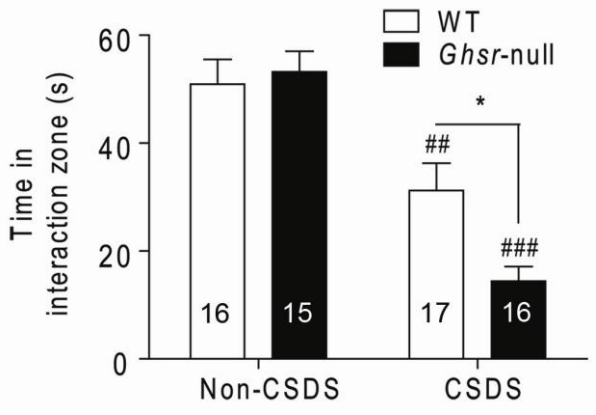
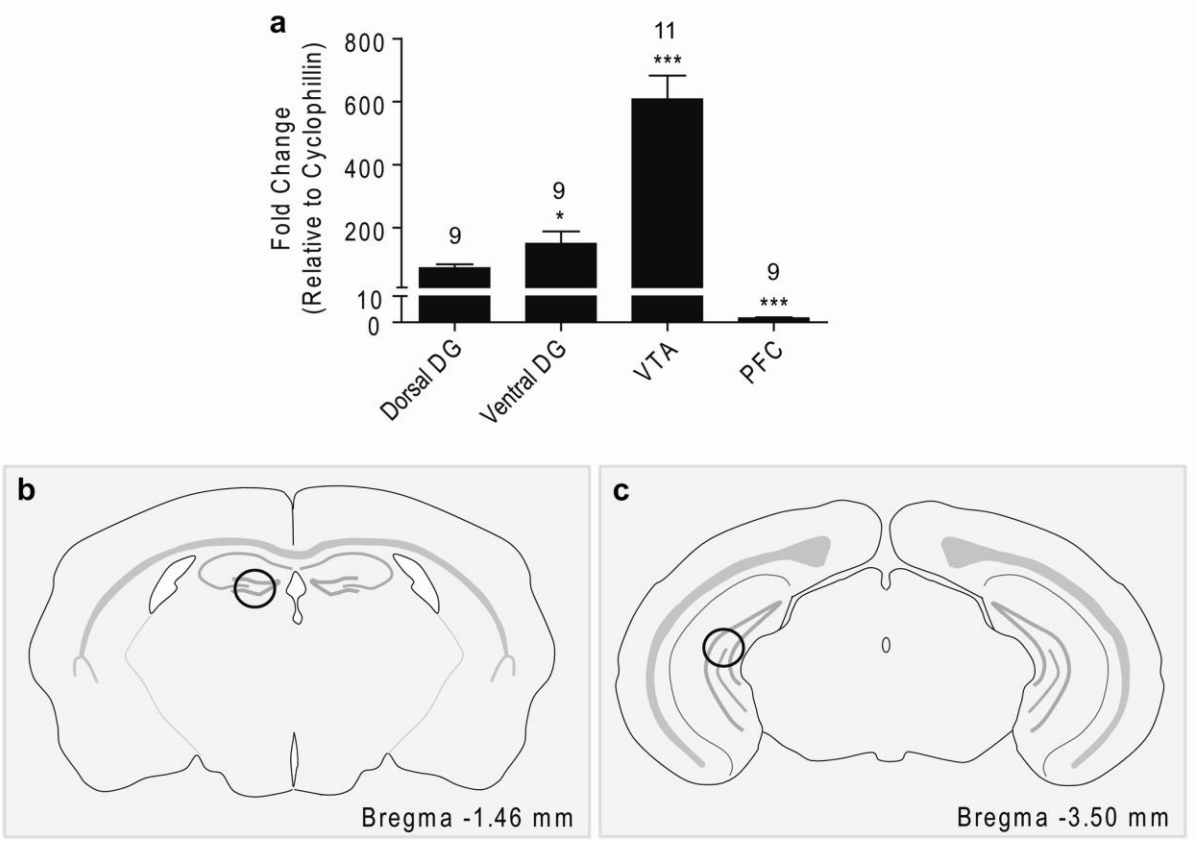
SUPPLEMENTARY MATERIAL

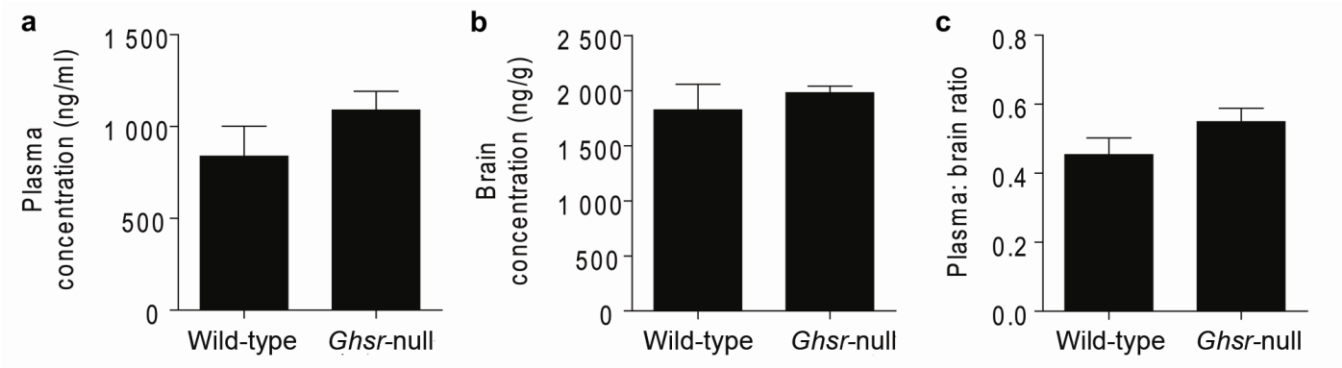
Supplementary Figures



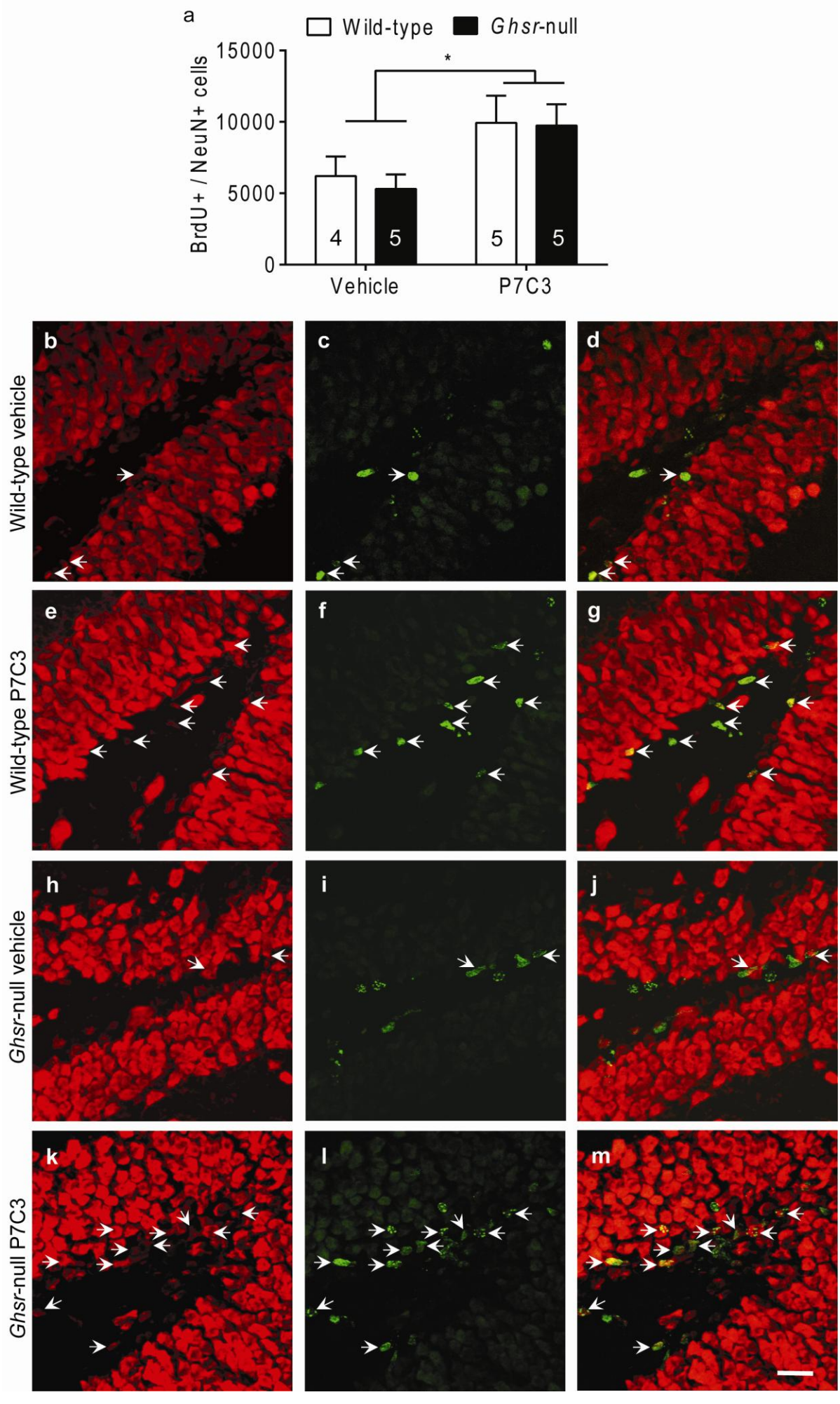
Supplementary Figure 1. Depressive-like behavior in *Ghsr*-null and wild-type littermates. Time spent in the social interaction zone when the target is present in CSDS-exposed and non-CSDS-exposed *Ghsr*-null and wild-type littermates. **P* < 0.05, comparing genotypes. ## *P* < 0.01, ### *P* < 0.001, as compared to non-CSDS controls.



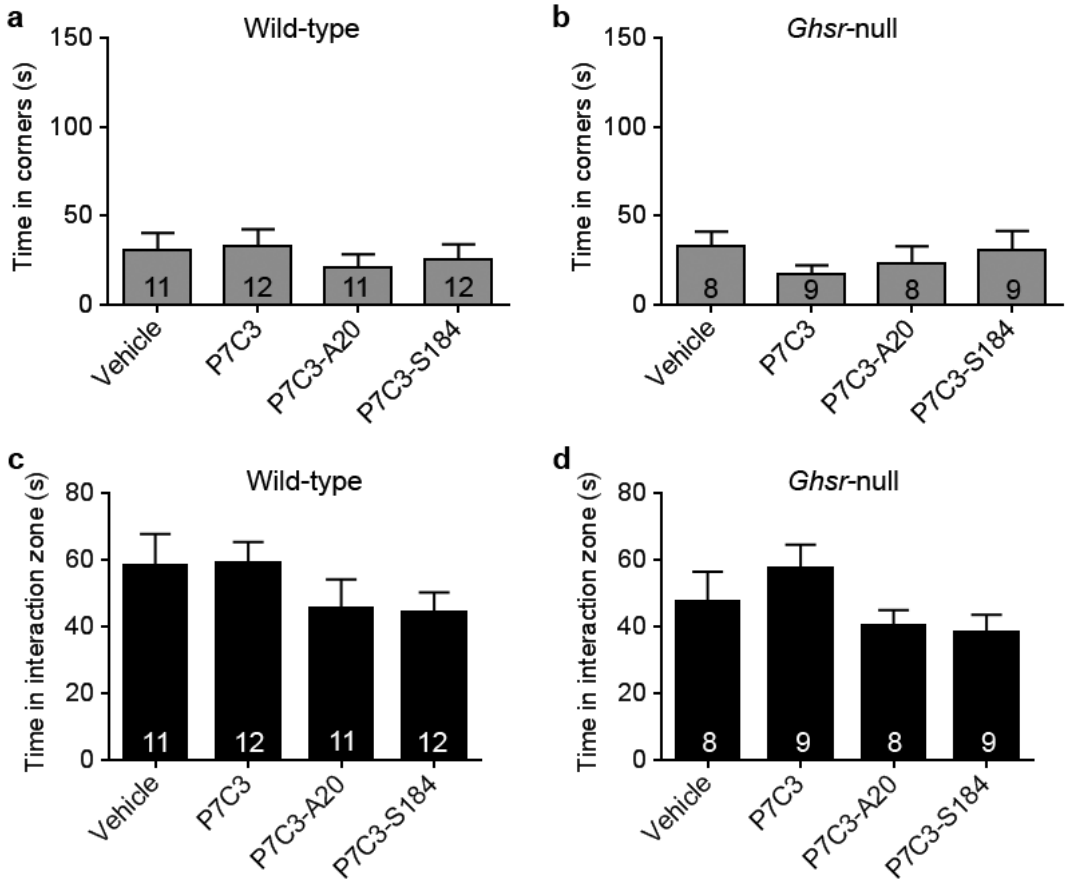
Supplementary Figure 2. Relative *Ghsr* mRNA expression across brain regions in wild-type mice. (a) Relative *Ghsr* mRNA expression in the dorsal DG, ventral DG, ventral tegmental area (VTA), and prefrontal cortex (PFC). (b-c) Image traces of coronal sections representing dorsal (b) and ventral (c) regions where punches were collected for qPCR analysis. Group sizes indicated above bars. $*P < 0.05$, *** $P < 0.001$, as compared to the dorsal DG.



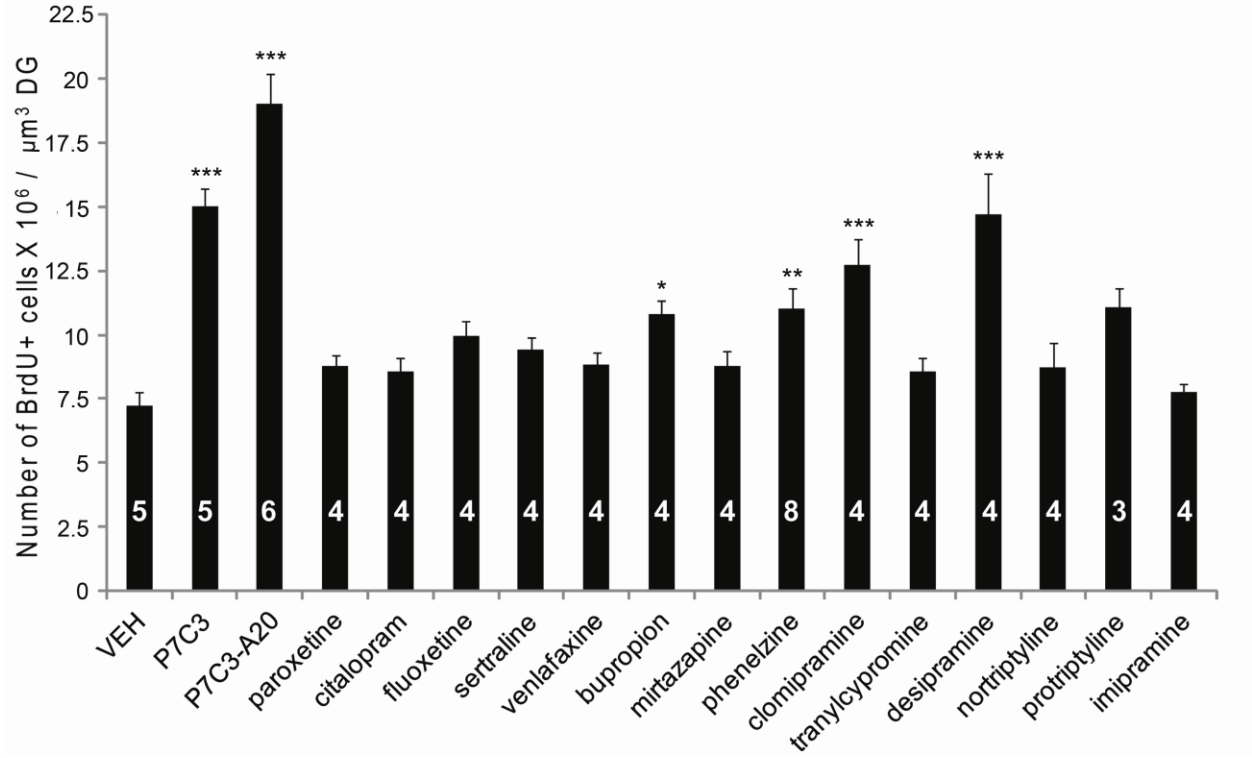
Supplementary Figure 3. P7C3 brain penetration. (a-b) P7C3 levels measured in the plasma (a) or brain (b) of *Ghsr*-null mice and wild-type littermates 6 hrs after final P7C3 injection. (c) Plasma to brain ratio of P7C3 levels in *Ghsr*-null mice and wild-type littermates. (N=3/group)


a
Wild-type
Ghsr-null
BrdU+ / NeuN+ cells
15000
10000
5000
0
*
4
5
5
5
Vehicle
P7C3
b
c
d
e
f
g
h
i
j
k
l
m
Wild-type vehicle
Wild-type P7C3
Ghsr-null vehicle
Ghsr-null P7C3

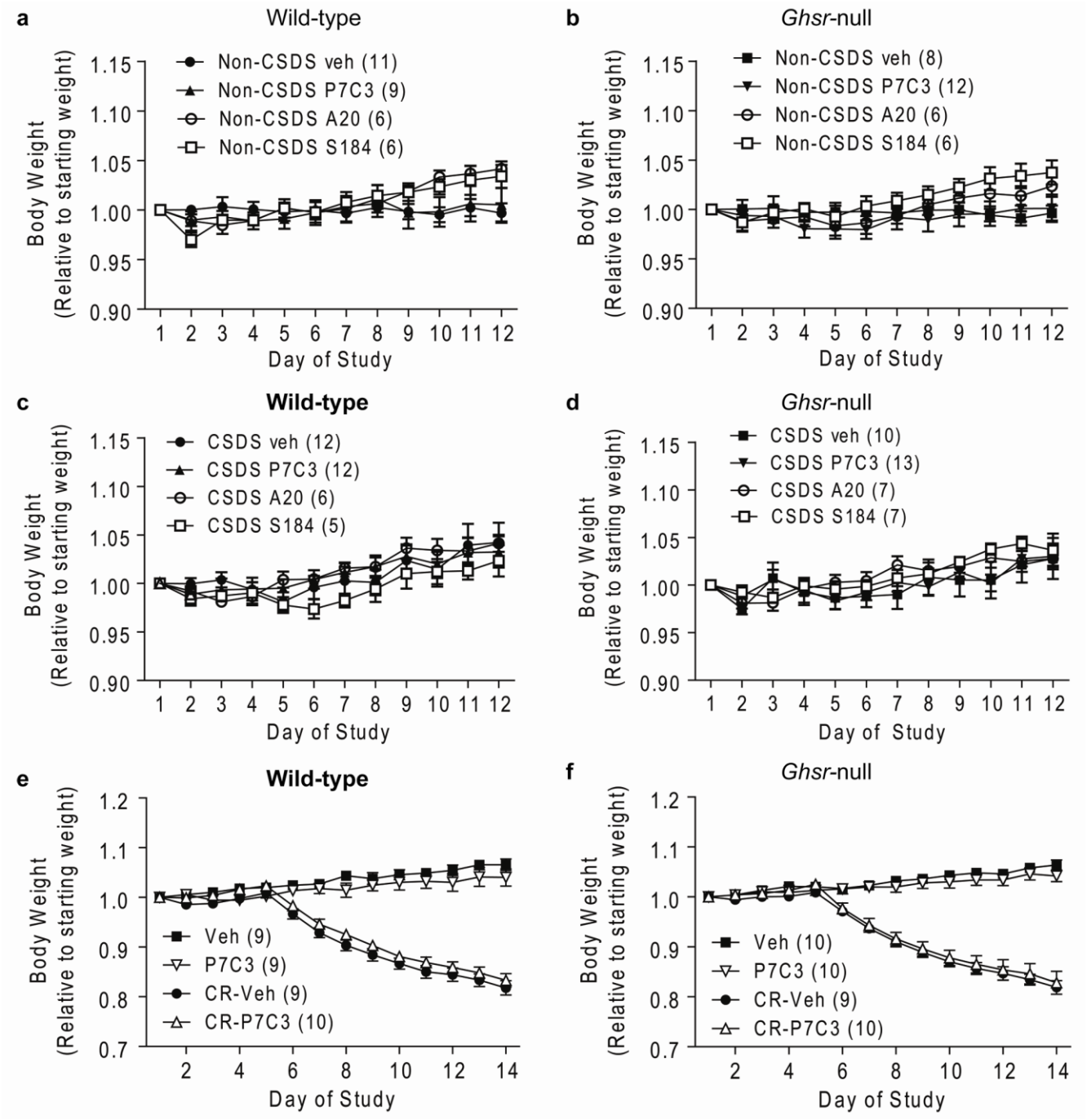
Supplementary Figure 4. Effect of P7C3 administration on mature neuron development. (a) Number of BrdU+/NeuN+ cells in wild-type and *Ghsr*-null mice administered vehicle or P7C3. Group sizes as indicated. (b-m) Representative photomicrograph images of DG sections with NeuN+ cells shown in red (b,e,h,k), BrdU+ cells shown in green (c,f,i,l), and colocalization of BrdU+/NeuN+ cells (d,g,j,m). Representative BrdU+/NeuN+ cells are distinguished by arrows. Scale bar in (e; 20 μm) pertains to (b-e). $*P < 0.05$ effect of compound by two-way ANOVA.



Supplementary Figure 5. Effect of P7C3 compounds on depressive-like behavior in non-CSDS exposed mice. (a-b) Time spent in the corners when the target is present for wild-type (a) and *Ghsr*-null (b) mice. (c-d) Time spent in the social interaction zone when target is present for wild-type (c) and *Ghsr*-null (d) mice. Group sizes as indicated.



Supplementary Figure 6. Proneurogenic efficacy of P7C3 compounds and antidepressants in DG. BrdU+ DG cell counts in wild-type mice receiving infusions of P7C3 compounds or antidepressants into the left ventricle. Group sizes indicated. *$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, as compared to vehicle-treated group.



Supplementary Figure 7. Effect of P7C3 compounds on body weight. (a-d) Relative body weights for non-CSDS-exposed wild-type (a) and *Ghsr*-null (b) mice as well as CSDS-exposed wild-type (c) and *Ghsr*-null (d) mice. (e-f) Relative body weights for calorie-restricted (CR) or *ad libitum*-fed wild-type (e) or *Ghsr*-null (f) mice. Group sizes are indicated within parentheses.

SUPPLEMENTARY ONLINE METHODS

Animal and housing

Male *Ghsr*-null and wild-type littermates on a pure C57/BL/6J genetic background were generated as described previously[1-2]. Mice were housed in a 12-hour-light/dark cycle with ad libitum access to water and regular chow (Teklad Global Diet 16% Protein Diet [2016]; Harlan Teklad, Madison, WI) unless otherwise stated. All procedures were performed according to protocols approved by The University of Texas Southwestern Medical Center Institutional Animal Care and Use of Committee guidelines.

P7C3 compounds

P7C3 was from Asinex (Moscow, Russia) and P7C3-A20 was prepared as described[45].

Behavioral testing

Behavioral tests were performed as described previously[2-3]. Injections of P7C3 compounds were administered at 9 a.m. and 5 p.m. daily, and BrdU was administered at 9 a.m., as per the schedule in the main text and Figures 3a and 5a. All mice were weighed each morning to calculate doses. Body weights were unaffected by administration of the compounds (Supplementary Fig. 7).

Chronic social defeat stress (CSDS)

CSDS was performed similar to methods used previously in the lab[2]. Test mice were housed individually in large cages paired with a CD1 aggressor mouse, separated by a plastic divider with holes so as to allow sensory contact. For 5 min a day, the plastic divider was removed, exposing test mice to the CD1 mouse. After 5 min of exposure to the CD1 mouse, test mice

were again separated and paired with a new CD1 mouse for the remainder of the 24 hours. This process was repeated for 10 days. Control mice were housed in equivalent cages with members of the same strain and handled daily. The social interaction test consists of placing mice in an arena with a small cage at one end. The movements of test mice were tracked for 2.5 min in the absence of another mouse, followed by 2.5 min in the presence of a novel CD1 mouse, or the "target". Ethovision 3.0 software (Noldus, Leesburg, Virginia) measured the duration spent in the interaction zone or in the corners. As done previously, data is presented as time spent in the interaction zone when the target is present or time spent in the corners when the target is present[2].

Focal cranial irradiation

Ionizing radiation was delivered to 8-12 week old mice via the X-RAD 225Cx self-contained irradiation system (Precision X-Ray). It has a custom collimator that delivers a 6 x 14 X-ray beam at a rate of 4 Gy/min (225 kV, 13 mA) for 3.44 min to achieve a cumulative dose of 15 Gy. All irradiated mice were anesthetized with isoflurane (1.5-2.5%) before positioning in the irradiator with the skull located directly under the collimator for cranial irradiation. Mice then received a single exposure of 15 Gy. For sham controls, the mice received isoflurane but no radiation. All animals were monitored post-procedurally for any ill effects and returned to normal housing after being observed to be fully alert and responsive. Four weeks after irradiation or sham treatment, the CSDS protocol was performed on these mice followed by the social interaction test.

Caloric restriction study

The 60% calorie restriction protocol was performed as previously[3]. This protocol, in which mice are provided daily with 60% of their usual daily calories, results in an 18-20% body weight loss in both *Ghsr*-null and wild-type littermates (Supplementary Fig. 7), as observed previously[3]. Mice received twice-daily injections of either P7C3 (20 mg/kg/d in divided doses) or vehicle 5 days prior to and during 10 days of calorie restriction or ad-lib feeding, replicating the injection protocol used for the CSDS studies. On Day 16, the forced swim test was performed for each mouse as done previously[3].

Immunohistochemistry and stereology

Immunohistochemistry and quantification were performed as described previously[2, 4-7]. Mice first were anesthetized and perfused transcardially with formalin (or paraformaldehyde for Fig. 6). Brains were extracted, coronally sectioned into 8 equal series at 25 μm thickness (or 5 equal series at 40 μm thickness for Fig. 6) using a sliding microtome, and then mounted onto Superfrost slides (Fisher Scientific, Richardson, TX), using previously described methods[1-2]. Standard procedures for immunolabeling of slides were employed consisting of antigen retrieval, 1 hr in blocking solution, overnight incubation in primary antibody solution, and 1 hr in appropriate secondary antibody solution(s)[4-6]. Image editing software Adobe Photoshop CS2 (San Jose, CA) was used to adjust brightness and contrast of photomicrographs and prepare figures.

<u>Ki67, AC3, and AC3/BrdU immunolabeling</u>

Primary antibodies included Rabbit-anti-Ki67 (1:1000, Vector Labs, Burlingame,CA), rabbit-anti-AC3 (1:450, Cell Signaling, Beverly, MA), and mouse-anti-BrdU (1:100, Roche Diagnostic,

Mannheim, Germany). For double immunolabeling of AC3 (rabbit-anti-AC3) and BrdU (mouse-anti-BrdU), primary antibodies were incubated simultaneously, and incubation in secondary antibodies occurred separately. Counting of labeled cells was done manually at a magnification of 40X on a Zeiss Axioskop 2 microscope (Carl Zeiss Inc., Thornwood, NY). For each brain, labeled cells were counted in all hippocampal sections from one of the 8 series (or one of the 5 series for Fig. 6). Final quantification of the whole DG of each mouse was obtained by adding labeled cell counts from all sections and multiplying by the number of series. Dorsal DG or ventral DG cell counts were obtained by adding labeled cell counts for either the septal and intermediate regions combined, which comprise the dorsal 2/3 of sections, or the temporal region which comprises the ventral 1/3 of sections [8-10]. The respective totals were then multiplied by the number of series. Photomicrograph images were taken using Axiovision software.

BrdU/NeuN immunolabeling

For double labeling BrdU (rat-anti-BrdU, 1:400, Accurate Chemicals, Westbury, NY) and NeuN (mouse-anti-NeuN, 1:500, Millipore, Billerica, MA), the tissue was incubated with the NeuN primary antibody overnight, followed by a fluorophore-conjugated secondary antibody. Full pretreatment for antigen unmasking was then performed, followed by the standard procedure for BrdU immunolabeling[7].

For proportional analysis, colocalization of signals was determined by scanning and optical sectioning in the Z plane of double-immunofluorescence labeled sections with a Zeiss Axiovert 200/LSM510 confocal microscope (emission wavelengths 488, and 633). 60 – 110 BrdU+/NeuN+ cells per animal were sampled from 3 sections across the longitudinal axis of the hippocampus (-0.82 mm to -4.24 mm from from Bregma), $n \geq 4$ per group) were analyzed. The

total number of BrdU+/NeuN+ cells was calculated by multiplying the total BrdU+ counts (Fig. 3) by the proportion of BrdU+/NeuN+ cells.

Quantitative RT-PCR

Mice were euthanized by live decapitation, and brains were processed with a brain matrix and a 15-guage blunt needle to excise 1-mm thick tissue punches from various sites. RNA was extracted from tissues, processed and reverse-transcribed, and the resulting cDNA was used as template for quantitative PCR, as previously described[2, 11]. Quantitative PCR was performed using iTaq SYBER Green Supermix with Rox (BioRad, Hercules, CA) and previously validated *Ghsr* and *cyclophilin* primer sets in an Applied Biosystems 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), as described[2, 11]. Relative mRNA levels were determined with *cyclophilin* used as the housekeeping gene and calculated with the comparative threshold cycle ($\Delta\Delta$Ct) method[2].

P7C3 brain penetration study

Mice were injected with either P7C3 (10 mg/kg) or vehicle twice every day at 9 am and 5 pm for a total of 19 days. Pharmacokinetic analysis was performed as done previously[4]. Six hours after the last injection of either P7C3 or 0.9% saline (for vehicle treated mice), mice were deeply anesthetized with chloral hydrate (500 mg/kg, i.p.) for blood and brain collection. Blood was collected transcardially using an EDTA-coated syringe, and blood was dispensed into EDTA-coated vials on ice. Whole brain was then removed, weighed, and snap-frozen in liquid nitrogen. Blood was spun down to separate plasma, which was kept at -80°C until analysis was performed.

Brain tissue was homogenized in a 3-fold volume of PBS to prepare lysates. Liquid chromatography and mass spectrometry were used to determine levels of P7C3.

Comparison of P7C3 compounds and antidepressant drugs

Proneurogenic efficacies in the DG of P7C3 and P7C3A20 were compared to those of vehicle (artificial cerebrospinal fluid) and several marketed antidepressant compounds (Sigma-Aldrich, St. Louis, MO) according to established methods[45-47]. In brief, mice were housed individually in cages without running wheels or any form of environmental enrichment, in order to create as low of a baseline level of hippocampal neurogenesis as possible. Compounds (10 μM, i.c.v.) were administered for 7 days via subcutaneously implanted Alzet osmotic minipumps connected to a cannula directed into the left lateral ventricle, with implantation day designated as day 0. Starting on day 1, mice also received daily injections of BrdU (50 mg/kg, i.p.) at 9 am daily, for 6 days. Twenty four hours after the sixth BrdU injection, mice were transcardially perfused as described above.

Statistical analyses

Two-way ANOVAs with Bonferonni post-hoc tests were performed when analyzing the effect of genotype and stress or genotype and injection/stress treatment on social interaction or cell counts for Ki67, AC3, and BrdU. One-way ANOVA with Dunnett's post-hoc test was performed when assessing the effect P7C3 analogs on social interaction, the effect of anti-depressants on BrdU cell number, or the effect of brain region on gene expression. If unequal variance was indicated by Bartlett's test, log transformation was performed prior to statistical analysis. Two-tailed unpaired t-tests were performed when analyzing P7C3 brain penetration data. Significant *p*-

value was defined as $p < 0.05$. GraphPad Prism 5.0 was used for all statistical analysis. Data are presented as mean ± s.e.m.

SUPPLEMENTARY ONLINE REFERENCES

1. Zigman JM, Jones JE, Lee CE, Saper CB, Elmquist JK. Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J Comp Neurol* 2006; **494:** 528-548.

2. Chuang JC, Perello M, Sakata I, Osborne-Lawrence S, Savitt JM, Lutter M, *et al.* Ghrelin mediates stress-induced food-reward behavior in mice. *J Clin Invest* 2011; **121:** 2684-2692.

3. Lutter M, Sakata I, Osborne-Lawrence S, Rovinsky SA, Anderson JG, Jung S, *et al.* The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. *Nature neuroscience* 2008; **11:** 752-753.

4. Pieper AA, Xie S, Capota E, Estill SJ, Zhong J, Long JM, *et al.* Discovery of a proneurogenic, neuroprotective chemical. *Cell* 2010; **142:** 39-51.

5. Malberg JE, Eisch AJ, Nestler EJ, Duman RS. Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2000; **20:** 9104-9110.

6. Walker AK, Park WM, Chuang JC, Perello M, Sakata I, Osborne-Lawrence S, *et al.* Characterization of gastric and neuronal histaminergic populations using a transgenic mouse model. *PLoS One* 2013; **8:** e60276.

7. Ables JL, Decarolis NA, Johnson MA, Rivera PD, Gao Z, Cooper DC, *et al.* Notch1 is required for maintenance of the reservoir of adult hippocampal stem cells. *J Neurosci* 2010; **30:** 10484-10492.

8. Thompson CL, Pathak SD, Jeromin A, Ng LL, MacPherson CR, Mortrud MT, *et al.* Genomic anatomy of the hippocampus. *Neuron* 2008; **60:** 1010-1021.

9. Moser MB, Moser EI. Functional differentiation in the hippocampus. *Hippocampus* 1998; **8:** 608-619.

10. Fanselow MS, Dong HW. Are the dorsal and ventral hippocampus functionally distinct structures? *Neuron* 2010; **65:** 7-19.

11. Walker AK, Ibia IE, Zigman JM. Disruption of cue-potentiated feeding in mice with blocked ghrelin signaling. *Physiol Behav* 2012; **108:** 34-43.