H), 4.26 - 4.54 (m, 2H), 7.27 (d, *J* = 8.69 Hz, 2H), 7.50 (dd, *J* = 8.69, 1.90 Hz, 2H), 8.08 (d, *J* = 1.90 Hz, 2H).

4-(3,6-dibromo-9H-carbazol-9-yl)-1-(phenylamino)butan-2-ol; (P7C3A35)

Following the procedure outlined for the synthesis of P7C3-OMe, P7C3A35 was isolated as a white solid in 31% yield.
^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.87 - 1.98 (m, 1H), 2.05 - 2.14 (m, 1H), 2.99 - 3.07 (dd, *J* =13.24, 3.43 Hz, 1H), 3.09 - 3.17 (dd, *J* = 13.24, 8.27 Hz, 1H), 3.60 - 3.74 (m, 1H), 4.39 - 4.48 (m, 1H), 4.51 - 4.60 (m, 1H), 6.57 (d, *J* = 7.71 Hz, 2H), 6.74 (t, *J* = 7.34 Hz, 1H), 7.15 (dd, *J* = 8.27, 7.59 Hz, 2H), 7.38 (d, *J* = 8.69 Hz, 2H), 7.56 (dd, *J* = 8.69, 1.90 Hz, 2H), 8.14 (d, *J* = 1.85 Hz, 2H)

m/z (ESI): 486.9 ($M + H^+$) ([M+1] for C22H20Br2N2O requires 467.0)

1-(3,6-dibromo-9H-carbazol-9-yl)-3-(3,5-dimethyl-1H-pyrazol-1-yl)propan-2-ol ((P7C3A29)



A solution of 1-(3,6-dibromo-9H-carbazol-9-yl)-3-hydrazinylpropan-2-ol (56 mg, 0.136 mmol, derived from opening epoxide **I** with hydrazine) and acetylacetone (13.7mg, 0.136mmol)

in 1 ml of EtOH was heated at 70 °C for 1 hour. The reaction mixture was cooled to room temperature and concentrated under reduced pressure. The crude residue was purified by chromatography (SiO_2, 0-50% EtOAc/Hexane) to afford the desired alcohol as an off-white solid (29 mg, 45%).
^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.12 (d, *J* = 1.9, 2H), 7.51 (dd, *J* = 1.9, 8.7, 2H), 7.14 (d, *J* = 8.7, 2H), 5.79 (s, 1H), 5.19-5.10 (br s, 1H), 4.51 (m, 1H), 4.38 (s, 1H), 4.36 (s, 1H), 3.92 (dd, *J* = 3.0, 13.8, 1H), 3.69 (dd, *J* = 6.4, 13.8, 1H), 2.21 (s, 3H), 1.85 (s, 3H).
ESI *m/z* 475.9 $([M+H])^+$, $C_{20}H_{20}Br_2N_3O$ requires 476.

Live cell imaging of mitochondrial membrane permeability: U2O cells were plated in culture media (DMEM from Hyclone, 10% FBS, 1% P/S) 24 hours before the assay. Primary cultured cortical neurons from embryonic rats (E14) were plated 6 days prior to the assay. At the start of the assay, media was aspirated and 1mL of fresh media containing compound was added, followed by incubation for 1 hour at 37°C with 5% CO_2 for one hour. Media was then aspirated and new media containing 25 nM TMRM dye as well as the respective test compound was added, followed by incubation for 25 minutes at 37°C, protected from light. Solution was then aspirated, cells washed in 1X warm media with the respective compound to remove residual dye, and then 1 mL of warm media containing 100uM $CaCl_2$, 10uM A23187 and the respective compound was added to the cells. Images were acquired at 2, 10, 20, 30 and 40 min thereafter, using an Olympus 60X/1.42 oil, PlanApo N lens, softWoRx software, CoolSNAP HQ camera from Photometric, and the EX: 555/28 EM: 617/73 filters, using the Applied Precision Deltavision RT Deconvolution Microscope. Each image was comprised of 17 z-stacks in which each section of 0.5 μM in depth.

Electrophysiologic analysis of synaptic transmission in hippocampal slices: Hippocampal slices were prepared from 3 month old mice. Mice were anesthetized with Isoflurane, the brain was removed, and the dissected hippocampus was sliced with a vibrating microtome (Leica VT 100S, Germany) into 400 μm sections. The dissection buffer contained 2.6 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, 212 mM sucrose, and 10 mM dextrose, and was gassed with 95% O_2 and 5% CO_2 prior to use. The CA3 region was further dissected from the hippocampal sections. Hippocampal slices were transferred to a chamber filled with aCSF containing 124 mM NaCl, 5 mM KCl, 1.25 mM Na_2PO_4, 26 mM $NaHCO_3$, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM dextrose and equilibrated with 95% O_2 and 5% CO_2. Dissected slices were then allowed to stabilize at 30°C for one or more hours prior to electrophysiological recordings.

For recordings, slices were transferred to a submerged recording chamber, maintained at 30°C, and perfused continuously with aCSF at a rate of 3 ml/min. In the dentate

gyrus, stimulating and recording electrodes were positioned in the outer molecular layer, which is innervated by axons of the perforant pathway originating from the entorhinal cortex. In the CA1 region of the hippocampus, stimulation and recording electrodes were positioned in the stratum radiatum, which is innervated by the Schaffer collateral axons of CA3 pyramidal cells. Stimulus intensity was increased in 5 μA increments, the slope of the decreasing part of field potentials was measured, and fEPSP was quantified relative to the amplitude of the fiber volley, which represents firing of action potentials in pre-synaptic axons. Field potentials (FPs) were evoked in Schaffer collateral afferents with a concentric bipolar tungsten stimulating electrode (Frederick Haer Company, Bowdoinham, ME) and recorded from the stratum radiatum of the CA1 region with an extracellular glass capillary electrode. In the dentate gyrus both stimulation electrode and recording electrode were located in the outer molecular layer. For input-output measurements stimulus intensity was increased in 5μA increments up to 100uA. FPs were filtered at 2 kHz, acquired, and digitized at 10 kHz on a computer using customized LabVIEW software (National Instruments, Austin, TX). The 20/80 slope of the FP was used in this experiment.

Morphometric Analysis of Hippocampal Size: Paraformaldehyde-fixed mouse brains were sectioned in the coronal plane, paraffin-embedded, sectioned (8 microns) and hematoxylin/eosin stained. Histological sections were obtained at 50-μm intervals. Measurements of the hippocampus, dentate granular cell layer, and forebrain were taken at the coronal level in which CA1 approaches the midline and the upper blade of the dentate gyrus runs parallel to the surface of the brain. An ocular lens fitted with an etched grid was used to measure the dentate, CA1 and CA3 height and neuronal size (60X) as well as hippocampal dimensions (2X). Measures were recorded as mean +/- S.E.M.

Analysis of P7C3 in Aged Rats: After arrival, rats were allowed to acclimate to their new environment for 1 week before any handling and behavioral testing began. Weight was recorded weekly beginning 3 weeks before drug or vehicle treatment began (baseline behavioral testing was conducted during this time). Behavioral tests were conducted prior to and 2 months after treatment. Rats received 10 mg/kg P7C3 i.p. or vehicle once daily at a concentration that produced an injection volume under 1.0 ml and received 50 mg/kg BrdU once weekly.

Morris water maze testing was conducted with a 143 cm-diameter, white plastic, circular pool filled to a depth of 33 cm with 22 ± 1°C water made opaque with commercially available black, nontoxic, liquid tempera paint. Posters of different

shapes and sizes on all of the surrounding walls served as cues for the rats and overhead room lights were turned off. Two external clip lamps were used instead to reduce glare on the water and hence any interference with the tracking system. Rats were placed in one of four starting locations (e.g. North, South, East, West) facing the pool wall and allowed to swim until they found a Plexiglas platform 15 cm in diameter submerged by 5 cm. A trial ended when rats found the platform and remained there for an additional 10 sec or after 120 sec had elapsed, whichever came first. If rats did not find the platform the experimenter gently guided the rat to the platform where they remained for 10 sec before being returned to the home cage. Rats received 4 trials a day for 5 consecutive days of training. On the 6th day, a single trial probe test was conducted in which the platform was removed from the pool and rats were allowed to swim for 120 sec. On the 7th day a visibility test was conducted as described except that all cues were removed from the walls before the test, overhead room lights were turned on and a visible cue was attached to the platform (a Plexiglas tube with black stripes painted on one end). The visible platform was moved to a new random location for each trial and rats were allowed 30 sec to find the platform. Rats were excluded from the data analysis if they failed to find the platform within an average of 30 sec over the 4 trials. The location of the platform position was different for each of the three water maze training periods. Latency to find the platform, percentage of time and frequency of crossings in each quadrant and platform zones, and swim speed were collected using automated video-tracking software from Ethovision (version 2.3.19, Noldus). Data were analyzed with 2-way repeated measures ANOVA (treatment X day for training and treatment X treatment time [pre and 2-months post] for probe tests).

To measure food intake the total weight of the rat chow was weighed on a Monday and again one week later. Animal husbandry was instructed to not alter food levels during this time. To obtain blood glucose levels, rats were fasted 12 hrs prior to collection of tail blood by a tail nick. Blood was analyzed with a blood glucose monitoring system (FreeStyle Freedom Lite, Abbott).

Figure S1. Pro-Neurogenic Screen Data Related to Figure 1. (See also Figure 1.) (A) The time course of neuron birth, death and migration in the dentate gyrus (DG) was determined in order to optimize the period of time during which the screen would be conducted. Following injection on day 0 with BrdU (50 mg/kg, i.p.), neural precursor cell proliferation in the DG subgranular zone (SGZ) and granular layer (GL) was monitored through immunohistochemistry for BrdU on days 1, 5, 10, 15, 20, and 25 post-injection. Four mice were evaluated at each time point, and 25-30 adjacent coronal sections through the hippocampus (progressing posteriorly from the point where the suprapyramidal and infrapyramidal blades are joined at the crest region and the dentate gyrus is oriented horizontally beneath the corpus callosum) from each mouse were examined. On days 1 and 5, almost 100% of BrdU-positive cells within the DG were localized in the SGZ. The total number of cells decreased approximately 40% between days 1 and 5, in accordance with the appearance of apoptotic cell bodies in the SGZ. These results indicated that daily injection of BrdU over a one week period of continuous molecule infusion would screen for both proliferation and survival of newborn cells in the SGZ. Data are expressed as mean +/- SEM. **(B)** The surgical procedure for the *in vivo* screen does not impact BrdU incorporation in the contralateral hemisphere. Mice infused with vehicle (artificial cerebrospinal fluid) over seven days by means of surgically implanted osmotic minipumps (n=5) display no difference in hippocampal neural precursor cell proliferation, as assessed by BrdU incorporation normalized for dentate gyrus volume, from mice treated identically except not having undergone surgery (n=4). When Alzet osmotic minipumps were loaded with fibroblast growth factor 2 (FGF-2; 10 mg/mL) (n=5), however, hippocampal neural precursor cell proliferation doubled with respect to both other groups (*, $p<0.001$, Student's *t* test). Data are expressed as mean +/- SEM. **(C)** Normal and ectopic BrdU incorporation are illustrated**.** Immunohistochemical staining of BrdU in the hippocampal field should normally be restricted to the SGZ of the dentate gyrus, as shown on the left. Very infrequently, some compounds exhibited non-specific BrdU incorporation in ectopic regions, such as CA3, CA1, cortex, and striatum, as shown on the right. Any molecules that demonstrated ectopic incorporation of BrdU were eliminated from the study. Both micrographs were taken at the same magnification. Scale bar = 1000 μM. **(D)** Subsequent to their initial identification, pools 7, 14, 18, 19, 41, 53, 54, 61, 69, and 70 were re-evaluated in 2 additional mice each. Results shown are average with SEM of all 4 mice evaluated for each compound. All pools significantly (*, $P<0.001$, Student's *t* test) stimulated neural precursor cell proliferation in the hippocampal dentate gyrus SGZ relative to vehicle control. Data are expressed as mean +/- SEM. **(E)** Re-supplied compounds were each evaluated in three 12-week old male C57/Bl6 mice at 10μM concentration to verify that the pro-proliferative effect on neural stem cells was not an

artifact of storage conditions in the UTSWMC chemical compound library. Re-supplied compounds were verified to be 99% pure by mass spectrometry and shown to retain significant pro-proliferative properties *in vivo* (*, P<0.001, Student's *t* test). Data are expressed as mean +/- SEM.

Figure S2. (See also Figure 3.) P7C3 stimulates formation of neurons in the dentate gyrus, and does not affect the abundance of astrocytes or oligodendrocytes in the hippocampus. (A) Co-localization of BrdU (blue) with the mature neuronal markers Prox1 (green) and NeuN (red) shows triple-labeled cells within the granular layer of the dentate gyrus from wild type and *npas3*-/- mice that have received P7C3 for an extended period of time (every day from embryonic day 14 up to 3 months of age). Results shown are representative of 5 sections from each of 3 animals in both groups. **(B)** Immunohistochemical detection of the astrocytic markers GFAP (red) and S100β (green) (Wang and Bordey, 2008) in the hippocampus demonstrates that there are no obvious differences in the abundance of astrocytes in this region irrespective of duration of exposure to P7C3 or vehicle-control. There are also no obvious differences between WT and *npas3*$^{-/-}$ mice, irrespective of their treatment paradigm. 'Extended treatment' describes mice that received P7C3 every day between embryonic day 14 and 3 months of age, as described in the text. **(B)** Likewise, immunohistochemical detection of the oligodendrocytic markers PDGFα (red) (Ndubaku and de Bellard, 2008) and GSTpi (green) (Tansey and Cammer, 1991) in this region also demonstrates that there are no differences among genotypes or treatment paradigms. Results shown are representative of 5 sections from each of 4 animals in every group. All micrographs were taken at the same magnification. Scale bar = 100 μm. In both (A) and (B) expanded insets are included to illustrate double labeling.

Supplemental Figure S3. (See also Figure 4.) Neural stem cell proliferation, apoptosis, and the efficacy of P7C3 in *npas3*$^{-/-}$ mice. (A) Quantification of short term (1 hour pulse) BrdU incorporation and CCSP3 formation in the dentate gyrus shows that *npas3*$^{-/-}$ mice have the same rate of proliferation of newborn cells in the dentate gyrus as wild type littermates (BrdU), but roughly twice the level of apoptosis (CCSP3) (*, P<0.001, Student's *t* test). Three 6 week old male mice (NPAS3-deficient or wild type littermates) in each group were evaluated. Data are expressed as mean +/- SEM. **(B)** Six 12-week old *npas3*$^{-/-}$ mice were orally administered vehicle or P7C3 (20 mg/kg/d) for 12 days, and also injected daily with BrdU (50 mg/kg). BrdU staining shows that P7C3 increases the magnitude of neural precursor cell proliferation in *npas3*$^{-/-}$ mice by roughly 4-fold (*, P<0.001, Student's *t* test). DCX staining shows that this results in enhanced neurogenesis, and that P7C3 also promotes more extensive process formation in developing hippocampal neurons in *npas3*$^{-/-}$ mice. Data are expressed as mean +/- SEM. Results shown for **(C)** and **(D)** are representative of 7-10 sections from each group of

four mice following immunohistochemical staining with an antibody specific for **(C)** synapsin 1/2 (1:250, Synaptic Systems, Germany) and **(D)** synaptobrevin 2 (1:500, Synaptic Systems, Germany). Synapsin 1/2 and synaptobrevin 2 levels are decreased in the molecular layer (ML) of *npas3*$^{-/-}$ mice, and restored following extended exposure to P7C3. All micrographs were taken at the same magnification. Scale bar = 50 μm. **(E)** Prolonged administration of P7C3 selectively increased the thickness of the *npas3*$^{-/-}$ dentate gyrus granular cell layer. 5 animals were analyzed in each group, and micrographs are representative of 15-20 sections analyzed per animal. All micrographs were taken at the same magnification. Scale bar = 50 μm. Data are expressed as mean +/- SEM.

Figure S4. P7C3 preserves mitochondrial membrane potential in cultured primary cortical neurons. (See also Figure 6.) Cortical neurons cultures from rats on embryonic day 14 were loaded with tetramethylrhodamine methyl ester (TMRM) dye after 6 days of maturation. The top panels (no calcium ionophore) show that the dye alone did not affect the health of neurons. The remaining panels are from cells that were exposed to the calcium ionophore A23187 at time zero. With vehicle-alone, cortical neuron mitochondrial membrane potential was rapidly lost after exposure to the ionophore. Escalating doses of P7C3 preserved mitochondrial membrane potential following exposure to the calcium ionophore A23187 in a dose dependent manner, with full protection achieved at 1 μM P7C3. The inactive P7C3 analog P7C3A29 failed to preserve mitochondrial membrane potential at any dose. Results shown are representative of 10 fields analyzed in each of 2 experimental runs for all conditions.

Supplemental Figure S5. (See also Figure 8.) P7C3 promotes hippocampal neurogenesis in aged rats, and does not affect important measures of control with respect to the data shown in Figure 8. (A) P7C3 (20 mg/kg/d, i.p.) and BrdU (50 mg / kg, i.p.) were administered daily for 7 days to 12-18 month old Fisher 344 rats (n=4 in each group). P7C3 promoted neural precursor cell proliferation by roughly 5 fold compared to vehicle. (*, $P < 0.001$, Students *t* test). DCX staining demonstrates that P7C3 specifically promoted neuronal differentiation and dendritic branching. These micrographs were taken at the same magnification. Scale bar = 50 μm. Data are expressed as mean +/- SEM. **(B)** Latency to find the hidden platform in the Morris water maze task, as well as **(C)** swim spend in aged rats treated with P7C3 or vehicle both before and after 2 months of treatment did not differ between groups. Data are expressed as mean +/- SEM. **(D)** Quantification of food intake and **(E)** fasting blood glucose levels in aged rats did not differ with respect to whether rats received P7C3 or vehicle. Data are expressed as mean +/- SEM.

Supplemental References

Abad, J.L., Guardiola, M., Casas, J., Sanchez-Baeza, F., and Messeguer, A. 1996. 2,3,18,19-Dioxidosqualene stereoisomers: characterization and activity as inhibitors of purified pig liver 2,3-oxidosqualene-lanosterol cyclase. J. Org. Chem. *60*, 3648-3656.

Asso, V., Ghilardi, E., Bertini, S., Digiacomo, M., Granchi, C., Minutolo, F., Rappaselli, S., Bortolato, A., Moro, S., and Macchia, M. (2008). Alpha-Naphthylaminopropano-2-ol derivatives as BACE1 inhibitor. ChemMedChem *3*, 1530-1534.

Ndubaku, U. and de Bellard, M.E. (2008). Glial cells: Old cells with new twists. Acta Histochem *110*, 182-195.

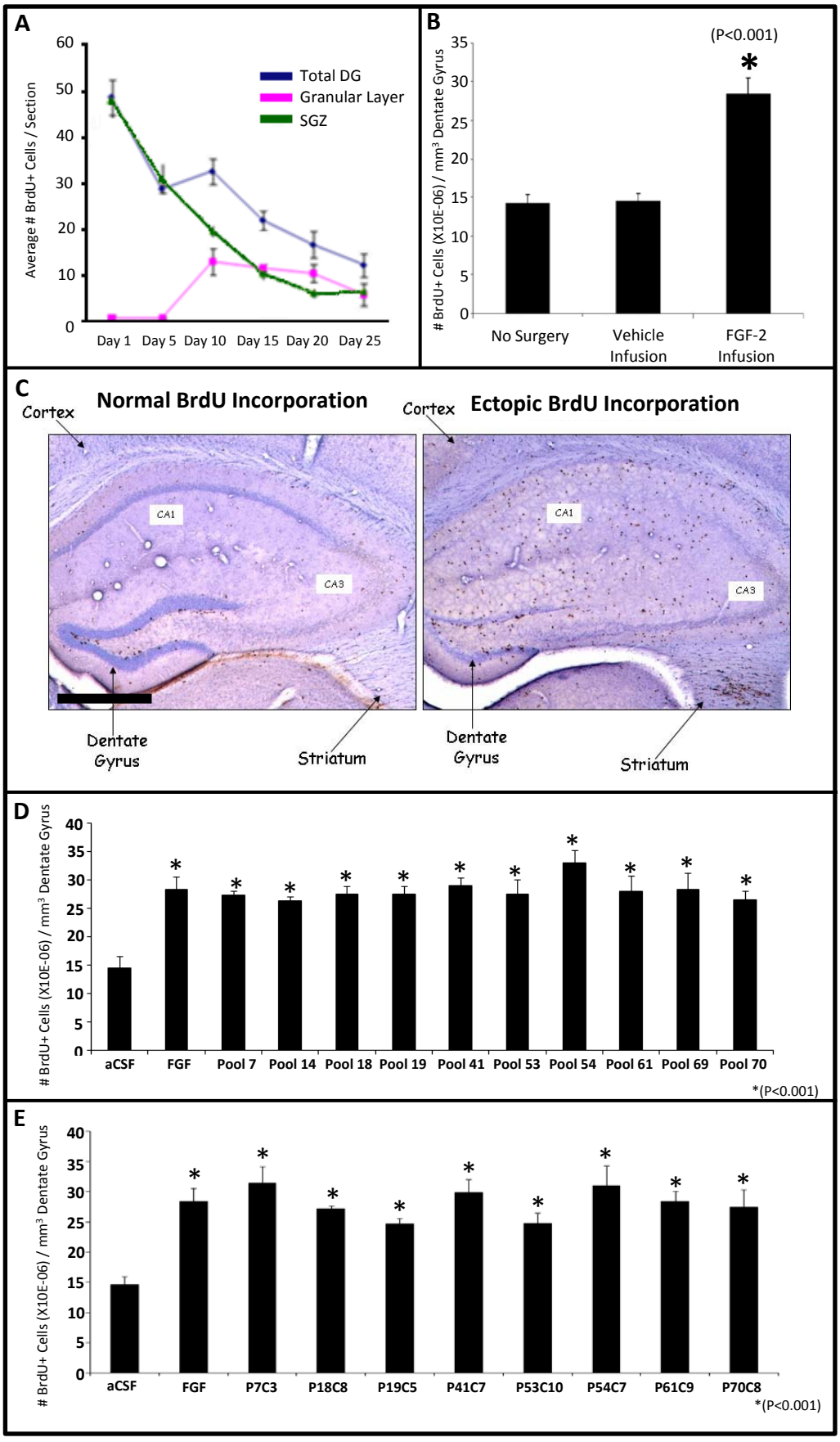
Tansey, F.A. (1991). A pi form of glutathione-S-transferase is a myelin- and oligodendrocyte-associated enzyme in mouse brain. J Neurochem *57*, 95-102.

Wang, D.D. and Bordey, A. (2008). The astrocyte odyssey. Prog Neurobiol *86*, 342-367.

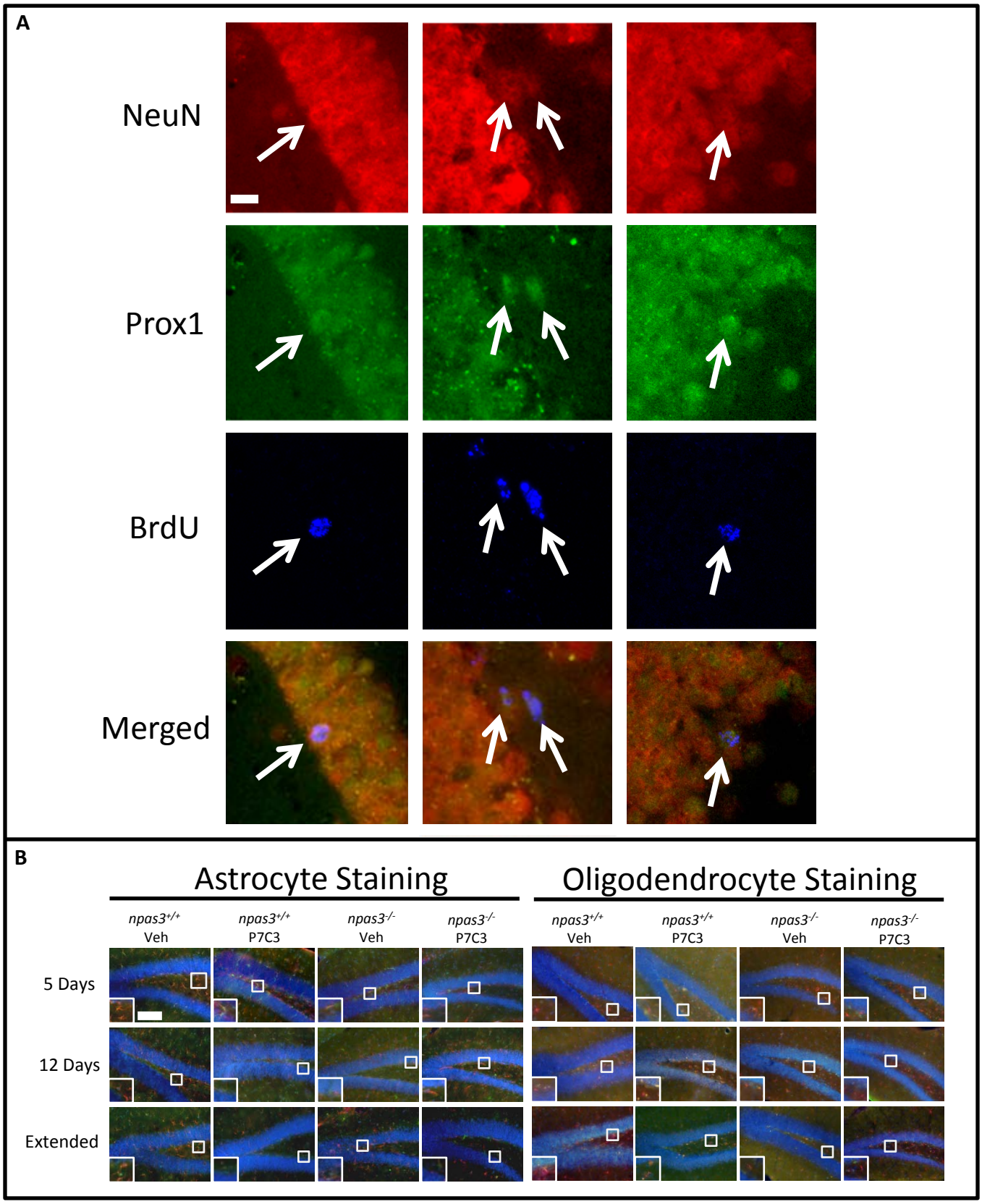
Supplemental Figure

Figure S1

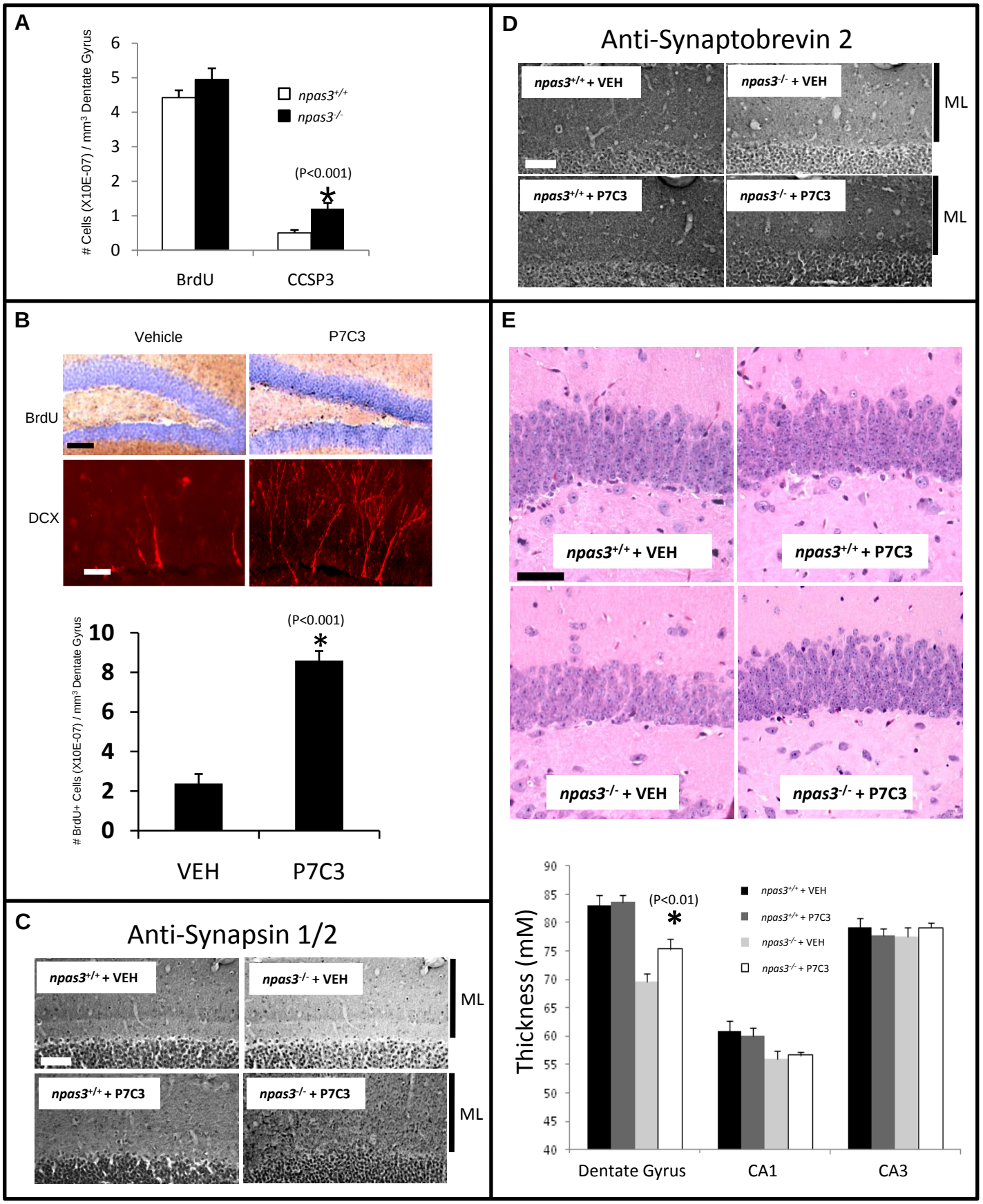
Click here to download Supplemental Figure: Pieper-McKnight Figure S1 pdf.pdf



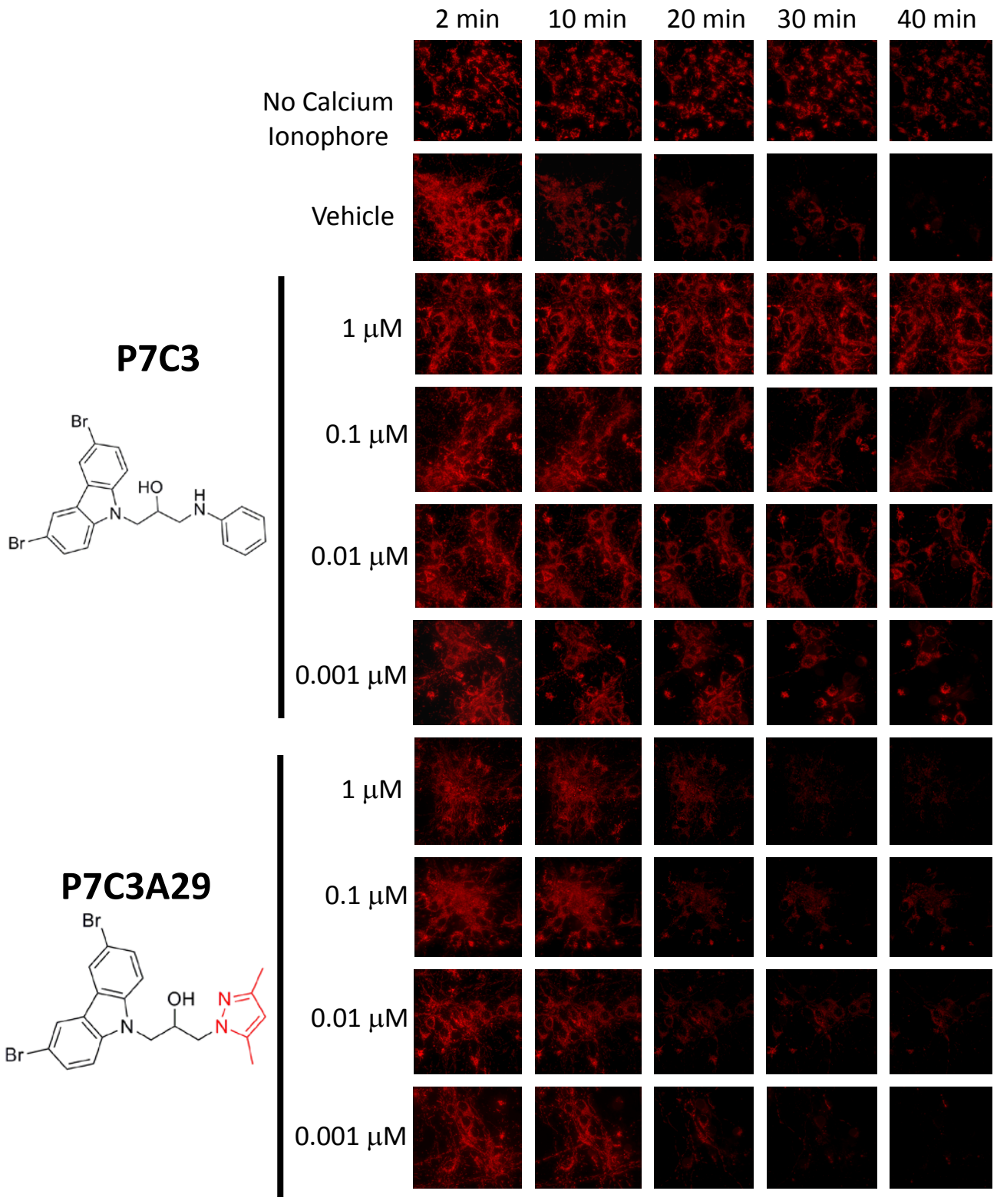
Supplemental Figure
Figure S2
Click here to download Supplemental Figure: Pieper-McKnight Figure S2 pdf.pdf



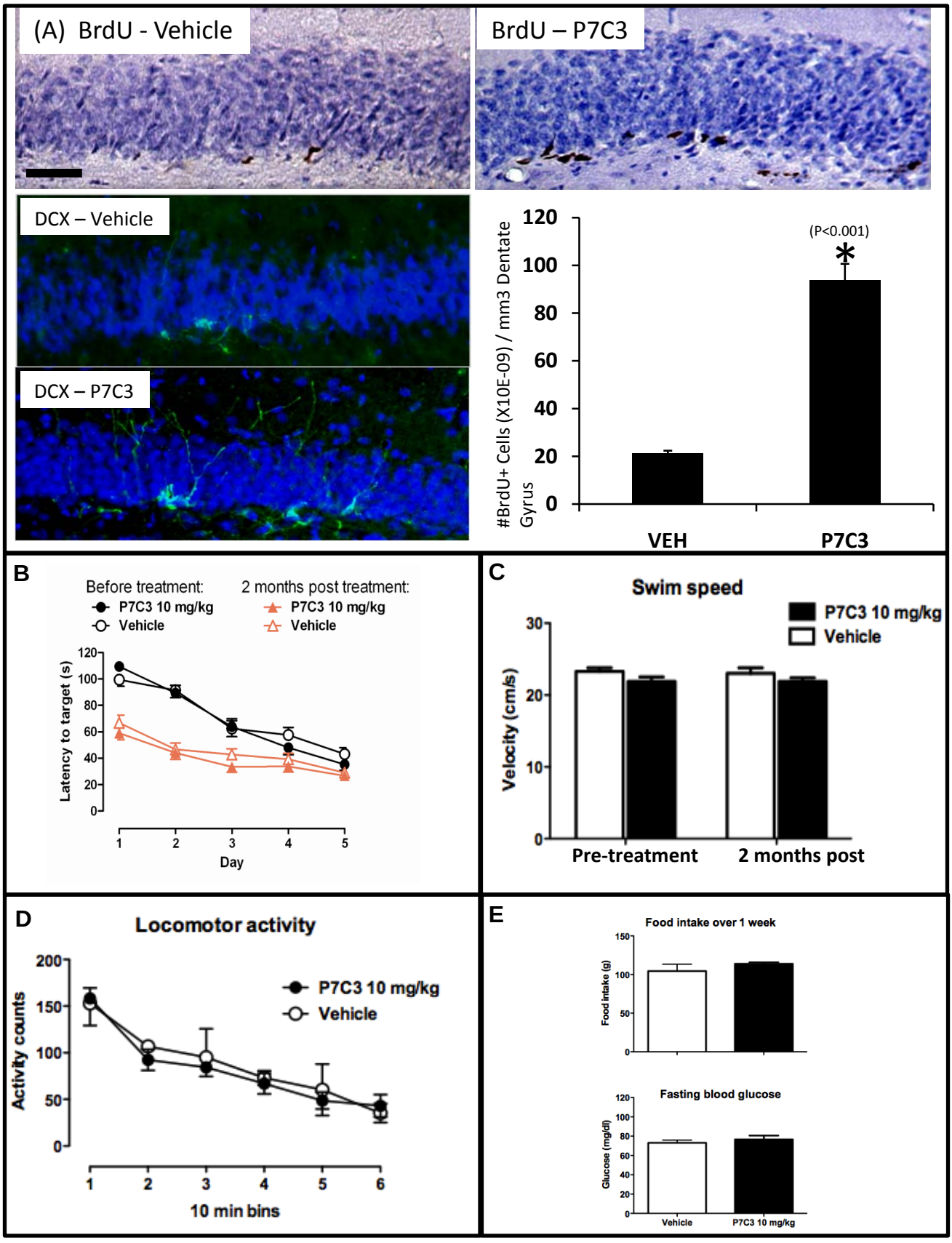
Supplemental Figure
Figure S3
Click here to download Supplemental Figure: Pieper-McKnight Figure S3 pdf.pdf



Supplemental Figure
Figure S4
Click here to download Supplemental Figure: Pieper-McKnight Figure S4 pdf.pdf

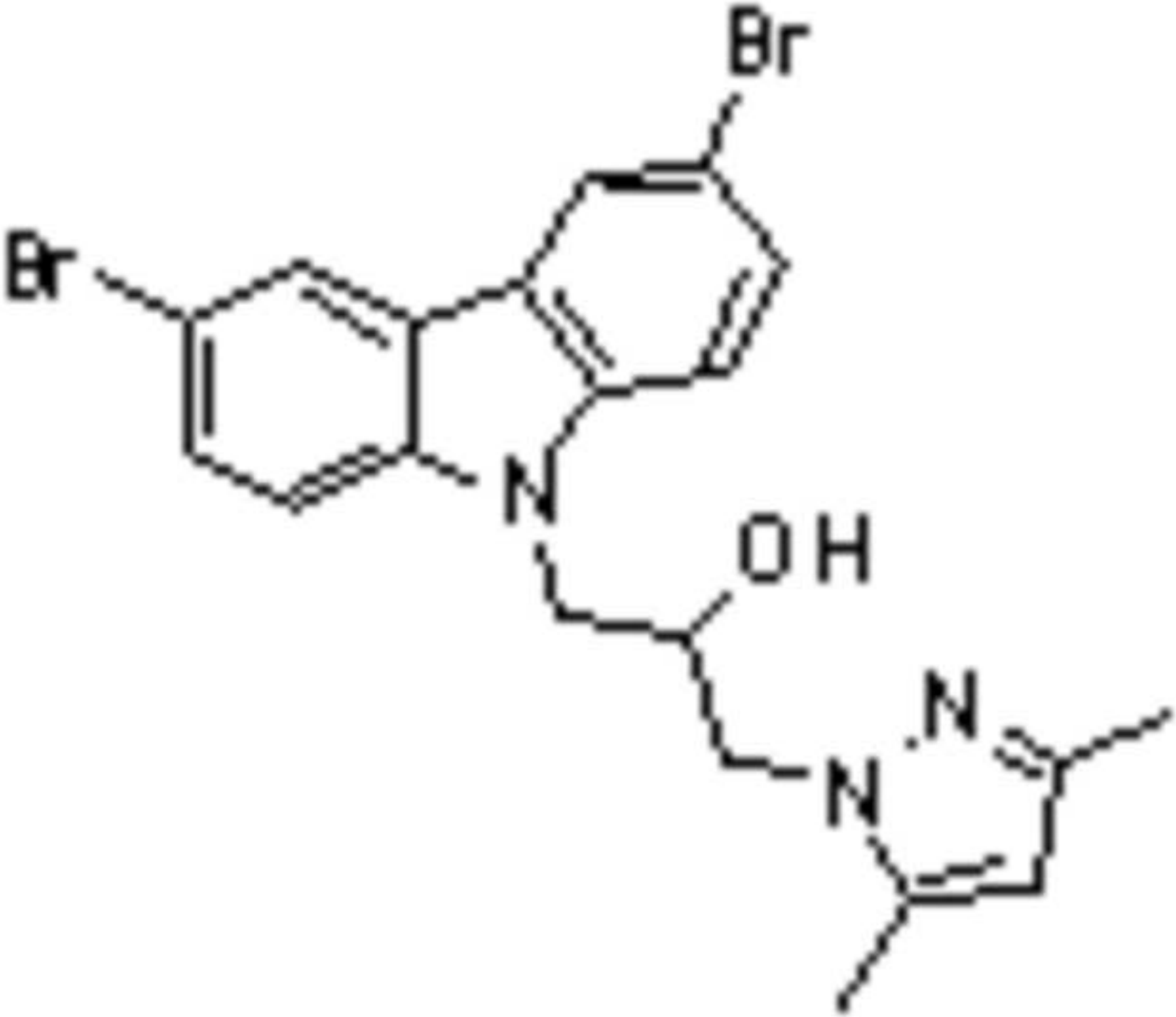


Supplemental Figure
Figure S5
Click here to download Supplemental Figure: Pieper-McKnight Figure S5 pdf.pdf



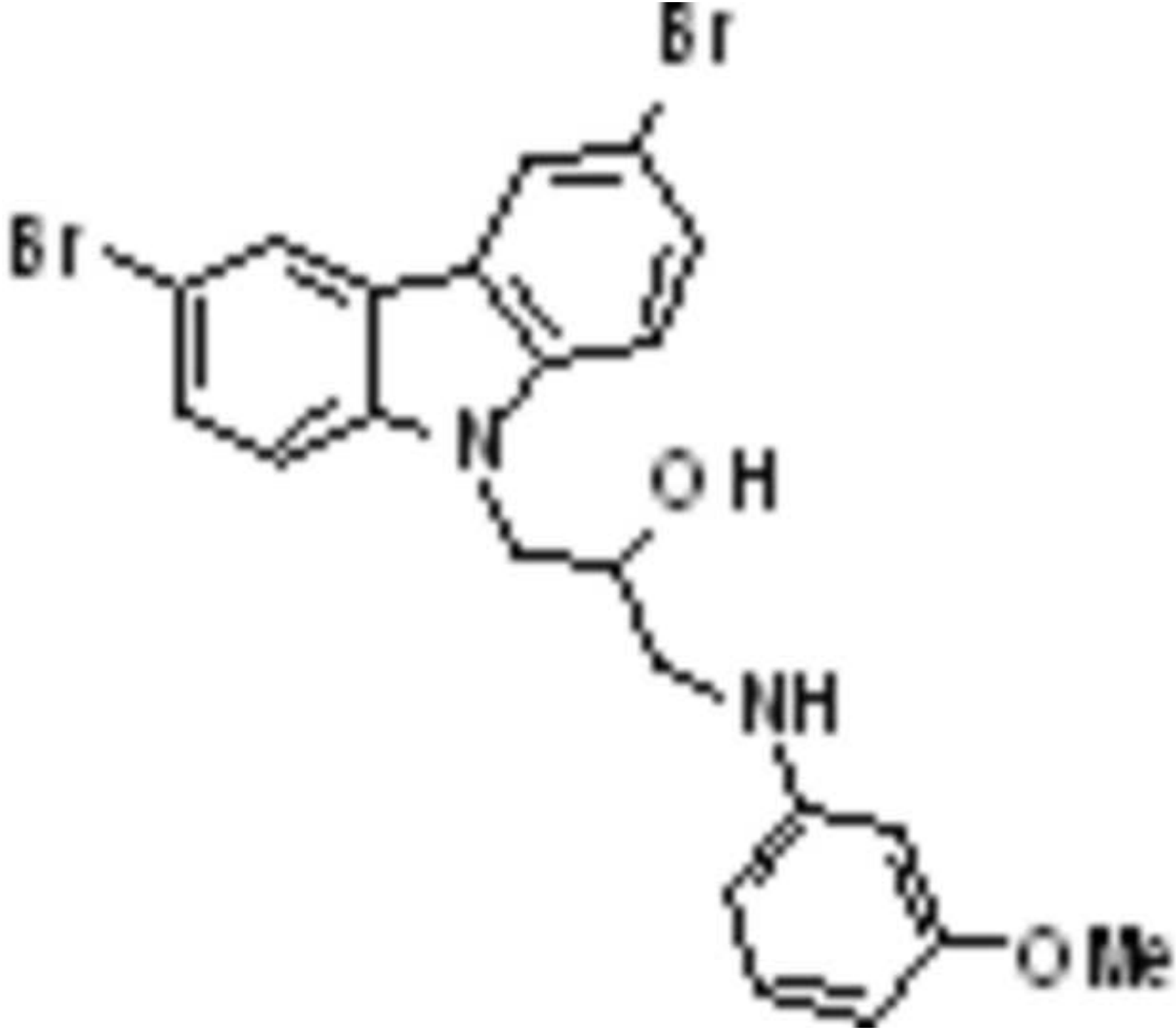
Supplemental Figure

Click here to download high resolution image



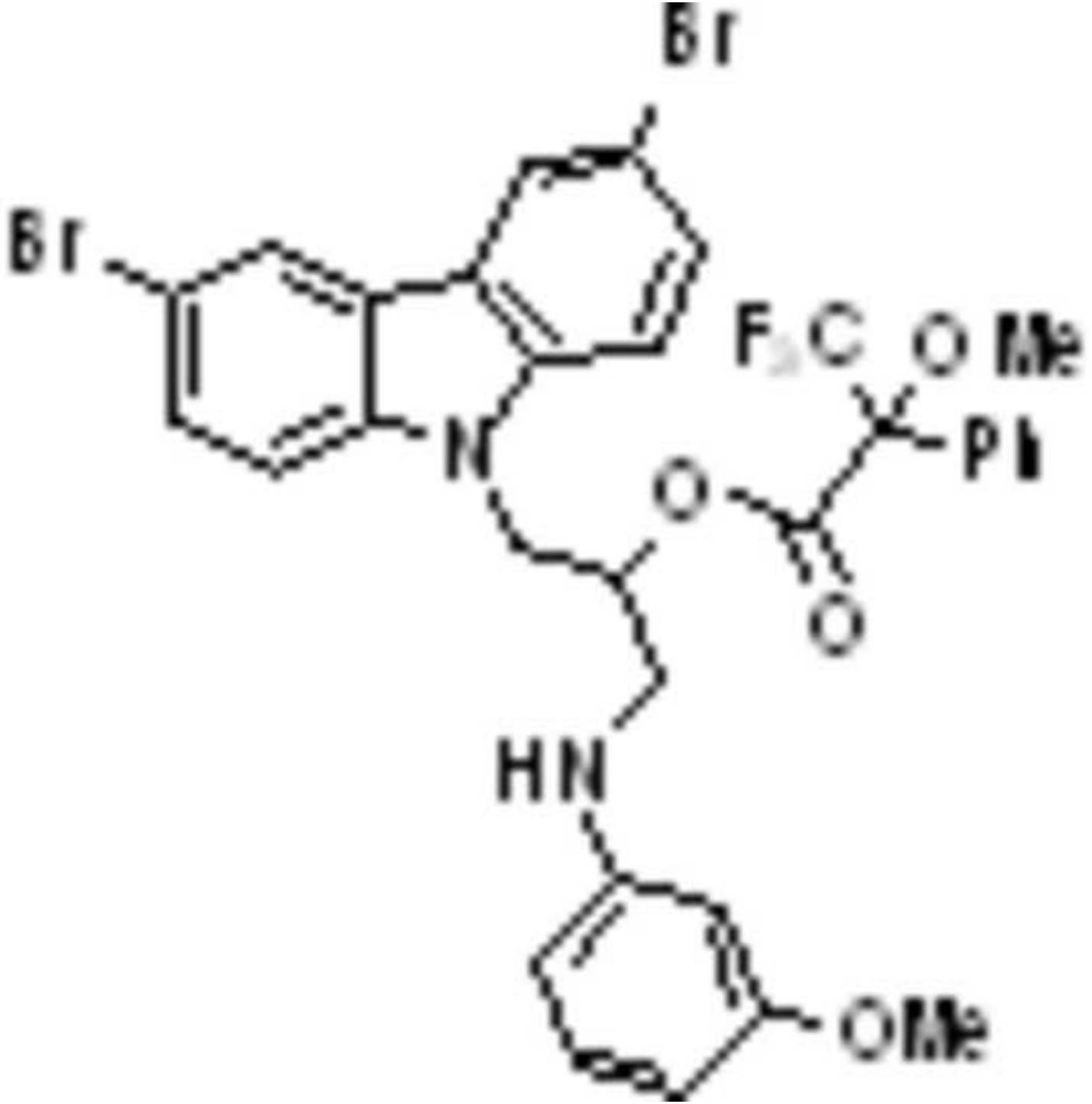
Supplemental Figure

Click here to download high resolution image



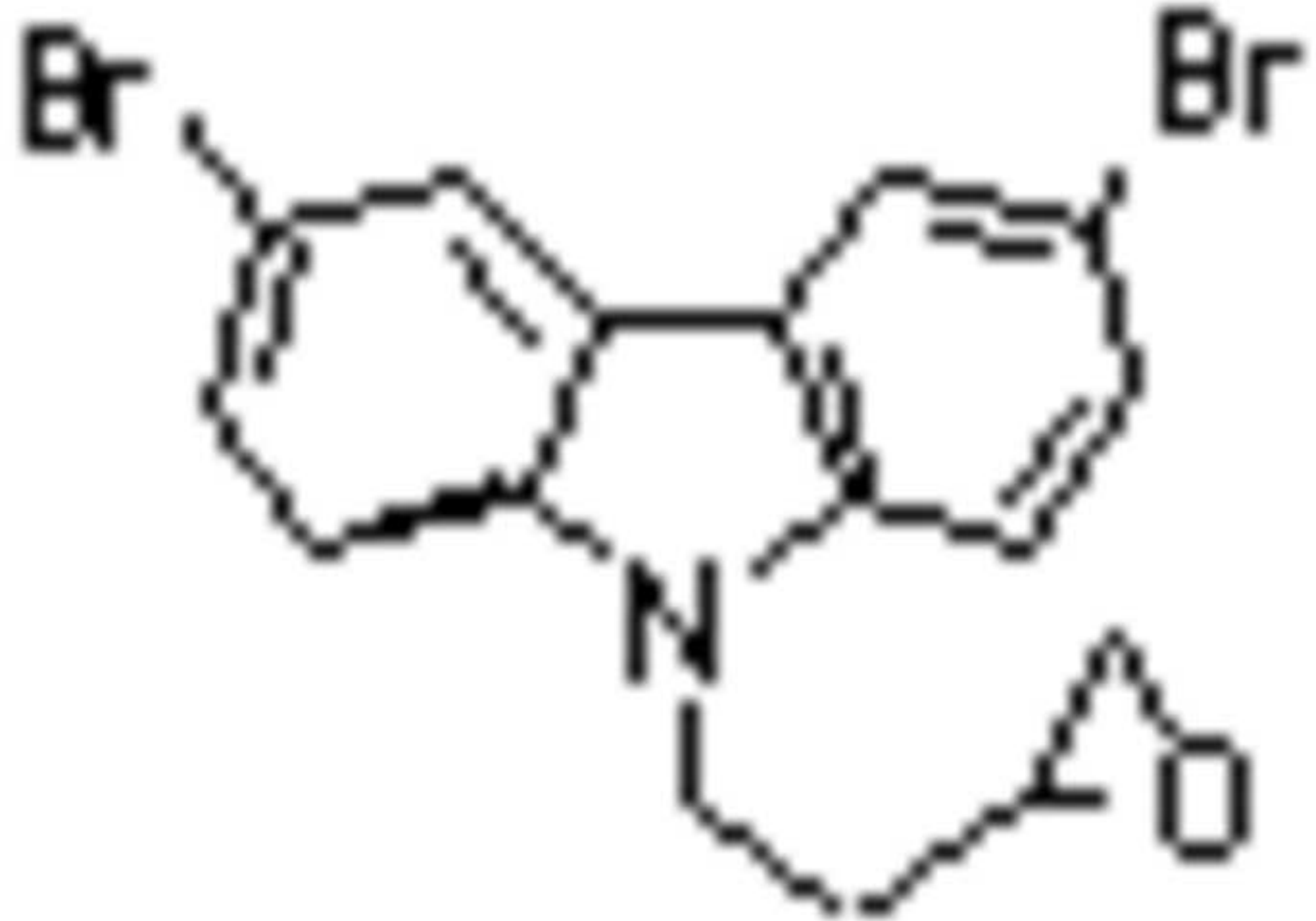
Supplemental Figure

Click here to download high resolution image



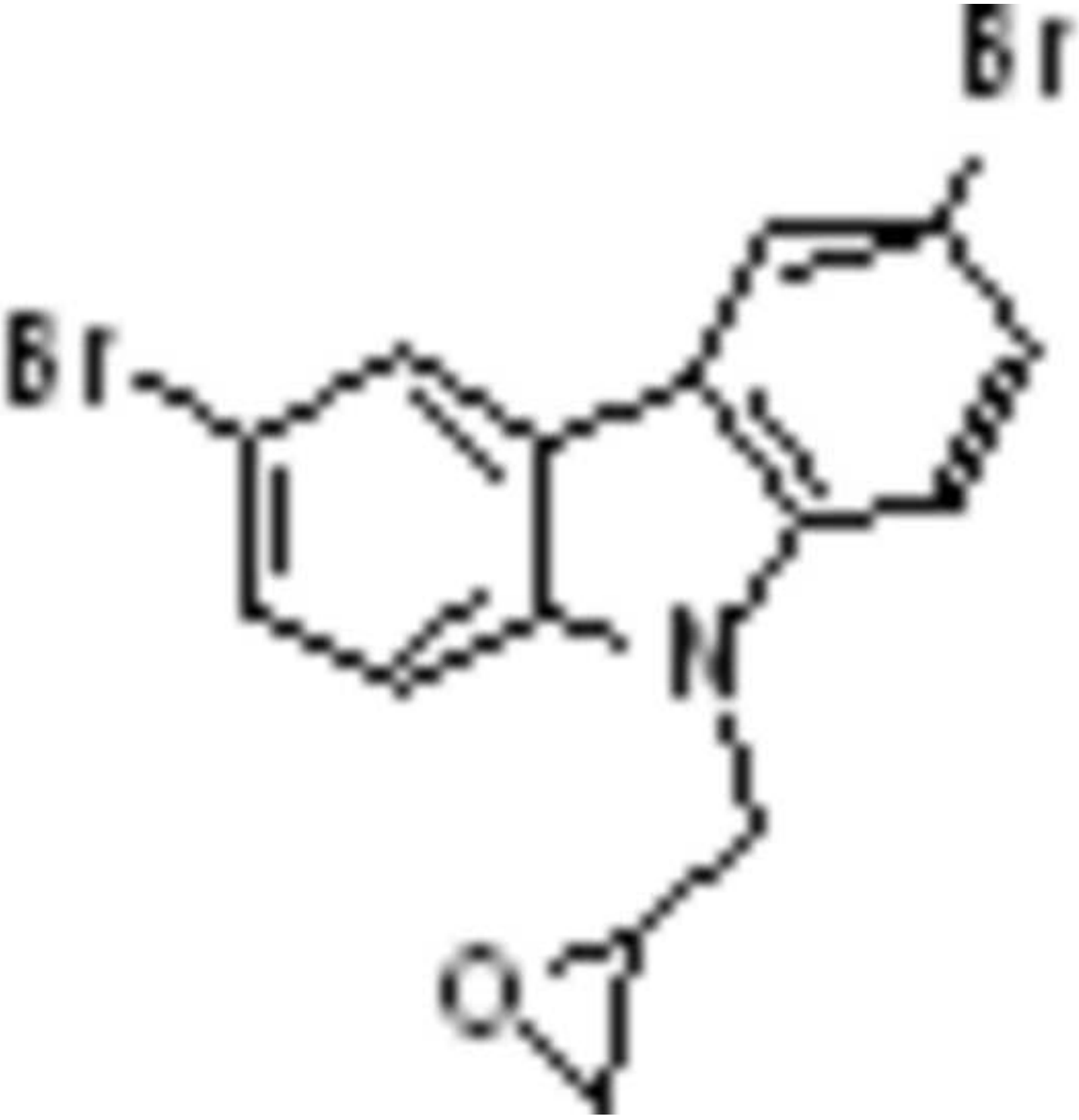
Supplemental Figure

Click here to download high resolution image



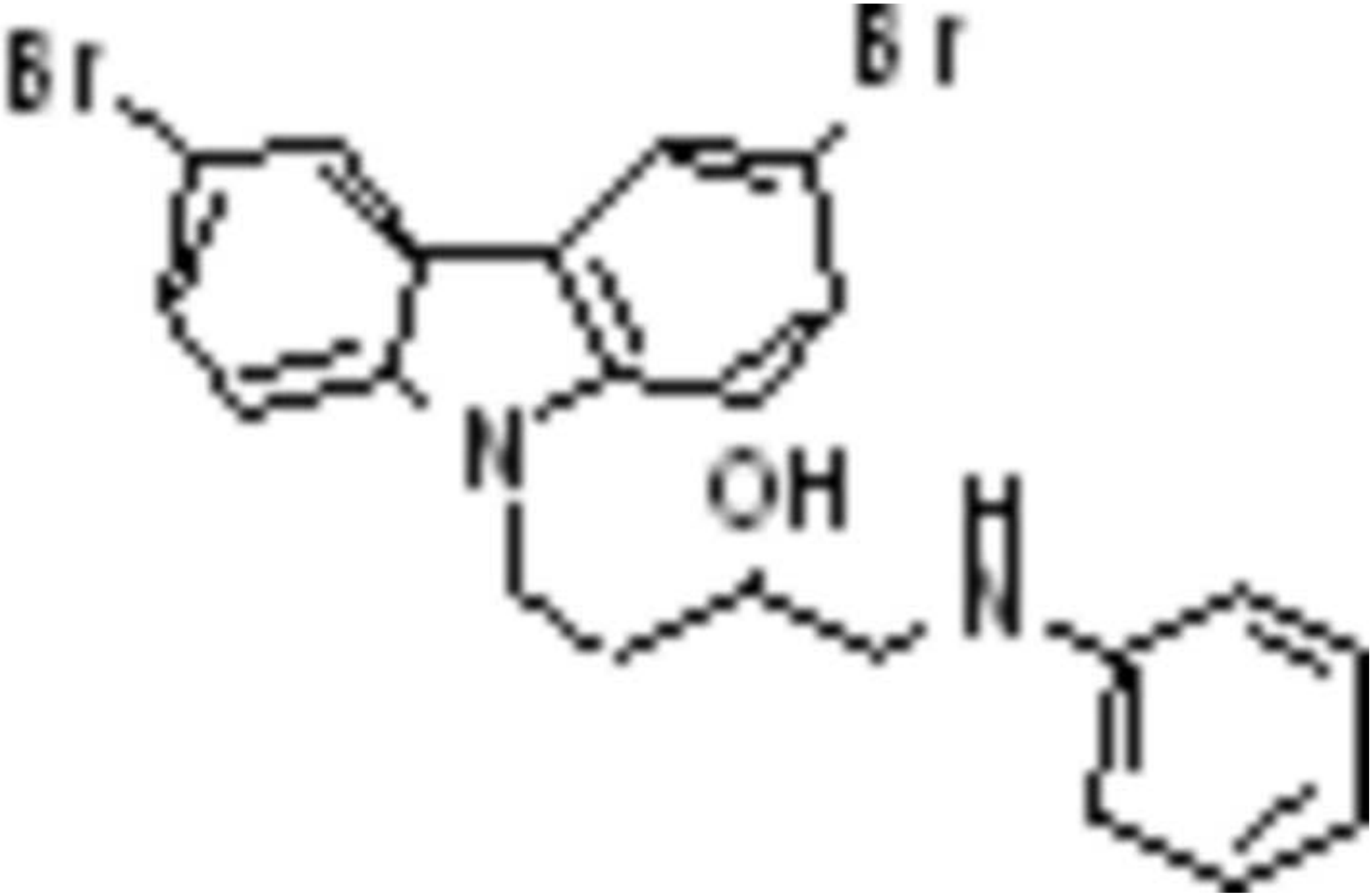
Supplemental Figure

Click here to download high resolution image



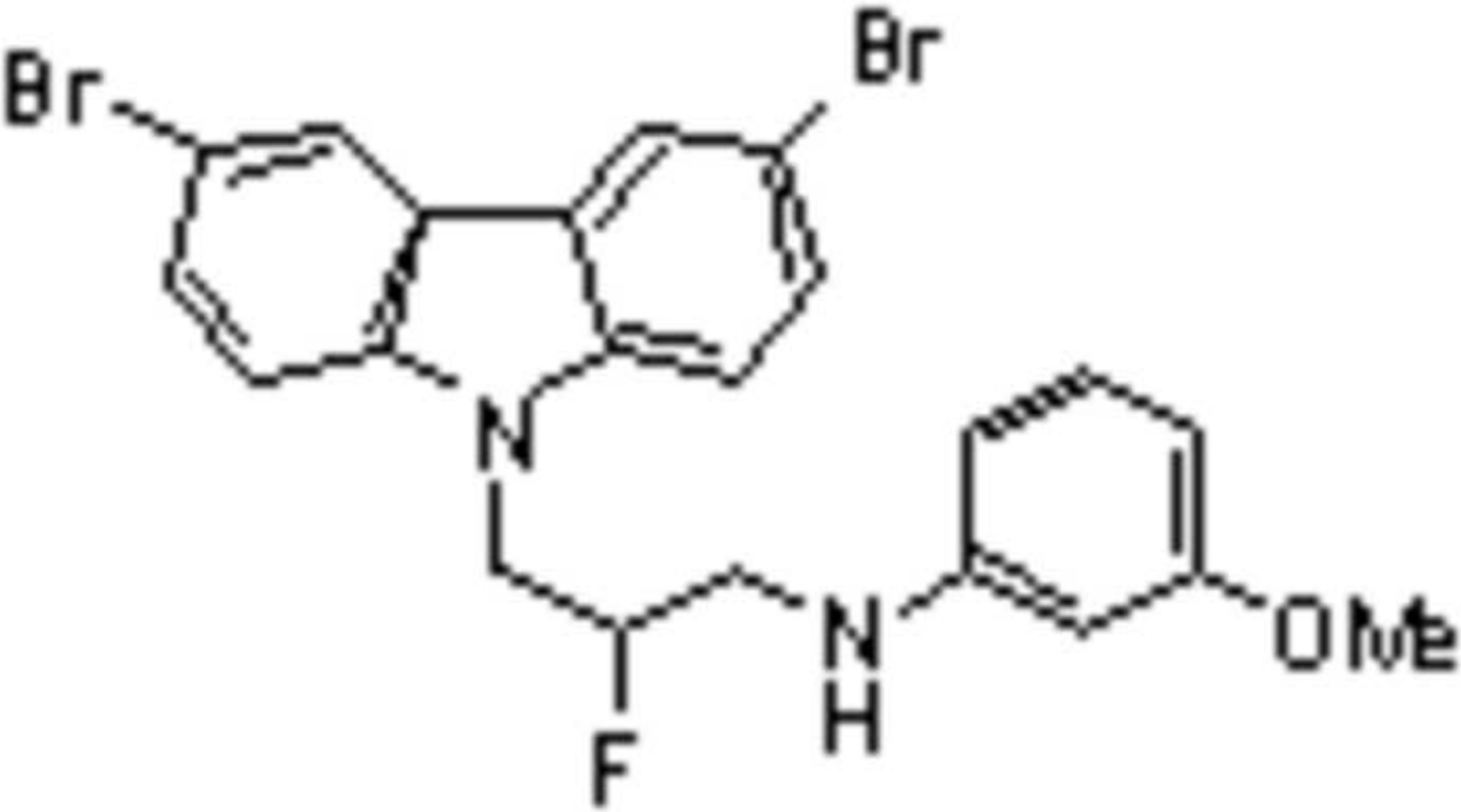
Supplemental Figure

Click here to download high resolution image



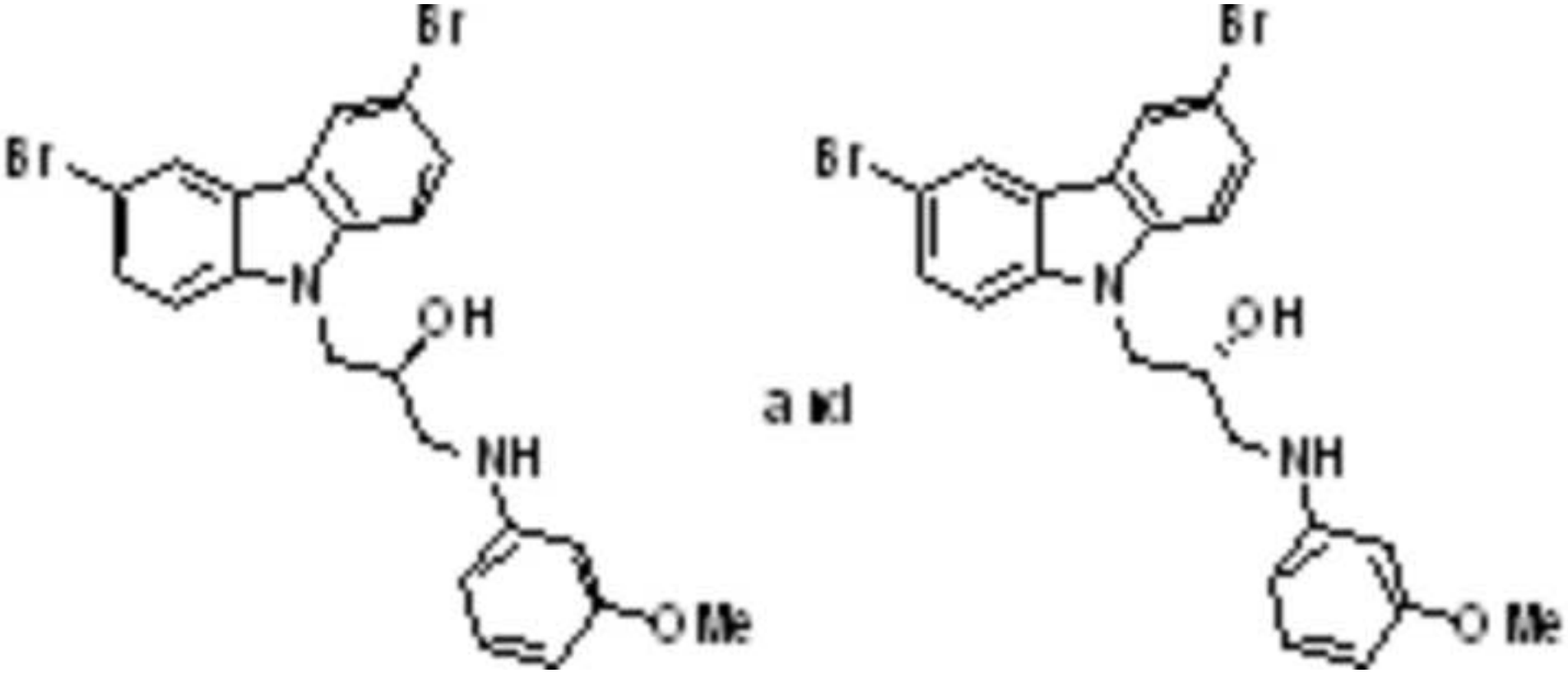
Supplemental Figure

Click here to download high resolution image



Supplemental Figure

Click here to download high resolution image



ETOC Paragraph (ACCEPTED papers only)

An unbiased screen was conducted in living mice to discover small molecules that might enhance hippocampal neurogenesis, blind to assumptions about mechanism. Out of 1000 compounds directly infused into the brains of mice, one compound, designated P7C3, was found to potently enhance hippocampal neurogenesis by protecting newborn neurons from apoptosis. Prolonged administration of P7C3 was safely tolerated by mice and rats, and corrected neuroanatomical and electrophysiological deficits in mutant mice that are virtually devoid of hippocampal neurogenesis. P7C3 also enhanced hippocampal neurogenesis, impeded neuron death, and preserved cognitive capacity as a function of terminal aging in rats.