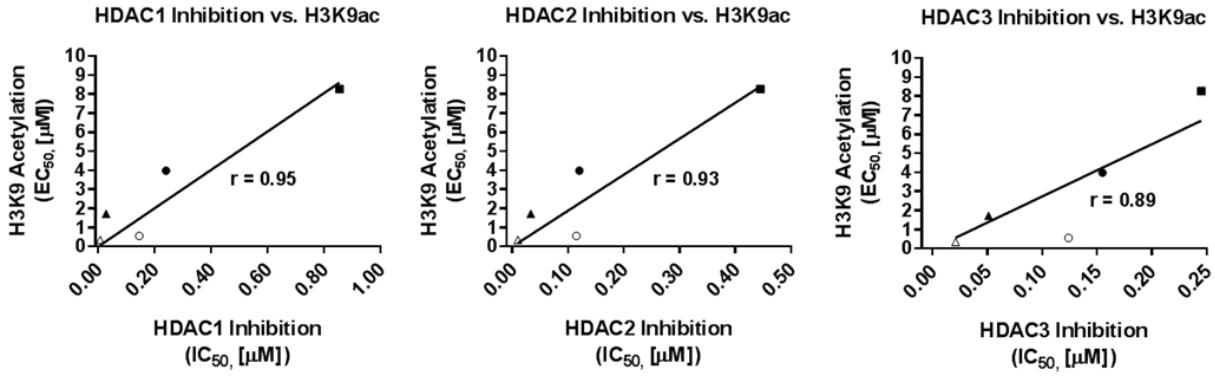
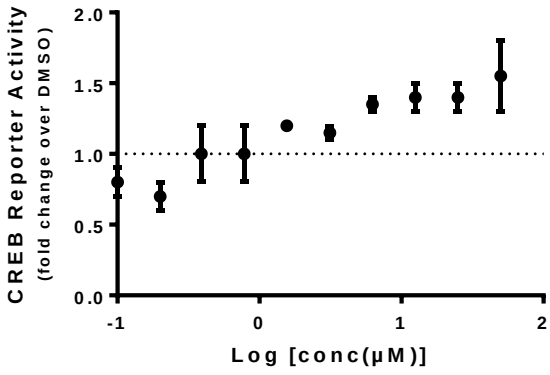
Supporting Information

Supplemental Figures

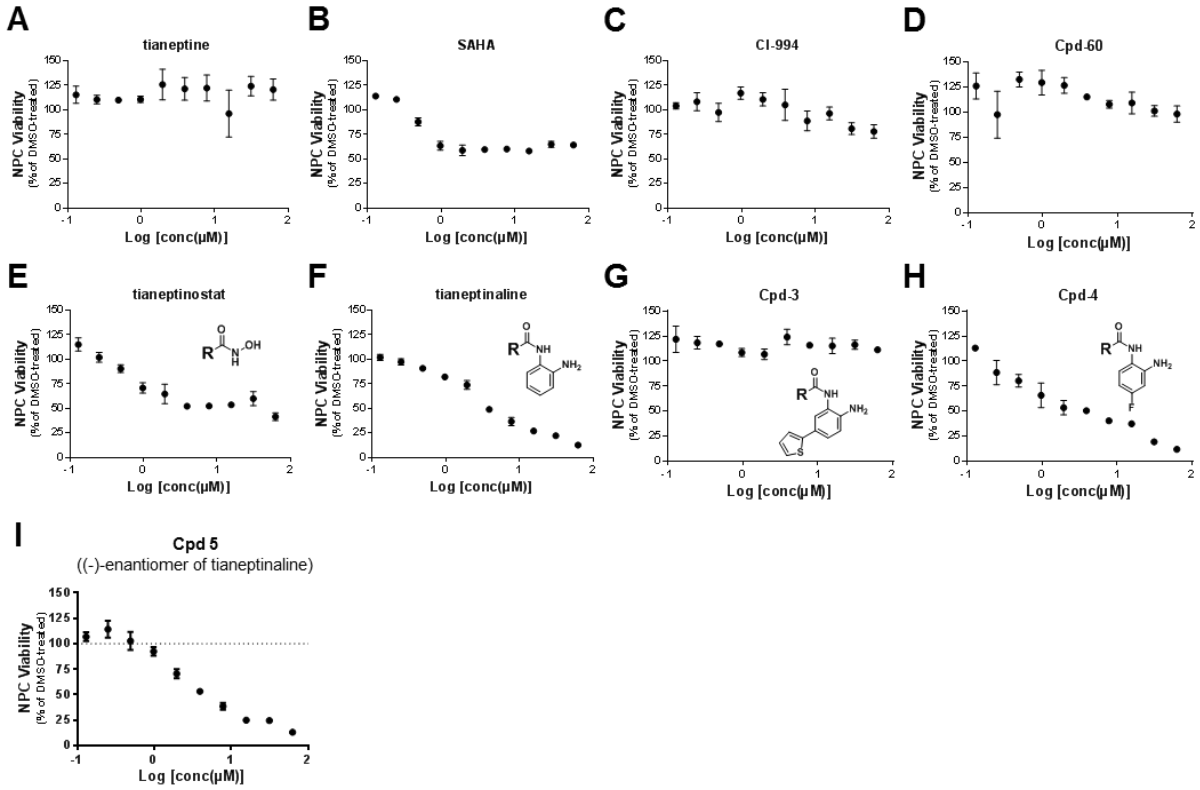


Supplemental Figure S1. Correlation between *in vitro* HDAC1/2/3 inhibitory activities and *ex vivo* neuronal H3K9 acetylation activities for tianeptine analogs. EC_{50} values of H3K9 acetylation are plotted against IC_{50} values of HDAC1/2/3 inhibition. The calculated correlation value r for HDAC1/2/3 is 0.95, 0.93, and 0.89, respectively. Δ – SAHA; o – CI-994; ▲ – tianeptinostat; ● – tianeptinaline; ■ – Cpd-4. Cpd-3 is omitted in plots due to low activity on H3K9ac resulting in the inaccurate calculation of EC_{50} value.

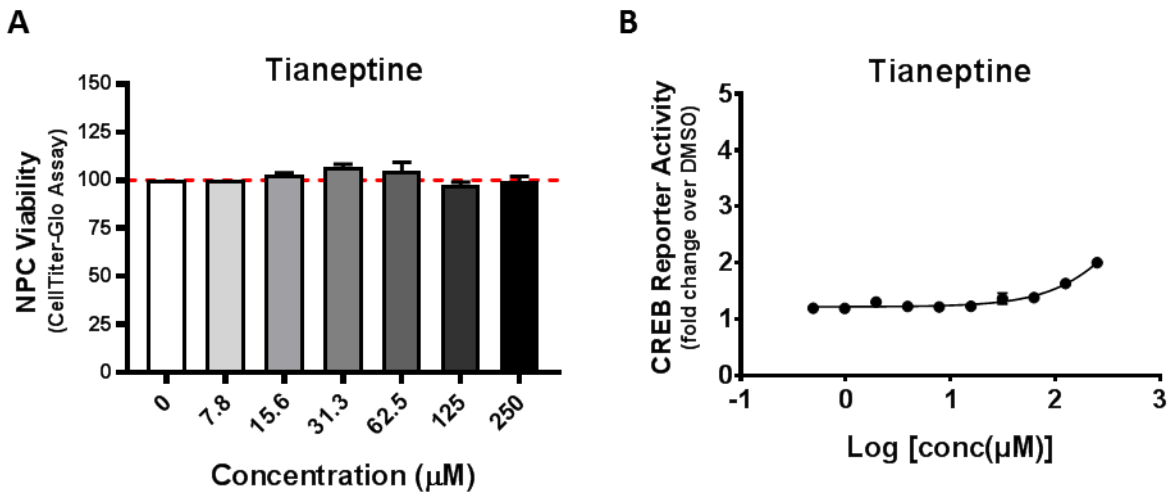


Supplemental Figure S2. Cellular activity of compound 5, (-)-enantiomer of compound 2, in induction of CREB signaling reporter. 10-point dose response tests were conducted in the presence of 2.5 μM forskolin. Experiments were repeated three

times. Each data point represents the mean of quadruplicate measurements in one biological replicate. Error bars display standard error of the mean (SEM).



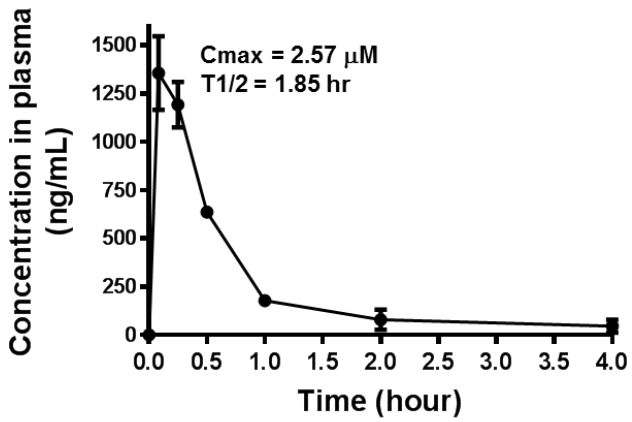
Supplemental Figure S3. Anti-proliferative effects of tianeptine analogs at high doses. Anti-proliferative effects of tianeptine analogs on human neural progenitor cells (NPCs) were tested with CellTiter-Glo reagent. NPCs were seeded and treated as for the CREB reporter assays. Each data point represents the mean ± SEM of duplicate measurements.



Supplemental Figure S4. Tianeptine shows modest induction on CREB reporter activity only at high doses. (A) No anti-proliferative effect was observed for tianeptine at high doses on human neural progenitor cells (NPCs), tested with CellTiter-Glo reagent. NPCs were seeded and treated as for the CREB reporter assays. Each data point represents the mean ± SEM of triplicate measurements. **(B)** Tianeptine modestly increased CREB signaling reporter activities at high doses tested (125 and 250 μM). 10-point dose response tests were conducted in the presence of 2.5 μM forskolin. Each data point represents the mean of quadruplicate measurements. Error bars display standard error of the mean (SEM).

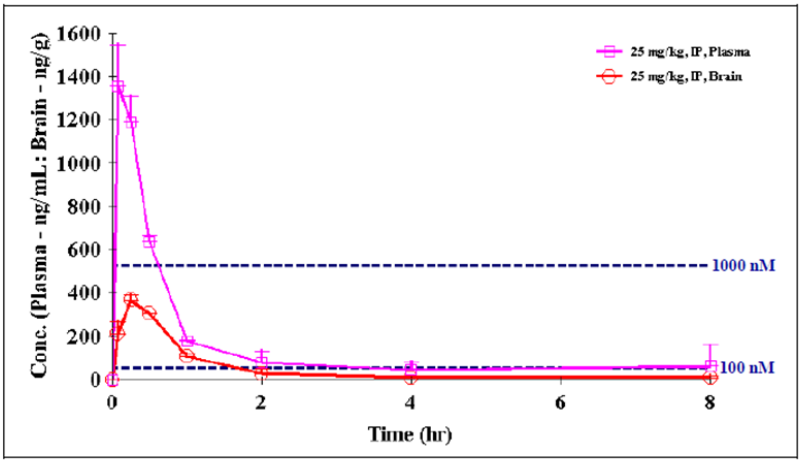
A

Plasma concentration – time profile of tianeptinaline



B

Plasma and brain concentration – time profiles of tianeptinaline



C

Brain to plasma C_{max} and exposure (AUC_{last}) ratios of tianeptinaline following a single intraperitoneal administration in male C57BL/6 mice (Dose: 25 mg/kg)

Compound	Matrix	Route	Dose (mg/kg)	C_{max} (µmol /L)	C_{max} ratio	AUC_{last} (hr*µmol /L)	AUC_{last} ratio
Tianeptinaline	Brain[c]	i.p.	25	0.69	0.27	0.73	0.33
	Plasma			2.57		2.23	

c - brain conc. and exposures expressed as µmol/kg and hr*µmol /kg, respectively; the density of brain was considered as 1 which is equivalent to plasma density (1)

Supplemental Figure S5. *In vivo* pharmacokinetics of tianeptinaline in C57BL/6 mouse brain following i.p. administration of tianeptinaline (25 mg/kg). (A) Plasma concentrations over time profile of tianeptinaline. **(B)** Plasma and brain concentration profiles in comparison. **(C)** Brain to plasma C_{max} and exposure (AUC_{last}) ratios of tianeptinaline.

Experimental Procedures

Chemical synthesis

Reagents and solvents were purchased from commercial suppliers and used as received unless otherwise indicated. Dry solvents were obtained according to the standard procedures. All reactions were performed under inert atmosphere (N_2) unless otherwise noted. Analytical silica gel 60 F_{254}-coated TLC plates were obtained from EMD Chemicals, Inc. and were visualized with UV light or by treatment with phosphomolybdic acid (PMA), Dragendorff's reagent or ninhydrin. Flash column chromatographic purifications were performed using Whatman Purasil® 60A silica gel 230-400 mesh. ^{1}H and ^{13}C nuclear magnetic resonance spectra were measured on Varian 400 MHz FT NMR spectrometer using tetramethylsilane (TMS) as an internal standard.

I. General procedure for amide coupling of tianeptine and mono protected ortho-amino aniline:

Tianeptine sodium salt and 1.1 equivalent of EDCI were added to a mixture of dichloromethane/pyridine (1:1) and stirred for 10 minutes at room temperature. Tert-butyl (2-aminophenyl)carbamate (1.1 eq.) and a catalytic amount of 4-DMAP were added and continued to stir for 2 to 12 hours at room temperature. The reaction mixture was evaporated under vacuum. The crude mixture was suspended in ethyl acetate and extracted from aqueous sodium bicarbonate solution. The combined organic layers were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was further purified by flash chromatography using hexane and ethyl acetate as mobile phase.

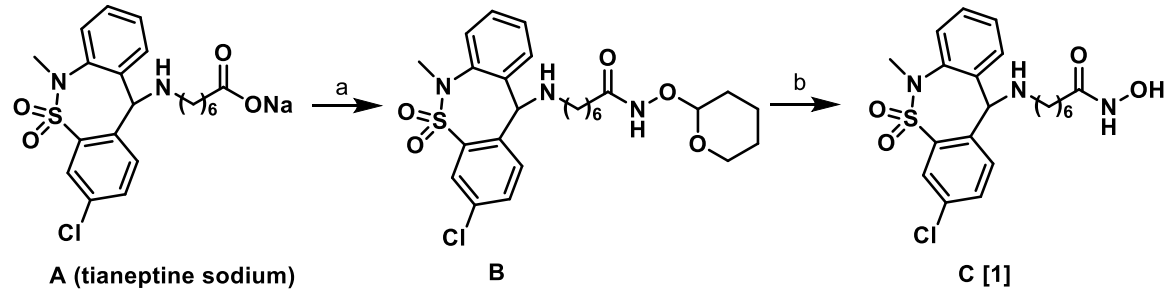
II. General procedure for nitro group reduction:

The nitro compound was reduced with hydrogen (1 atm) in the presence of 10% palladium on charcoal (catalytic amount) in methanol at room temperature for 30 min. The crude product was filtered through a pad of Celite, and the filtrate was evaporated to yield the desired compound sufficiently pure to be used for the next reaction.

III. General procedure for Boc-deprotection:

The Boc-protected aniline was dissolved in dichloromethane and cooled to 0°C. Trifluoroacetic acid diluted with an equal volume of dichloromethane was added and the reaction mixture was stirred on ice-bath for 5 minutes and stirring was continued for another 25 minutes at room temperature. Completion of the reaction was monitored by TLC. The reaction mixture was evaporated under reduced pressure and the residue was suspended in ethyl acetate, washed with aqueous sodium bicarbonate solution and brine. The evaporation of ethyl acetate layer gave sufficiently pure amine deprotected compound.

Scheme 1:



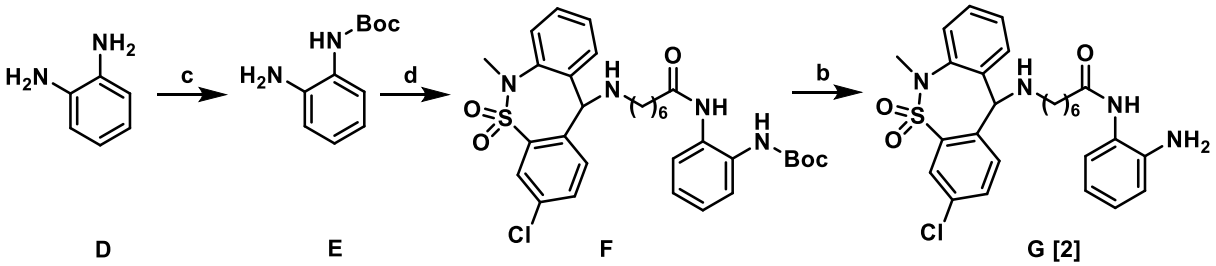
Reagents and conditions: (a) o-THP-hydroxyl amine, PyBOP, DIPEA (N,N'-diisopropylethyldiamine), CH_2Cl_2, 1h, rt. (b) TFA:CH_2Cl_2 (1:1), rt, 30 min

Synthesis of 7-((3-chloro-6-methyl-5,5-dioxido-6,11-dihydrodibenzo[c,f][1,2]thiazepin-11-yl)amino)-N-hydroxyheptanamide (C):

Tianeptine sodium salt (0.150 g, 0.3274 mmol) and *O*-(tetrahydro-2H-pyran-2-yl)hydroxylamine (0.037 g, 0.3601 mmol) were added in dichloromethane. PyBOP (0.205 g, 0.393 mmol) and DIPEA (0.051 g, 0.393 mmol) were added to the reaction mixture. The reaction was continued with stirring at room temperature for one hour. The mixture was diluted with dichloromethane and washed with water. The organic layer was dried over sodium sulfate and the organic solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography on silica with dichloromethane/methanol/triethylamine (95:4:1) to yield compound **B** that was subjected to THP-deprotection using a (1:1) mixture of trifluoroacetic acid and

dichloromethane following general procedure III. The reaction solvent was evaporated and the crude residue was suspended in ethyl acetate and washed with aqueous sodium bicarbonate. The organic layer was dried over sodium sulfate and evaporated under reduced pressure to yield sufficiently pure compound **C** (0.088 g, final yield was 59.7%). ^{1}H NMR (400 MHz, DMSO-d6) δ 10.29 (s, 1H), 8.62 (s, 1H), 7.77 (s, 1H), 7.71 (s, 2H), 7.49 (t, *J* = 7.4Hz, 2H), 7.38 (dt, *J* = 19.7, 7.3 Hz, 2H), 5.17 (s, 1H), 3.37 (s, 3H), 2.41-2.39 (m, 2H), 1.85-1.82 (m, 2H), 1.49-1.13 (m, 8H). ^{13}C NMR (101 MHz, DMSO-d6) δ 169.54, 141.22, 140.82, 138.31, 133.14, 132.54, 132.06, 129.53, 128.92, 128.66, 127.19, 47.97, 40.55, 39.48, 38.65, 32.68, 29.77, 29.52, 28.99, 26.99, 25.54. LC-MS calculated for expected $C_{21}H_{26}ClN_3O_4S$ [M]: 451.13; Found [M-H]$^{-}$: 450.11.

Scheme 2:



Reagents & conditions: (c) Boc_2O, 4-DMAP, THF, 2h, rt. (d) Tianeptine sodium, EDCI, 4-DMAP(cat), CH_2Cl_2:pyridine (1:1), rt, 2h. (b) CH_2Cl_2:TFA (1:1), rt, 30 min.

Synthesis of *tert*-butyl (2-aminophenyl)carbamate (Boc protected ortho amino aniline) (E):

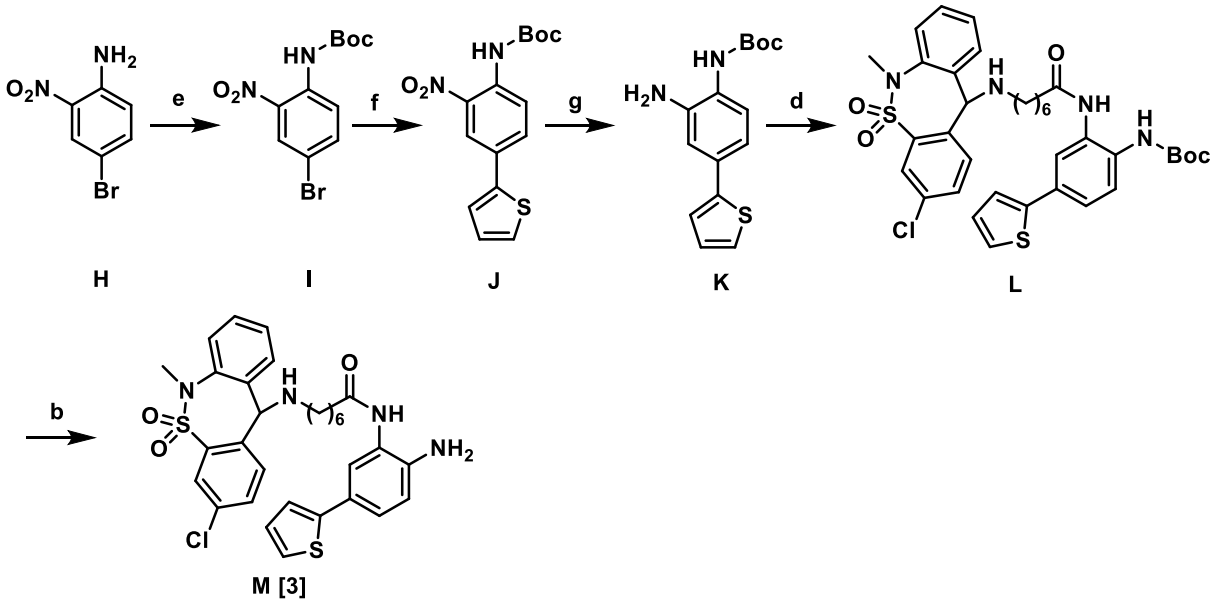
o-phenylenediamine (**D**, 0.200 g, 1.849 mmol) was added in tetrahydrofuran (THF). Di-*tert*-butyl dicarbonate (0.404 g, 1.849 mmol) was added to the reaction mixture. A catalytic amount of 4-DMAP was added and the reaction mixture was stirred for 2 hours under a nitrogen atmosphere at room temperature. The reaction mixture was evaporated and suspended in ethyl acetate, followed by the addition of a sodium bicarbonate solution. The aqeous layer was extracted with ethyl acetate. The combined organic layers were dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica using hexane and ethyl acetate as a mobile phase to give *tert*-butyl (2- aminophenyl)

carbamate (**E**, 96 mg, 25% yield). ^{1}H NMR (400 MHz, DMSO-d6) δ 9.15 (s, 1H), 7.96 – 6.80 (m, 4H), 6.27(s, 2H), 1.40 (s, 9H).

Synthesis of N-(2-aminophenyl)-7-((3-chloro-6-methyl-5,5-dioxido-6,11-dihydrodibenzo[c,f][1,2]thiazepin-11-yl)amino)heptanamide (G):

Tianeptine sodium salt (0.150 g, 0.326 mmol) and *tert*-butyl (2-aminophenyl) carbamate (0.075 g, 0.359) were coupled following general procedure I to yield compound **F**, which after purification by column chromatography with ethyl acetate and hexane as eluent, was deprotected following general procedure III to yield compound **G** without further purification (0.096 g, 55.8% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.05 (s, 1H), 7.78 (d, *J*=1.4Hz, 1H), 7.71(d, *J*=1.3Hz, 2H), 7.53(m, 2H), 7.44-7.33 (m, 2H), 7.15 (d, *J*=7.8Hz, 1H), 6.89 (s, 1H), 6.72 (dd, *J*=8.0, 1.3Hz, 1H), 5.18 (s, 1H), 4.79 (s, 2H), 3.39 (s, 3H), 2.41 (bs, 2H), 2.39 (m, 2H), 1.61-1.11 (m, 8H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.58, 142.30, 141.23, 140.83, 138.31 (d, *J* = 2.8 Hz), 133.14, 132.53, 132.07, 129.54, 128.93, 128.66, 127.19, 126.12, 125.68, 124.07, 116.64, 116.37, 63.82, 48.00, 40.65, 40.44, 38.66, 36.20, 29.79, 29.07, 27.07. LC-MS calculated for expected $C_{27}H_{31}ClN_4O_3S$ [M]: 526.18; Found $[M-H]^-$: 525.09.

Scheme 3:



Reagents & conditions: (e) i.Boc_2O, 4-DMAP, THF, 18h, ii. NaOH, H_2O, THF, 4h, rt to 70 ^oC; 4h. (f) Ar-$B(OH)_2$, *tri-o*-tolyl-phosphine,K_2CO_3, $Pd(PPh_3)_4$(0), DME, H_2O, 80 ^oC, 20 min (microwave). (g) H2-balloon, Pd/C, MeOH, rt, 2h. (d) Tianeptine sodium, EDCI, 4-DMAP(cat), CH_2Cl_2:pyridine (1:1), rt, 2h. (b) DCM:TFA (1:1), rt, 30 min.

Synthesis of *tert*-butyl(4-bromo-2-nitrophenyl)carbamate (I):

4-bromo-2-nitro aniline (0.200 g, 0.921mmol) and di-*tert*-butyl dicarbonate (0.221 g, 1.013 mmol) were dissolved in tetrahydrofuran (THF), followed by the addition of a catalytic amount of 4-DMAP. The reaction mixture was stirred for 90 minutes at room temperature and the solvent was evaporated under reduced pressure to yield a thick oil. The crude reaction mixture was dissolved in THF and stirred at 70°C. After 18h solid sodium hydroxide (0.037 g, 0.921mmol) was added and the reaction mixture was stirred for an additional 4h at the same temperature. The solvent was evaporated and the remaining oil was suspended in ethyl acetate and washed with water. The ethyl acetate layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude compound was purified by column chromatography on silica using ethyl acetate and hexane (10:90) as a mobile phase to yield the desired product *tert*-butyl(4-bromo-2-nitrophenyl)carbamate (0.216g, 74% yield). ^1H NMR (400 MHz, DMSO-d_6) δ (ppm) 9.62 (s, 1H), 7.98 (d, *J*=2.2 Hz, 1H), 7.76 (dd, *J*=8.6, 2.2 Hz, 1H), 7.55 (d, *J*=8.8 Hz, 1H), 1.44 (s, 9H).

Synthesis of *tert*-butyl(2-nitro-4-(thiophene-2-yl)phenyl)carbamate (J):

2-thiophene boronic acid (0.200 g, 1.56 mmol), *tert*-butyl(4-bromo-2-nitrophenyl) carbamate (**I**) (0.248 g, 0.781 mmol), tri-*o*-tolyl-phosphine (0.062 g, 0.203 mmol) and potassium carbonate (0.028 g, 0.203 mmol) were mixed in degassed dimethoxyethane and water. A catalytic amount of tetrakis (triphenylphosphine) palladium (0) was added and the reaction mixture was heated in CEM microwave reactor at 80°C for 20 minutes. The mixture was cooled and diluted with ethyl acetate, washed with brine and dried over $MgSO_4$. The organic layer was evaporated and subjected to column purification on silica using ethyl acetate and hexane (10:90) as a mobile phase to yield the desired product *tert*-butyl(2-nitro-4-(thiophene-2-yl)phenyl)carbamate (**J**, 0.205 g, 81% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 9.59 (s,1H), 8.10 (d, *J*=2.2 Hz, 1H), 7.91 (dd, *J*=8.4, 2.2Hz, 1H), 7.71 (d, *J*=8.6, 1H), 7.63 (dd, *J*=4.3, 2.4Hz, 2H), 7.19 (dd, *J*=4.9, 3.8Hz, 1H), 1.44 (s, 9H).

Synthesis of *tert*-butyl (2-amino-4(thiophen-2-yl)phenyl)carbamate (K):

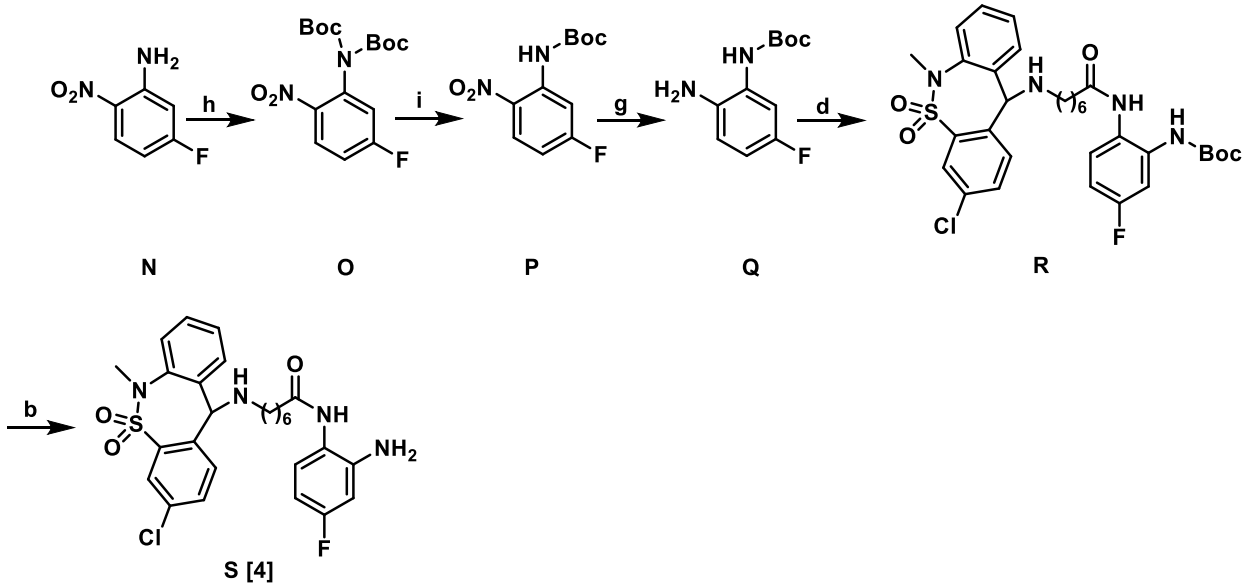
tert-butyl(2-nitro-4-(thiophene-2-yl)phenyl)carbamate (**J**, 0.350 g, 1.093 mmol) was dissolved in methanol and a catalytic amount of palladium on charcoal (Pd/C, 10% w/w) was added. The mixture was stirred for 2 hours at room temperature under a hydrogen atmosphere (1 atm). The mixture was filtered through pad of celite and the filtrate was evaporated to yield the product *tert*-butyl (2-amino-4(thiophen-2-yl)phenyl)carbamate (**K**, 0.286 g, 90% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 8.31 (s, 1H), 7.39 (d, *J*=5.1Hz, 1H), 7.28-7.20 (m, 2H), 7.04 (t, *J*=4.4Hz, 1H), 6.92 (s, 1H), 6.80 (d, *J*=8.2, 1H), 5.01 (bs, 2H), 1.43 (s, 9H).

Synthesis of N-(2-amino-4-fluorophenyl)-7-((3-chloro-6-methyl-5,5-dioxido-6,11-dihydrodibenzo[c,f][1,2]thiazepin-11-yl)amino)heptanamide (M):

Tianeptine sodium salt (0.100 g, 0.217 mmol) and *tert*-butyl (2-amino-4(thiophen-2-yl)phenyl)carbamate (K, 0.069 g, 0.239 mmol) were coupled following general procedure I to yield compound **L**, which was further purified by column chromatography

using ethyl acetate and hexane as eluent. Compound **L** was deprotected following general procedure III to yield final compound **M** without further purification (0.051 g, 38.5% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.22 (s, 1H), 7.93 – 6.98 (m, 13H), 5.12 (d, *J* = 7.2 Hz, 2H), 3.35 (s, 3H), 2.42 (m, 2H), 2.01 (s, 1H), 1.83 – 0.90 (m, 10H). ^{13}C NMR (101 MHz, DMSO) δ 171.81, 144.84, 142.03, 141.24, 138.31, 133.15, 132.54, 129.54, 128.94,128.65, 127.19, 124.18, 123.64, 123.54, 122.79, 122.65, 121.39, 116.59, 48.00, 40.65, 40.44, 40.23,40.02, 39.91, 39.81, 39.61, 38.67, 36.24, 29.78, 27.07, 25.65. LC-MS calculated for $C_{31}H_{33}ClN_4O_3S_2$ expected [M]: 608.17; Found $[M-H]^-$: 607.14

Scheme 4:



Reagents & conditions: (h) Boc_2O, 4-DMAP, 2-Me-THF, 12h, rt (i). 3%TFA in CH_2Cl_2, rt, 30 min. (g) H_2-balloon, Pd/C, MeOH, rt, 2h. (d) Tianeptine sodium, EDCI, 4-DMAP(cat), CH_2Cl_2:pyridine (1:1), rt, 2h. (b) CH_2Cl_2:TFA (1:1), rt, 30 min.

Synthesis of 5-fluoro-2-nitro-di Boc aniline (O):

Di-tert-butyl dicarbonate (0.307 g, 1.409 mmol) in 2-methyl tetrahydrofuran was added to 5-fluoro-2-nitro aniline (0.200 g, 1.28 mmol) in 2-methyl tetrahydrofuran followed by the addition of a catalytic amount of 4-DMAP. After stirring overnight at room temperature the reaction mixture was evaporated, suspended in ethyl acetate and washed with sodium bicarbonate solution. The ethyl acetate layer was dried over Na_2SO_4 and the solvent was removed under reduced pressure. The crude compound

was purified by column chromatography on silica using ethyl acetate and hexane as eluent to yield intermediate **O** (0.355 g, 76% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.17 (s, 1H), 8.02-7.68 (m, 2H), 1.49 (s, 18H)

Synthesis of *tert*-butyl (5-fluoro-2-nitrophenyl)carbamate (P):

Compound **O** (0.300 g, 0.821 mmol) was treated with 3% trifluoroacetic acid in dichloromethane at room temperature for 30 minutes with continuous monitoring of the reaction progress by TLC. The reaction mixture was washed with aqueous sodium bicarbonate solution and extracted with dichloromethane to yield *tert*-butyl (5-fluoro-2-nitrophenyl) carbamate **P** (0.170 g, 78.8% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.98 (s, 1H), 8.13 (s, 1H), 8.22-7.78 (m, 2H), 1.45 (s, 9H)

Synthesis of *tert*-butyl (2-amino-5-fluorophenyl)carbamate (Q):

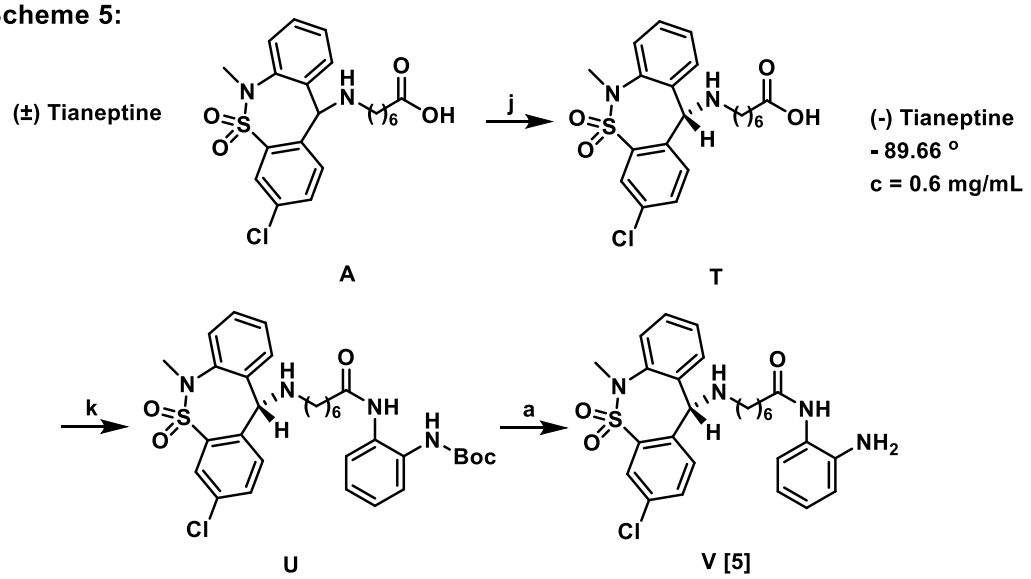
Tert-butyl (5-fluoro-2-nitrophenyl) carbamate **P** (0.150 g, 0.585 mmol) was dissolved in methanol and a catalytic amount of palladium on charcoal (Pd/C, 10% w/w) was added. The mixture was stirred for 2 hours at room temperature under a hydrogen atmosphere (1 atm). The mixture was filtered through pad of Celite and methanol was evaporated to yield sufficiently pure *tert*-butyl (2-amino-5-fluorophenyl) carbamate, **Q** (0.113 g, 85% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.85 (s, 1H), 7.78-6.72 (m, 3H), 5.97 (s, 2H), 1.39 (s, 9H).

Synthesis of N-(2-amino-5-(thiophen-2-yl)phenyl)-7-((3-chloro-6-methyl-5,5-dioxido-6,11-dihydrodibenzo[c,f][1,2]thiazepin-11-yl)amino)heptanamide (S):

Tianeptine sodium salt (0.100 g, 0.217 mmol) and *tert*-butyl (2-amino-5-fluorophenyl) carbamate (Q, 0.054 g, 0.239 mmol) were coupled following general procedure I to yield compound **R** after purification by column chromatography on silica using ethyl acetate and hexane as eluent. The pure intermediate was deprotected following general procedure III to yield final compound **S** without further purification (0.070 g, 59% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.00 (s, 1H), 7.96 – 6.91 (m, 8H), 6.60 – 6.13 (m, 2H), 5.48 (bs, 3H), 3.52 – 3.28 (m, 4H), 2.35-1.81 (m, 3H), 1.00- 1.65 (m9H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 144.58, 138.27, 132.44, 129.55, 128.66, 127.08, 99.99, 63.79, 47.95,

40.67, 40.52, 40.45, 40.32, 40.24, 40.11, 40.03, 39.91, 39.83, 39.70, 39.62, 39.50, 39.41, 39.30, 39.28,38.55, 36.08, 28.98. LC-MS calculated for expected $C_{27}H_{30}ClFN_4O_3S$ [M]: 544.17; Found $[M-H]^-$: 543.21.

Scheme 5:



Reagents & conditions: (j) Chiral column HPLC. (k) Boc protected *O*-amino aniline, EDCI, 4-DMAP(cat), CH_2Cl_2:pyridine (1:1), RT, 2h. (b) CH_2Cl_2:TFA (1:1), rt, 30 min.

Synthesis of *(S)-N*-(2-aminophenyl)-7-((3-chloro-6-methyl-5,5-dioxido-6,11-dihydrodibenzo[c,f][1,2]thiazepin-11-yl)amino)heptanamide (V):

Racemic tianeptine was separated enantiomerically by HPLC using chiral column. Chromatographic parameter were mobile Phase: 0.1% TFA in hexane : isopropyl alcohol (80 : 20 v/v), Column: Chiralpak AD3 (250 X 4.6 mm) 3 uM, Flow Rate: 1.5 ml/min, at ambient temperature to get (-) tianeptine which was used as a starting material synthesis of **V**. (-) enantiomer of tianeptine (0.200 g, 0.457 mmol) and tert-butyl (2- aminophenyl) carbamate (0.099 g, 0.479) were reacted following general procedure I to yield compound **U** after purification by column chromatography on silica using ethyl acetate and hexane as eluent. The intermediate was deprotected following general procedure III using trifluoroacetic acid and dichloromethane mixture (1:1) to yield final compound **V** without further purification (0.134 g, 85% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.05 (s, 1H), 7.76 (d, *J*=1.4Hz, 1H), 7.73(d, *J*=1.3Hz, 2H), 7.52(m, 2H), 7.47-7.39 (m, 2H), 7.14 (d, *J*=7.8Hz, 1H), 6.91 (s, 1H), 6.69 (dd, *J*=7.2, 1.4Hz, 1H), 5.01

(s, 1H), 4.78 (s, 2H), 3.37 (s, 3H), 2.43 (m, 2H), 2.36 (m, 2H), 1.58-1.09 (m, 8H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.58, 142.30, 141.23, 140.83, 138.31 (d, *J* = 2.8 Hz), 133.14, 132.53, 132.07, 129.54, 128.93, 128.66, 127.19, 126.12, 125.68, 124.07, 116.64, 116.37, 63.82, 48.00, 40.65, 40.44, 38.66, 36.20, 29.79, 29.07, 27.07. LC-MS calculated for $C_{27}H_{31}ClN_4O_3S$ expected [M]: 526.18; Found $[M-H]^-$: 525.13

***In vitro* HDAC enzymatic assays**

HDAC activities were measured *in vitro* using recombinant human HDACs 1-3, 5, 6 (BPS Bioscience). The trypsin coupled enzyme assays were performed under conditions appropriate for Michaelis-Menten kinetic analysis with the use of tripeptide acetylated substrate at K_m that is amide coupled to 7-amino-4-methylcoumarin (MAZ1600). HDAC assay buffer contains 100 mM KCL, 50 mM HEPES, pH 7.4 (Gibco, #15630-114), 0.05% BSA (Invitrogen, P2489), 0.001% Tween-20 (Zymed, #00-3005) in Milli-Q H_2O. Detailed procedures are as described in our previous publications [1] [2].

Histone acetylation assays using iPSC-derived human neuronal cultures

To prepare human neuronal cultures for evaluation of cellular HDAC inhibitory activities of chemical compounds, human iPSC-derived neural progenitor cells were differentiated into post-mitotic neurons as previously described [3] in T75 flasks for 3 weeks. The resulting neuronal cultures were dissociated to single cell suspension and re-plated to poly-ornithine/laminin-coated 96-well plates (Corning 3904). To dissociate and re-plate neuronal cultures, cultures in T75 flasks were washed once with PBS, treated with 3 mL of Accutase (Sigma, A6964) for total 10 min at 37°C, during which time cells were gently triturated multiple times. After dilution with fresh media, suspended cells were passed through 40 μm cell strainer (BD Falcon, 352340) to remove cell clumps. Cells were counted and then spun down. Re-suspended cells were seeded at the density of 50,000 cells per well in 96-well plates. Re-plated neuronal cultures were allowed to recover for one week before treatment with compounds. 24-hour treated cultures were fixed and immune-stained for histone acetylation marks H3K9ac and H4K12ac (Millipore, #07-352 and #04-119), as well as neuronal dendritic marker MAP2 (EnCor Biotechnology, CPCA-MAP2). 4-9 images were taken from each well in 96-well plates on automated

confocal microscope IN Cell Analyzer 6000 (GE Healthcare). Immunofluorescent intensities of H3K9ac or H4K12ac in nuclei were quantified by high-content image analysis (IN Cell Analyzer Workstation 3.7.2, GE Healthcare). HDAC inhibitory activities were reported as increased H3K9ac and H4K12ac intensities normalized to DMSO-treated samples.

CREB signaling reporter assay

Activities of CREB signaling stimulation were measured with CREB reporter stable NPC line. Generation of the stable CREB reporter NPC line and conduction of assays followed the procedures described in Zhao et al [4]. The CRE reporter encodes the firefly luciferase gene under the control of a minimal CMV promoter and tandem repeats of the CRE transcriptional response element. Ready-to-transduce lentiviral particles (CLS-002L) were purchased from SABiosciences, and applied to NPCs followed by subsequent continuous puromycin (1 μg/mL) selection to establish the stable cell line. For assays, single cell suspensions of the reporter NPCs were dispensed into pre-coated 384-well plates at 9000 cells per well in 30 μL culture media. 24 hrs later, 10 μL of media or media containing forskolin was dispensed to each well to achieve media-only or 2.5 μM forskolin in final concentration, followed by compound treatment. After 6 hr treatment, cells were lysed by dispensing 15 μL of SteadyGlo reagent (Promega) and read for luminescence. Activities of CREB signaling stimulation are expressed as fold change over DMSO-treated samples.

Nuclear Arc expression and acetylation of H3K9 in mouse cortical neurons

For mouse primary cortical cultures, forebrains of E16.5 C57BL/6 mouse embryos were dissected and then dissociated in trypsin/DNAse solution for 10 min followed by three washes with phosphate-buffered saline (PBS). Single cell suspension was achieved by gentle pipet trituration. Neurons were seeded on poly-L-lysine/laminin coated coverslips in 24-well plates at a density of 0.1×10^7 per well and maintained in Neurobasal medium containing B27 supplement (2%, Invitrogen), penicillin (50U/mL, Invitrogen), streptomycin (50 μg/mL, Invitrogen) and glutamine (1 mM, Sigma). Compounds and BDNF (100 ng/mL, Millipore Corps) were added directly to the culture medium at DIV14

for 6 hrs. Preparation of mouse primary forebrain cortical neuron cultures was approved by the Animal Care and Use Committee of the Massachusetts General Hospital and carried out according to institutional guidelines.

After experimental treatment, cells were fixed for 30 min at room temperature with 4% paraformaldehyde in PBS. After fixation, cells were washed twice with PBS, permeabilized with PBST (PBS and 0.25% Triton X-100) for 20 min, blocked in blocking solution (5% goat nonimmune serum in PBS) for another 30 min, and finally incubated overnight at 4°C with all three primary antibodies (MAP2 (Millipore, AB5543), Arc (Synaptic Systems, #156005), and acetyl-H3K9 (Millipore, #07-352)) in blocking solution simultaneously. The next day, coverslips were extensively washed with PBS and then incubated for 2 hours at room temperature with a cocktail combining the appropriate fluorophore-conjugated secondary antibodies in blocking solution. Cell nuclei were finally counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and coverslips were mounted on glass slides with ProLong Antifade reagent (Invitrogen, Molecular Probes).

Cells cultured on coverslips from two independent biological replicates were imaged with an IN Cell 6000 high-performance laser-based confocal imaging system and a 40x objective (GE Healthcare Life Sciences). CellProfiler was used for automated pixel intensity measurement of nuclear Arc and acetyl-H3K9 immunofluorescence signals. Image preparation and assembly were performed with ImageJ and Photoshop CS. Change in contrast and evenness of the illumination was applied equally to all images presented in the study.

In vivo pharmacokinetic study

Pharmacokinetics of tianeptinaline was evaluated in mice (Sai Life Sciences Limited). 27 male C57BL/6 mice were administered intraperitoneally at 25 mg/kg dose with solution formulation of 10% DMSO and 45% PEG400 in 45% saline. Blood samples (approximately 60 µL) and brains were collected from a set of three mice at pre-determined time points (Pre-dose, 0.08, 0.25, 0.5, 1, 2, 4, 8 and 24 hr). Immediately after collection of blood, brains were collected from a set of three mice at the same time point. Plasma was harvested by centrifugation of blood and stored below -70ºC prior to analysis. Brain samples were homogenized using ice-cold phosphate buffer saline (pH

7.4) and homogenates were stored below -70ºC prior to analysis. Total homogenate volume was three times the tissue weight. The plasma and brain concentration-time data for tianeptinaline were used for the pharmacokinetic analysis. Brain concentrations were converted to ng/g from ng/mL considering total homogenate volume and brain weight. Pharmacokinetic analysis was performed using NCA module of WinNonlin Enterprise Version 5.2.

Mouse contextual fear conditioning test

C57BL/6J mice were housed and cared for following standard procedures. 9 to 10-week-old mice were injected daily intraperitoneally with either vehicle alone (5% DMSO, 30% Cremophor, 65% saline) or test compound dissolved in vehicle for consecutive 10 days, followed by a training day and a test day. For training, mice were placed in a training chamber for a period of 3 min followed by two foot shocks (2 s; 0.8 mA constant current) applied 30 s apart, and kept for additional 15 s before being returned to their home cage. 24 hours later, mice were put back to the training chamber to test their memory on the foot shocks. Freezing or lack of movement with an associated crouching posture was recorded every 10 s over the total 3 min test time, and expressed as a percentage of the number of positive freezing incidences over the total number of sampling intervals (18). Exploratory open field locomotion was recorded for 3 min as horizontal movement (distance traveled in cm) in the training chamber prior to foot shocks on the training day, using the TSE Fear Conditioning software package. Three groups of mice (vehicle, 25 mg/kg of tianeptinaline, or 5 mg/kg of CI-994) with total 22 mice in each group were tested over two trials, and the data was graphed with combined effects. All procedures were performed in accordance with the Committee for Animal Care of the Division of Comparative Medicine at the Massachusetts Institute of Technology and with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals: Eighth Edition.

Supplemental References

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