**SUPPLEMENTARY INFORMATION**

# Active, phosphorylated fingolimod inhibits histone deacetylases and facilitates fear extinction memory

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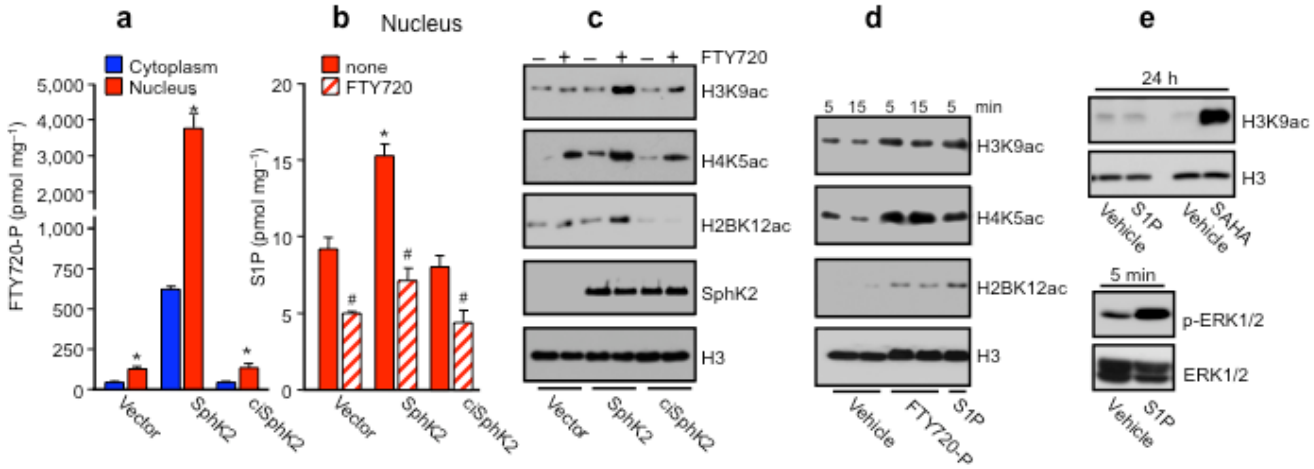
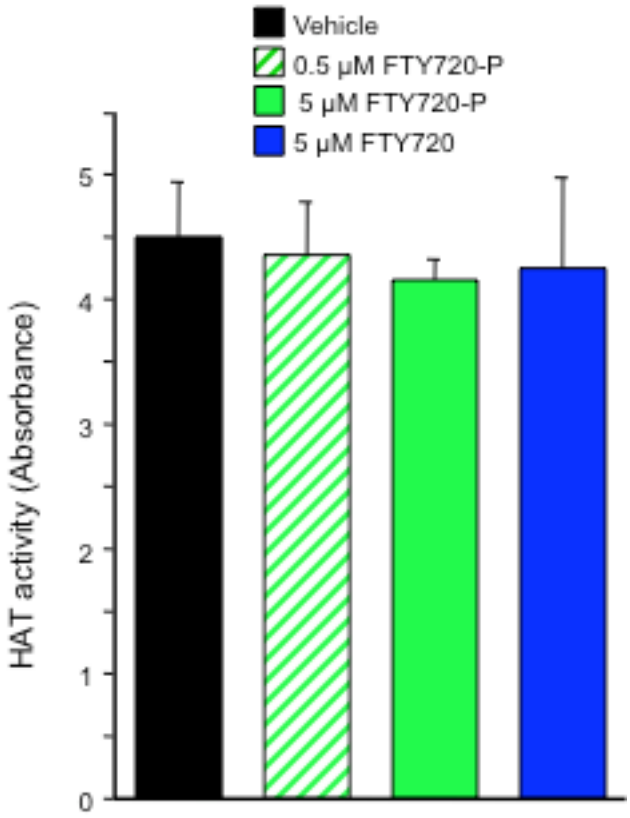
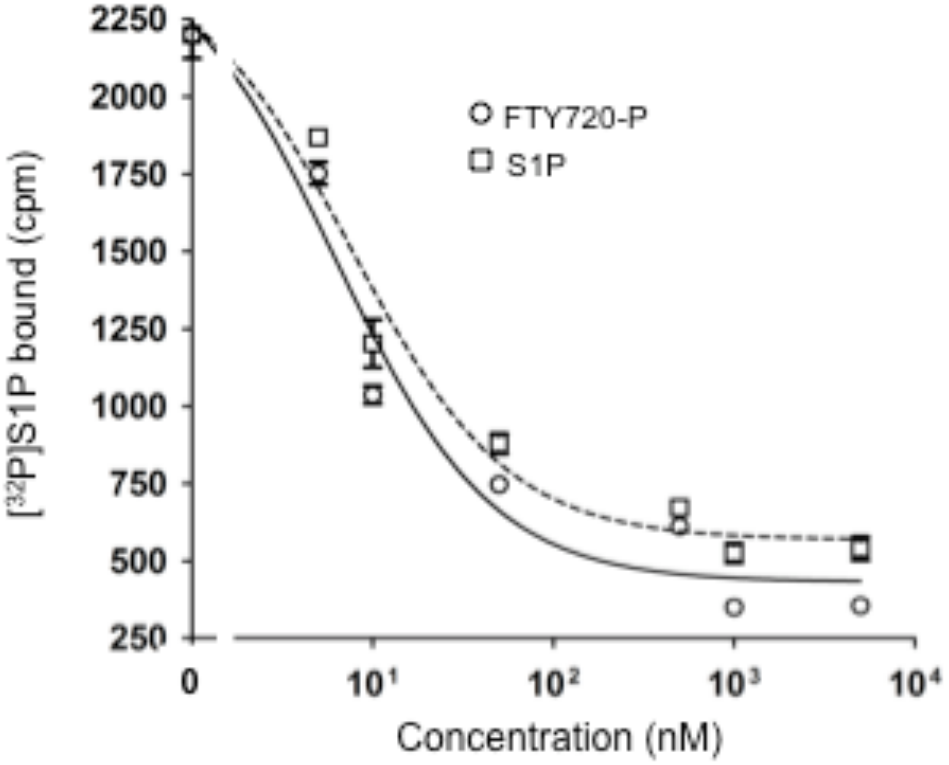


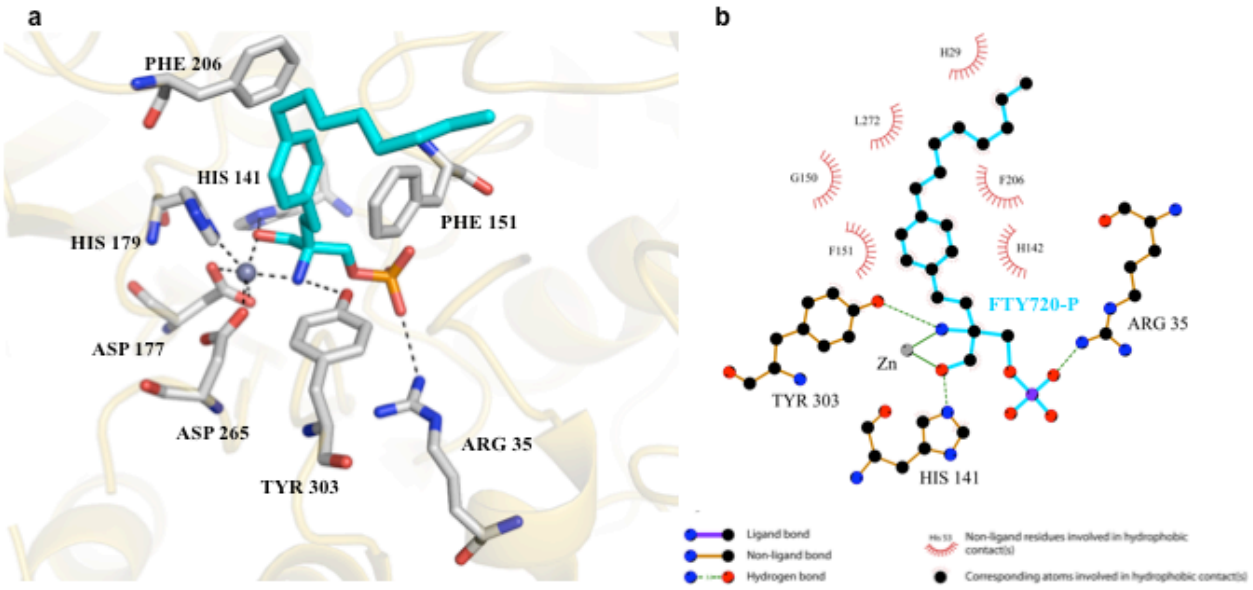

**Supplementary Figure 1. FTY720 is phosphorylated by nuclear SphK2 and enhances histone acetylations in HeLa cells.** (**a-c**) HeLa cells transfected with vector, SphK2, or catalytically inactive SphK2$^{G212E}$ (ciSphK2) were treated without or with FTY720 (5 μM) for 6 h (N = 3 per group). Nuclear and cytoplasmic levels of FTY720-P (**a**) and S1P (**b**) were determined by LC-ESI-MS/MS. Data are means ± s.d. *P < 0.01 compared to vector. $^{\#}$P < 0.01 compared to untreated (unpaired two-tail t test). (**c**) Histone acetylations in nuclear extracts were detected by immunoblotting with the indicated antibodies. (**d**) Purified nuclei from HeLa cells were incubated for the indicated times with vehicle, S1P (1 μM), or FTY720-P (1 μM) and histone acetylations determined. (**e**) Naïve HeLa cells were treated with vehicle, S1P (100 nM), or SAHA (1 μM) for the indicated times and proteins were analyzed by western blotting.



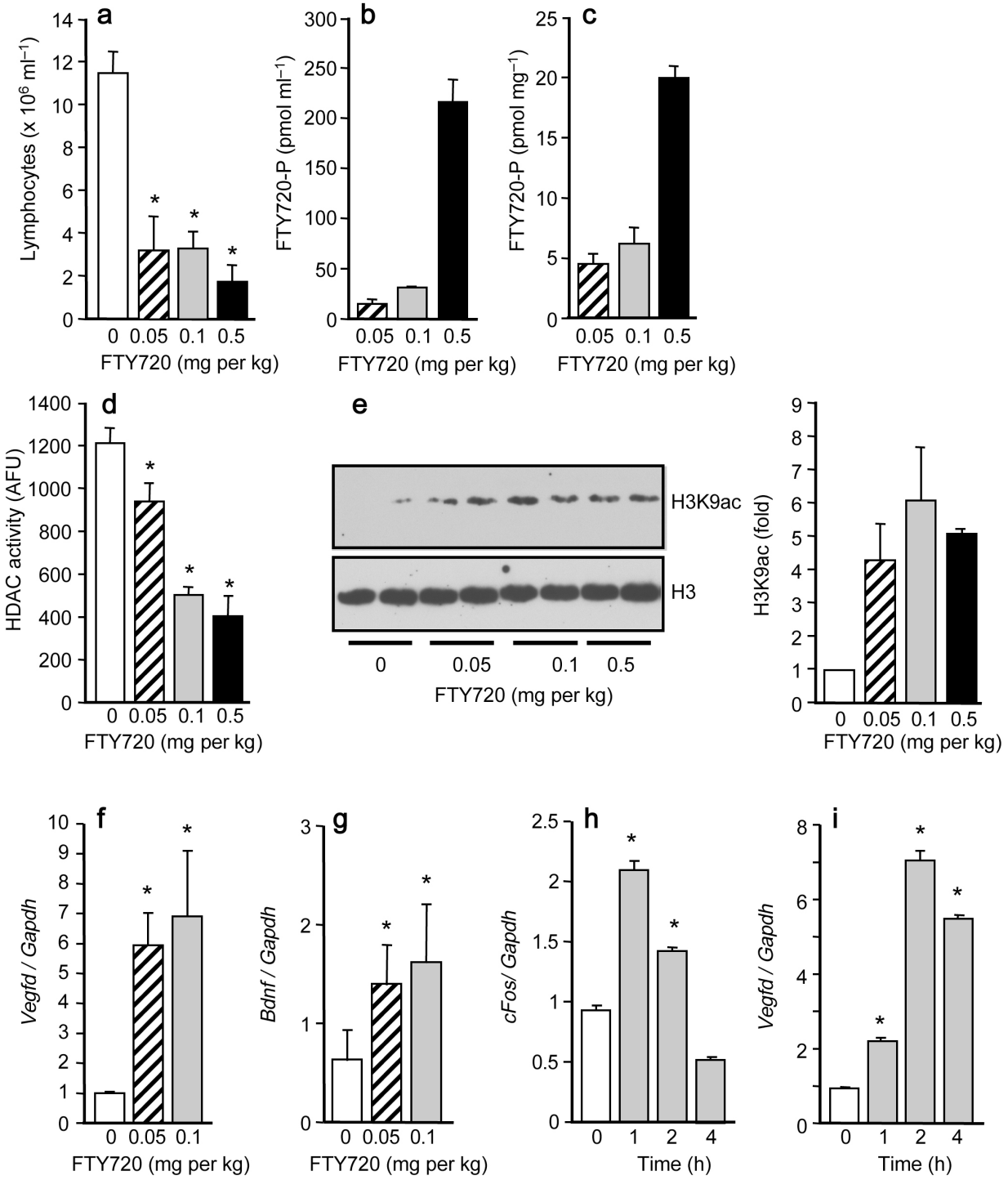

**Supplementary Figure 2. FTY720-P does not affect HAT activity.** HAT activity in nuclear extracts was determined with a colorimetric HAT activity assay in the presence of vehicle, FTY720-P (0.5 or 5 μM) or FTY720 (5 μM). HAT activities are averages of triplicate determinations ± s.d. and expressed as OD at 440 nm per mg.



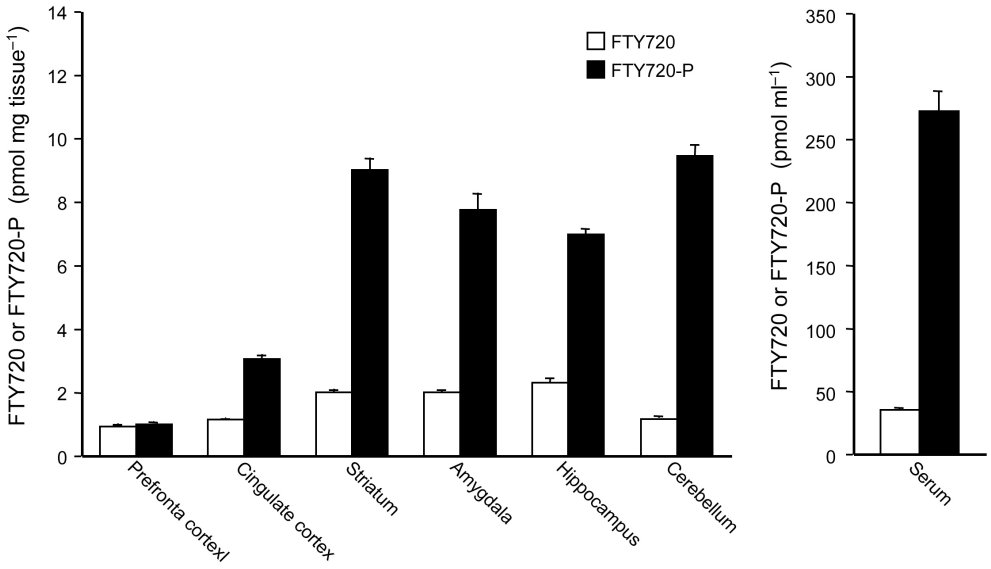

**Supplementary Figure 3. High-affinity binding of S1P and FTY720-P to HDAC1.** Recombinant His-tagged HDAC1 immobilized to Ni-NTA-resin was incubated with [$^{32}$P]S1P (0.1 nM) in the absence or presence of increasing concentrations of unlabeled S1P or FTY720-P. Beads were washed extensively and [$^{32}$P]S1P bound to HDAC1 was eluted with 500 mM imidazole and radioactivity detected by scintillation counting. Data were fit to a one-site Scatchard model using GraphPad Prism. S1P and FTY720-P bind to HDAC1 with apparent Kd values of 7.5 and 6.2 nM, respectively. $R^2$ values for displacement by S1P and FTY720-P were 0.9581 and 0.9610, respectively.



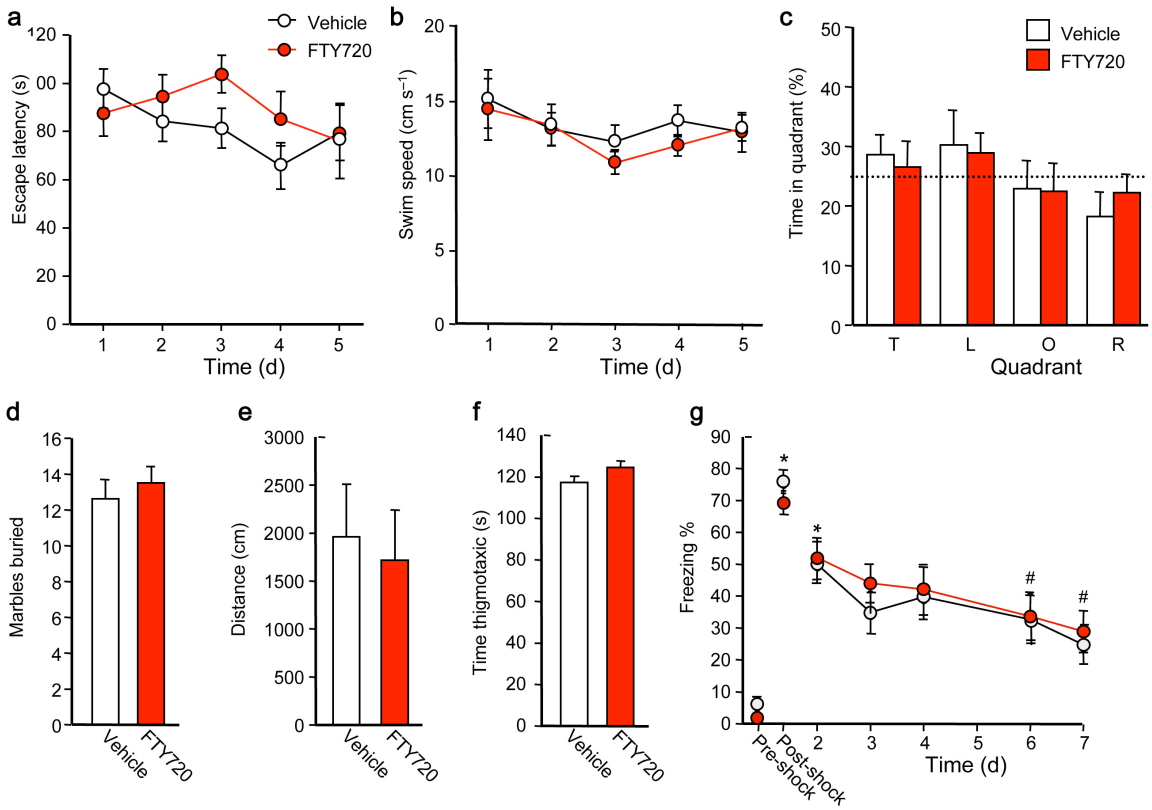

**Supplementary Figure 4. Docking of FTY720-P into the active site of HDAC2.**
**(a)** Binding mode between FTY720-P and the substrate-binding pocket of HADC2. Active site residues that are involved in the interaction with small molecules are shown as sticks and the Zn atom is shown as a sphere. The polar contacts between active site residues, Zn, and small molecules are represented by black dashed lines. **(b)** Schematic representation of the interaction of FTY720-P with HDAC2 calculated by Ligplot. Thatched semi-circles indicate van der Waals contacts between hydrophobic amino acid residues and FTY720-P. Hydrogen bonds are shown as green dashed lines.



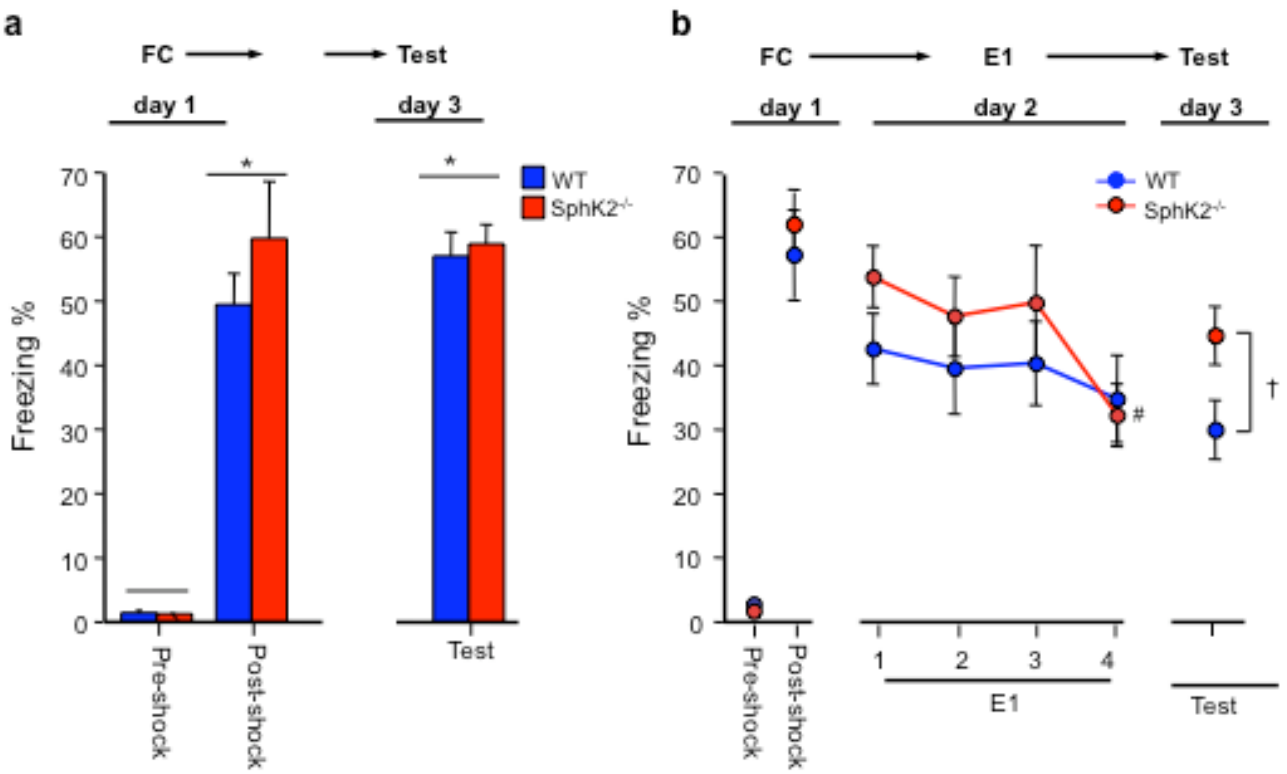

**Supplementary Figure 5. Formation of FTY720-P, inhibition of HDACs, and enhanced histone acetylation and gene expression in the hippocampus following oral administration of FTY720 to C57Bl/6 mice.** (**a-e**) FTY720 was administered by gavage to C57Bl/6 mice at the indicated doses. 24 h later, lymphocytes in blood were counted (a), FTY720-P levels in serum (**b**) and hippocampal nuclei (**c**) measured by LCESI-MS/MS, and hippocampus HDAC activity (**d**) and H3K9 acetylation (**e**) were determined. (**f-i**) QPCR analysis of *Vegfd*, *Bdnf*, and *cFos* expression after treatment of mice with different doses of FTY720 for 24 h (**f,g**) or with 0.1 mg/kg for the indicated times (**h,i**). *P < 0.01, compared to untreated (unpaired two-tailed t test).



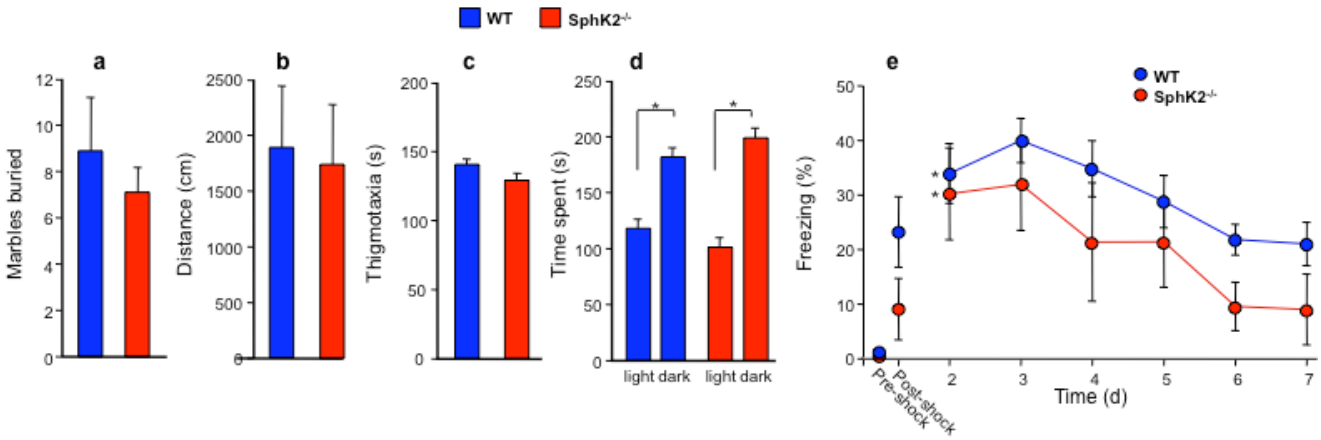

**Supplementary Figure 6. Accumulation of FTY720-P in various brain regions.** SCID mice were treated daily with FTY720 as described in Fig. 5d. Blood was collected and brain regions were isolated and levels of FTY720 and FTY720-P were measured by LC-ESI-MS/MS.
Note: Assuming brain is 75% water, the concentration of FTY720-P in hippocampus for example is ~9 μM compared to 0.27 μM in blood.




**Supplementary Figure 7. FTY720 does not affect performance of SCID mice in the Morris water maze, marble burying, exploratory activity and cued fear conditioning test.** SCID mice were treated orally each day by gavage with saline or FTY720 (1 mg/kg) starting 16 h prior to the **(a-c)** MWM paradigm, **(d-f)** behavioral testing, and **(g)** tone-shock paired conditioned fear testing. **(a-c) FTY720 did not affect the performance of SCID mice in MWM. (a)** Escape latencies (two-way repeated measures ANOVA; interaction: $F(4,60) = 1.43$; $P = 0.23$; day: $F(4,60) = 1.96$; $P = 0.11$; treatment: $F(1,60) = 0.64$; $P = 0.44$) and **(b)** swim speeds (two-way ANOVA; interaction: $F(4,60) = 0.26$; $P = 0.90$; day: $F(4,60) = 1.68$; $P = 0.17$; treatment: $F(1,60) = 0.33$; $P = 0.57$) of FTY720 and saline treated mice did not significantly differ on the five days of fixed platform training. **(c)** The percentage of time spent in the in each quadrant (T, target quadrant; L, left quadrant; O, opposite quadrant; R, right quadrant) during a 120 s probe trial. 24 h after the fifth day of training also did not differ in mice treated with FTY720 or saline (two-way repeated measures ANOVA; interaction: $F(3,45) = 0.10$; $P = 0.96$; quadrant: $F(3,45) = 1.15$; $P = 0.34$; treatment: $F(1,45) = 0.93$; $P = 0.35$). T, target quadrant; L, left quadrant; O, opposite quadrant; R, right quadrant. **(d)** In the marble burying test, SCID mice buried a similar number of marbles regardless of FTY720 treatment (t-test: $t(1,18) = 0.63$; $P = 0.54$). (**e-f**) FTY720 did not affect (e) distance traveled (t-test: $t(1,18) = 0.32$; $P = 0.75$) or (**f**) time thigmotaxic during a 3 min exposure to a novel environment (t-test: $t(1,18) = 1.61$; $P = 0.13$). **(g) SCID mice treated with FTY720 or saline displayed similar freezing and extinction in a tone-shock paired conditioning paradigm**. Data represent percent time freezing during the 3 min before tone-shock pairings (pre-shock), 60 s after tone-shock pairings (post-shock), and during 200 s tone on extinction days. *$P < 0.001$, vs. pre-shock (Bonferroni post hoc test). $^{\#}P < 0.001$, compared to day 2, the first day of extinction (Bonferroni post hoc test). Both treatment groups had similar levels of freezing pre-shock, post-shock, and on extinction day 1 (two-way ANOVA; interaction: $F(2,36) = 0.51$; $P = 0.61$; time: $F(2,36) = 146.54$; $P < 0.0001$; treatment: $F(1,36) = 0.67$; $P = 0.42$) and exhibited similar rates of extinction (two-way repeated measures ANOVA, interaction: $F(4,72) = 0.28$; $P = 0.89$; extinction session: $F(4,72) = 8.33$; $P < 0.0001$; treatment: $F(1,72) = 0.23$; $P = 0.64$). Tests in (d-g) were performed in the same cohort of mice. N = 7-10 mice/group. Data are mean ± s.e.m.

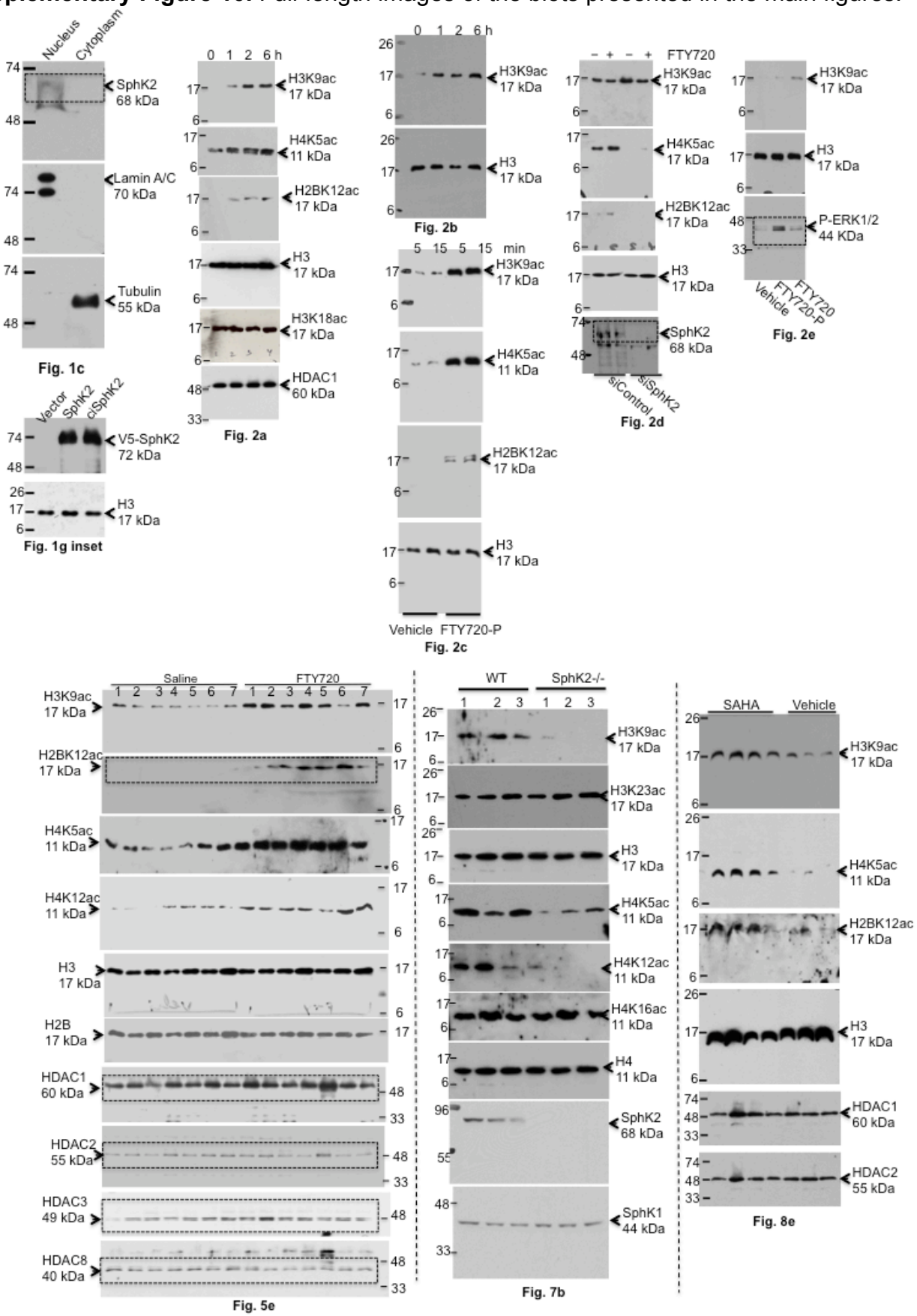



**Supplementary Figure 8. *Sphk2*$^{-/-}$ mice show decreased fear extinction in the contextual fear paradigm.** (**a)** SphK2$^{-/-}$ and WT mice do not forget the association between the context and footshock 48 h after conditioning. Mice were subjected to three footshocks on the conditioning day and received a 2.5 min test 48 h later. SphK2$^{-/-}$ and WT mice exhibited similar levels of freezing pre-shock, post-shock, and 48 h after conditioning (i.e., no extinction sessions) (two-way repeated-measures ANOVA; interaction: $F(2,30) = 0.77$; $P = 0.47$; time: $F(2,30) = 100.95$; $P < 0.0001$; genotype: $F(1,30) = 0.87$; $P = 0.37$). Percent time freezing in both genotypes was significantly increased post-shock and at 48 h compared to pre-shock (*$P < 0.001$, Bonferroni post hoc test). N = 8 for WT; N =9 for SphK2$^{-/-}$ mice. (**b)** SphK2$^{-/-}$ mice display extinction learning deficits in the contextual fear paradigm. SphK2$^{-/-}$ and WT mice were subjected to three footshocks on day 1 and received one extinction session (10 min) 24 h later (E1, consecutive 2.5 min bins are indicated by 1, 2, 3, 4). Mice were then evaluated on day 3 in a 2.5 min test. Both genotypes exhibited similar levels of freezing pre-shock, post-shock, and 24 h after conditioning (two-way repeated-measures ANOVA; interaction: $F(2,32) = 1.44$; $P = 0.26$; time: $F(2,32) = 110$; $P < 0.0001$; genotype: $F(1,32) = 1.87$; $P = 0.19$). Significant increases in freezing were found post-shock ($P < 0.001$) and in the first 2.5 min bin of the extinction session (E1) ($P < 0.001$) as compared with pre-shock levels of freezing (Bonferroni post hoc test). In the 10 min extinction session both genotypes exhibited similar freezing behavior (two-way repeated measures ANOVA; interaction: $F(3,48) = 2.01$; $p = 0.13$); extinction time: $F(3,48) = 6.22$; $P < 0.0012$; genotype: $F(1,48) = 1.6$; $P = 0.22$). $^{\#}P < 0.01$ indicates a significant difference from the first bin of the extinction session for both genotypes (Bonferroni post hoc test), $^{\dagger}P < 0.05$ indicates a significant difference between WT and SphK2$^{-/-}$ mice on test day 3 (Bonferroni post hoc test). N = 9 per group. Data are presented as mean ± s.e.m.




**Supplementary Figure 9. Anxiety-like behavior, exploratory activity, and performance in the cued fear conditioned tests were similar in WT and *Sphk2*$^{-/-}$ mice. (a-c)** WT and SphK2$^{-/-}$ mice show similar responses in the marble-burying test and for exploratory behavior in a novel environment. (**a**) No genotype differences were found for the number of marbles buried (unpaired t-test: t(1,17) = 0.73; P = 0.48), as well as (**b**) for the distance traveled (unpaired t-test: t(1,17) = 0.19; P = 0.86) and (**c**) time spent thigmotaxic in a novel environment (unpaired t-test: t(1,17) = 1.73; P = 0.10). **(d)** WT and SphK2$^{-/-}$ mice were evaluated in the Light/Dark Box test to assess anxiety-like behavior. Both genotypes spent significantly more time in the dark compartment than the light compartment (paired Student's t test: WT: t(1,8) = 3.91; P < 0.01; SphK2$^{-/-}$: t(1,8) = 5.69; P< 0.01), but did not differ between each other in the dark (unpaired t-test: t(1,16) = 1.43; P = 0.17) or light (unpaired Student's t test: t(1,16) = 1.43; P = 0.17) compartments. **(e) WT and SphK2$^{-/-}$ mice displayed similar freezing behavior and extinction in tone-paired conditioning paradigm.** WT and SphK2$^{-/-}$ mice had similar levels of freezing during the 3 min prior to tone-shock pairing (pre-shock), 60 s after tone-shock paring (post-shock), and during 200 s tone presentation without shock 24 h after conditioning (day 2) (two-way repeated measures ANOVA; interaction: F(2,34) = 2.03; P = 0.15; time: F(2,34) = 15.8; P < 0.0001; genotype: F(1,34) = 3.0; P = 0.10). *P < 0.001, vs. pre-shock (Bonferroni post hoc test). Both genotypes exhibited similar decreases in freezing across the six days of extinction training (two-way ANOVA; interaction: F(5,85) = 0.52; P = 0.76; genotype: F(1,85) = 1.52; P = 0.23; test day: F(5,85) = 13.8; P < 0.0001). P < 0.001 for days 6 and 7 as compared to day 2 (Bonferroni post hoc test). Tests in (a-d) were performed in the same cohort of mice. N = 9-10 mice/group. Data are expressed as mean ± s.e.m.

**Supplementary Figure 10.** Full-length images of the blots presented in the main figures.



**Supplementary Table 1 Bioinformatics analysis of memory related genes that were differentially regulated by FTY720 during fear extinction (FDR < 5%).**

| Functions Annotation | p-Value | Regulation z-score | # Molecules | Molecules |
|---|---|---|---|---|
| behavior | 3.22E-10 | -1.163 | 66 | ADCY6,ADORA2A,ATF2,ATG7,B2M,CADPS2,CARTPT,CHRD,CNR1,CRY1,DDC,DNAJC5,DRD2,EGR1,EGR2,FA2H,FEZF2,FOS,FIGF,GABRD,GPD2,GRIA1,GRP,HCN1,HSPA1A/HSPA1B,HTR1A,IDO1,ILF3,KHDRBS1,KIT,LHX8,LPAR1,MAP2,MUT,NCOA1,NETO1,NEUROD1,NOV,NPTX2,NPY,NPY2R,NR4A2,NXPH3,PCP4,PDE1B,PDYN,PENK,PPP1R1B,PRKCG,PTN,RARB,RGS9,SEMA3A,SEZ6,SLC11A2,SLC17A6,SLC1A2,SLC26A4,SNCA,SNRPN,SST,TAC1,TACR3,TRPC5,VIP,ZIC1 |
| cognition | 5.48E-08 | -0.778 | 29 | ADORA2A,CHRD,DRD2,EGR1,FA2H,GABRD,HCN1,HTR1A,KIT,LHX8,NCOA1,NETO1,NPTX2,NPY2R,NR4A2,NXPH3,PCP4,PDE1B,PLP1,PPP1R1B,PRKCG,PTN,RARB,SEZ6,SLC11A2,SNCA,SST,TAC1,TACR3 |
| learning | 6.12E-08 | -0.515 | 27 | CHRD,DRD2,EGR1,FA2H,GABRD,HCN1,HTR1A,KIT,LHX8,NCOA1,NETO1,NPTX2,NPY2R,NR4A2,NXPH3,PCP4,PDE1B,PPP1R1B,PRKCG,PTN,RARB,SEZ6,SLC11A2,SNCA,SST,TAC1,TACR3 |
| emotional behavior | 1.10E-05 | -0.253 | 20 | ADORA2A,B2M,CADPS2,CARTPT,CNR1,DRD2,GABRD,GRIA1,HSPA1A/HSPA1B,HTR1A,KHDRBS1,MAP2,NPY,NPY2R,NR4A2,PDYN,PENK,SLC17A6,TACR3,TRPC5 |
| conditioning | 1.96E-05 | -1.31 | 17 | ADORA2A,CARTPT,CNR1,DRD2,FEZF2,GABRD,GMFB,GRIA1,HCN1,MAP2,NPY,PDYN,PENK,PPP1R1B,PRKCG,SLC17A6,TIMP2 (includes EG:21858) |
| addiction behavior | 5.16E-05 | -0.842 | 6 | ADORA2A,CNR1,DRD2,NR4A2,PENK,PPP1R1B |
| anxiety | 5.86E-05 | 0.106 | 17 | ADORA2A,CADPS2,CARTPT,CNR1,DRD2,FKBP5,GABRD,GRIA1,HTR1A,NPY,NPY2R,PDYN,PENK,PRKCG,RASD2,TAC1,TRPC5 |
| spatial learning | 1.69E-04 | | 15 | DRD2,FA2H,HCN1,HTR1A,KIT,NCOA1,NETO1,NPY2R,NXPH3,PDE1B,PPP1R1B,PRKCG,RARB,SEZ6,TACR3 |
| motor learning | 2.50E-04 | | 5 | FA2H,HCN1,PCP4,PRKCG,SST |
| exploratory behavior | 2.92E-04 | 0.133 | 7 | ADORA2A,CADPS2,CHRD,CNR1,DRD2,GRIA1,PDE1B |
| feeding | 3.72E-04 | -1.174 | 21 | CARTPT,CNR1,DRD2,EGR2,FEZF2,FOS,FIGF,GPD2,GRP,HTR1A,ILF3,LPAR1,MUT,NPY,NPY2R,NR4A2,PDYN,SEMA3A,SNRPN,TACR3,ZIC1 |
| place preference | 4.81E-04 | -0.903 | 6 | CARTPT,CNR1,DRD2,GRIA1,PDYN,PPP1R1B |
| aversive behavior | 2.04E-03 | | 5 | CARTPT,DRD2,NPY,PDYN,SLC17A6 |
| locomotion | 2.25E-03 | -0.142 | 19 | ADORA2A,ATG7,CARTPT,CNP,CNR1,DRD2,FEZF2,GPR37,GRIA1,HSPA1A/HSPA1B,MYO6,NPY2R,NR4A2,PDE1B,PPP1R1B,RARB,RASD2,RELN,SNCA |
| long-term memory | 3.59E-03 | | 6 | DRD2,EGR1,GRIA1,GRP,NPY2R,TAC1 |
| fear | 7.02E-03 | 1.681 | 6 | HTR1A,MAP2,NPY,NPY2R,PENK,TRPC5 |
| memory | 7.38E-03 | -0.771 | 13 | ADORA2A,CADPS2,CNR1,DRD2,EGR1,GABRD,GRIA1,GRP,HTR1A,NETO1,NPY,NPY2R,TAC1 |