Supplementary Information

Serotonin 1A Receptor-Mediated Signaling Through ERK and PKCα is Essential for Normal Synaptogenesis in Neonatal Mouse Hippocampus

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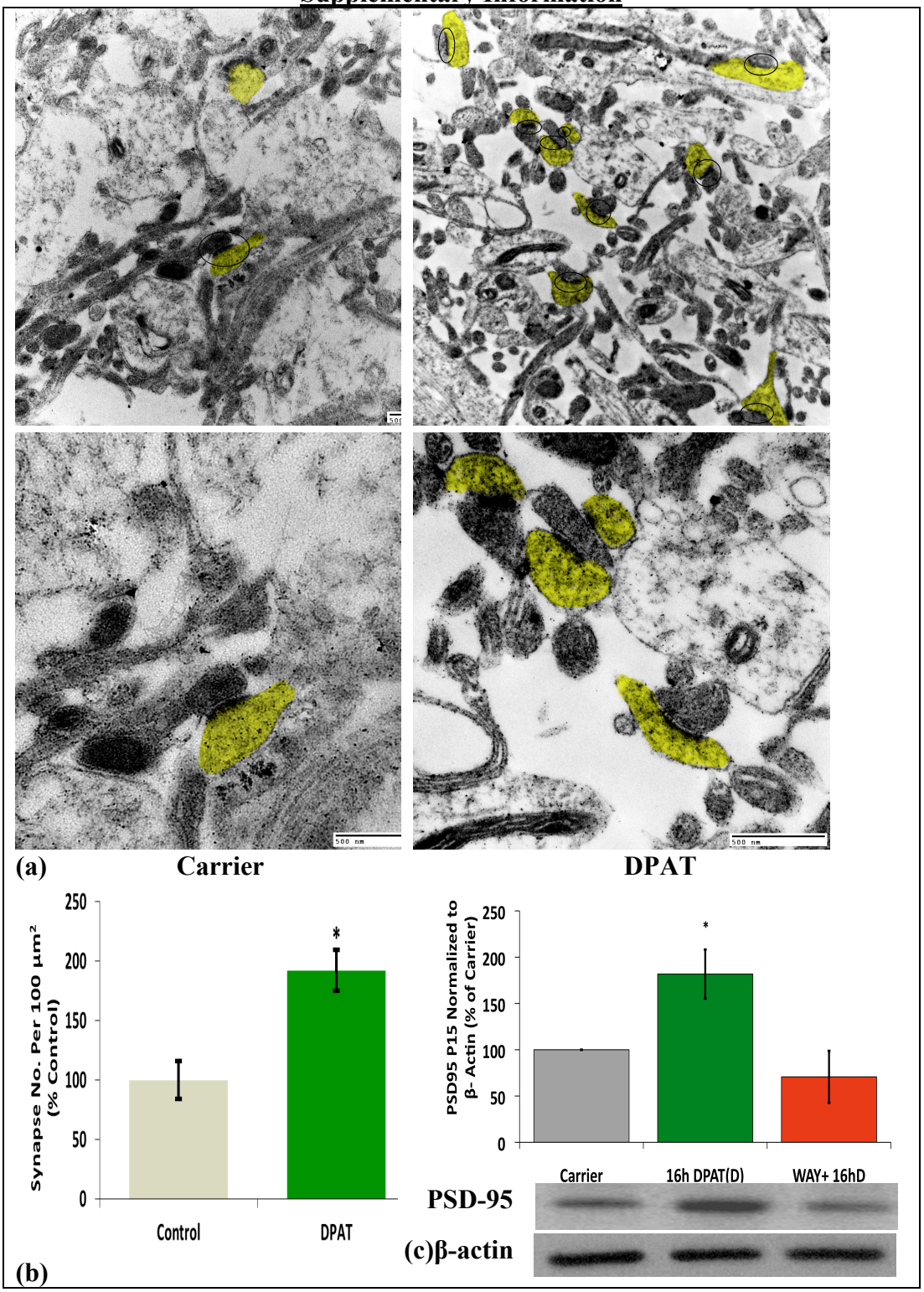
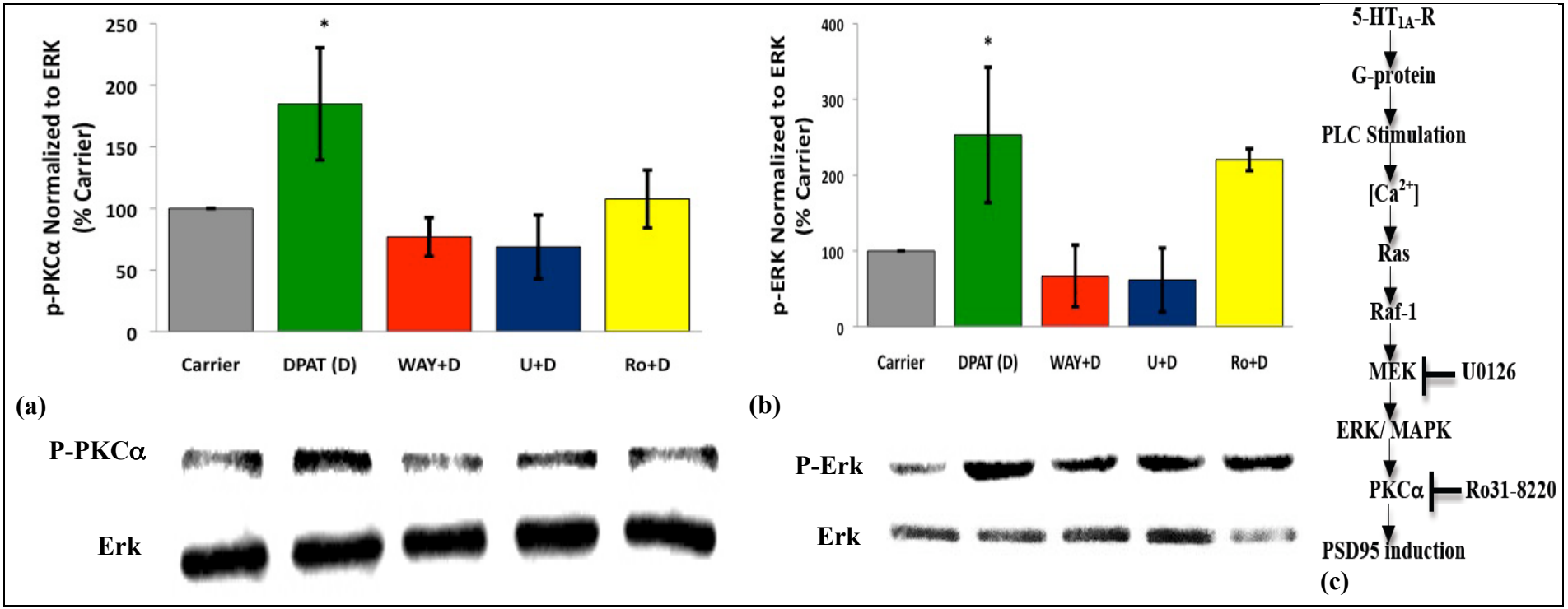
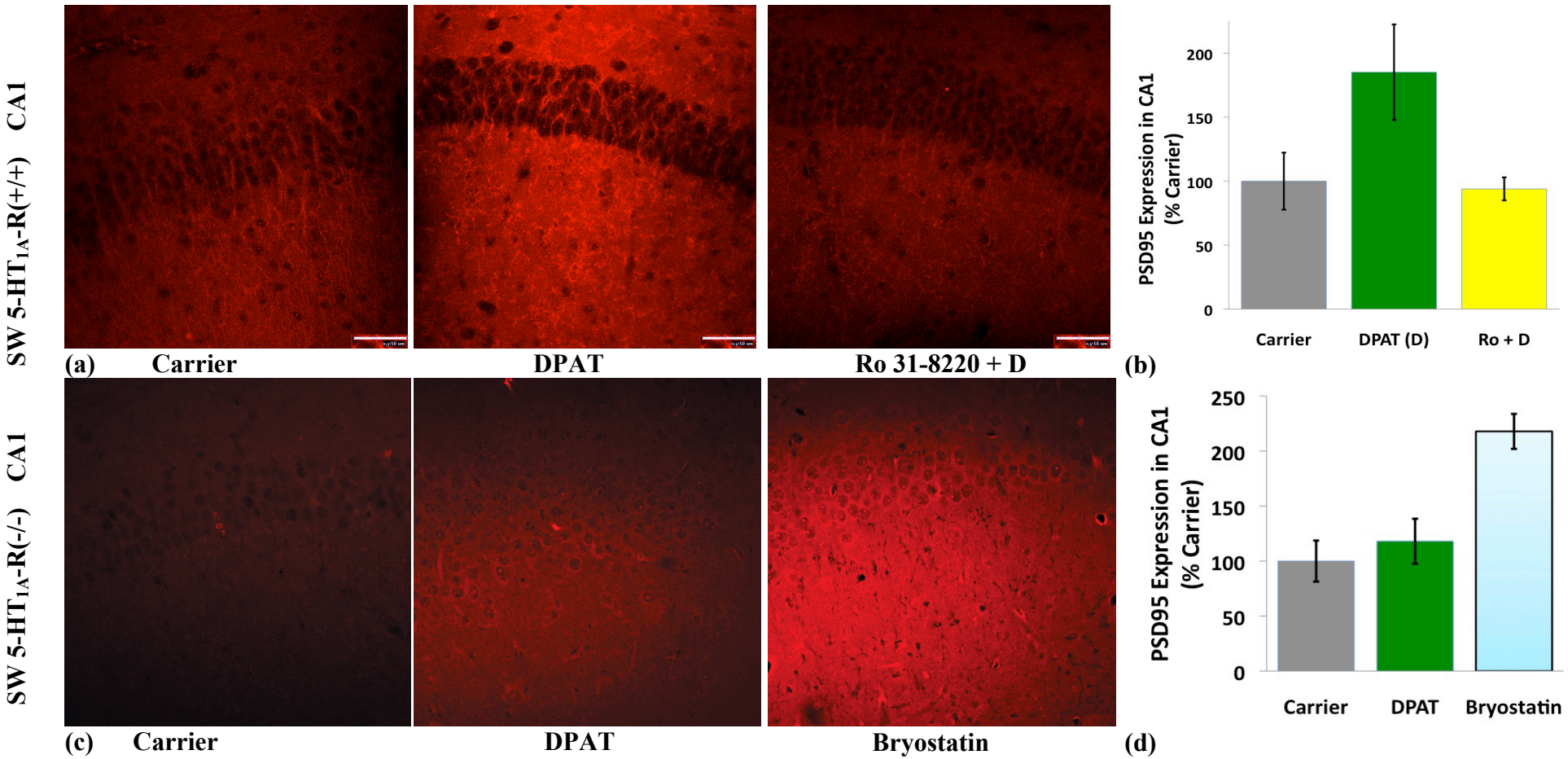


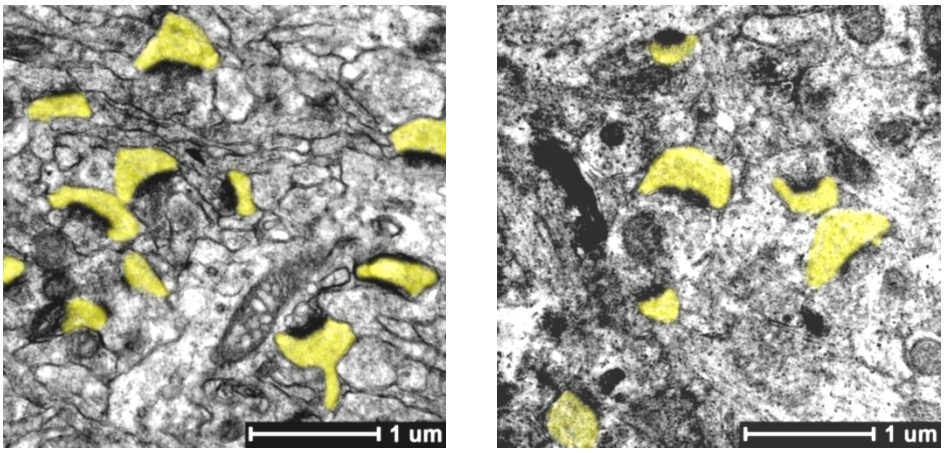
Supplementary Figure 1: Treatment of cultured hippocampal slices from P15 mice with a 5-HT$_{1A}$-R agonist causes increased synaptogenesis and PSD95 expression. Organotypic cultures of P15 mouse hippocampal slices at 6DIV were treated with carrier or 100 nM DPAT for 16 h, following which the slices were fixed and subjected to electron microscopy. **(a)** Synapses with post-synaptic density were counted using three stained sections and four fields of view per section (the post-synaptic terminals highlighted in yellow) (Scale bar: 500 nm). **(b)** Quantification showed a significant increase in the number of synapses following DPAT treatment (*P< 0.001 DPAT vs Carrier). **(c)** Western blot analysis of the treated slices showed a significant increase PSD95 expression following 100-nM DPAT treatment, which was eliminated in the presence of WAY100635 (4 µM) (*P ≤ 0.004 for DPAT vs other sets, obtained from three discrete experiments; n = 3).



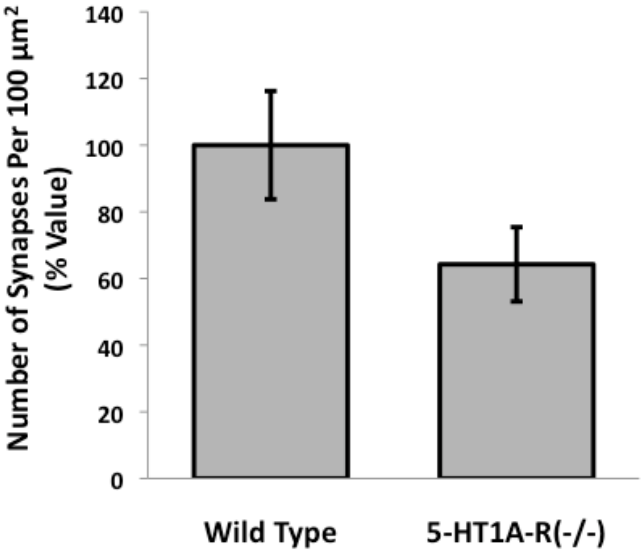
Supplementary Figure 2: Serotonin 1A receptor-mediated sequential stimulation of Erk and PKCα in whole hippocampal tissue from treated mice. **(a)** and **(b)**. The PKCα stimulation is blocked in the presence of both U0126 (inhibits MEK) as well as Ro 31-8220 (Ro) (inhibits only PKCα ≤10 nM), but the activation of Erk is not blocked by Ro 31-8220 (Ro). **(c)** Data presented in (a) and (b) place Erk above PKCα in a signaling pathway, as elucidated in our earlier reports (12, 25). (a, *P< 0.0001 DPAT versus all other groups; n = 3); (b, *P<0.005 DPAT versus Carrier; P< 0.001 DPAT versus WAY100635 and U0126; P=1 DPAT versus Ro 31-8220; n = 3).



Supplementary Figure 3. The 5-HT$_{1A}$-R and PKCα-mediated induction of PSD95 also occurs in SW mice: downstream stimulation of PKC with bryostatin elicits induction of PSD95 in SW 5-HT$_{1A}$-R(-/-) mice. (a) DPAT-infusion caused an increase in PSD95 expression, which was eliminated in the presence of WAY100635 (Scale bar: 50 μm). **(b)** Quantification of volume-rendered images from three experiments revealed that this induction was significant (P< 0.0001 DPAT versus all other groups; n = 3). **(c, d)** In the 5-HT$_{1A}$-R deficient mice, bryostatin but not DPAT infusion caused a significant increase in PSD95 expression in the CA1 region (P< 0.0001 Bryostain versus all other groups; n = 3) (Scale bar 50 μm).

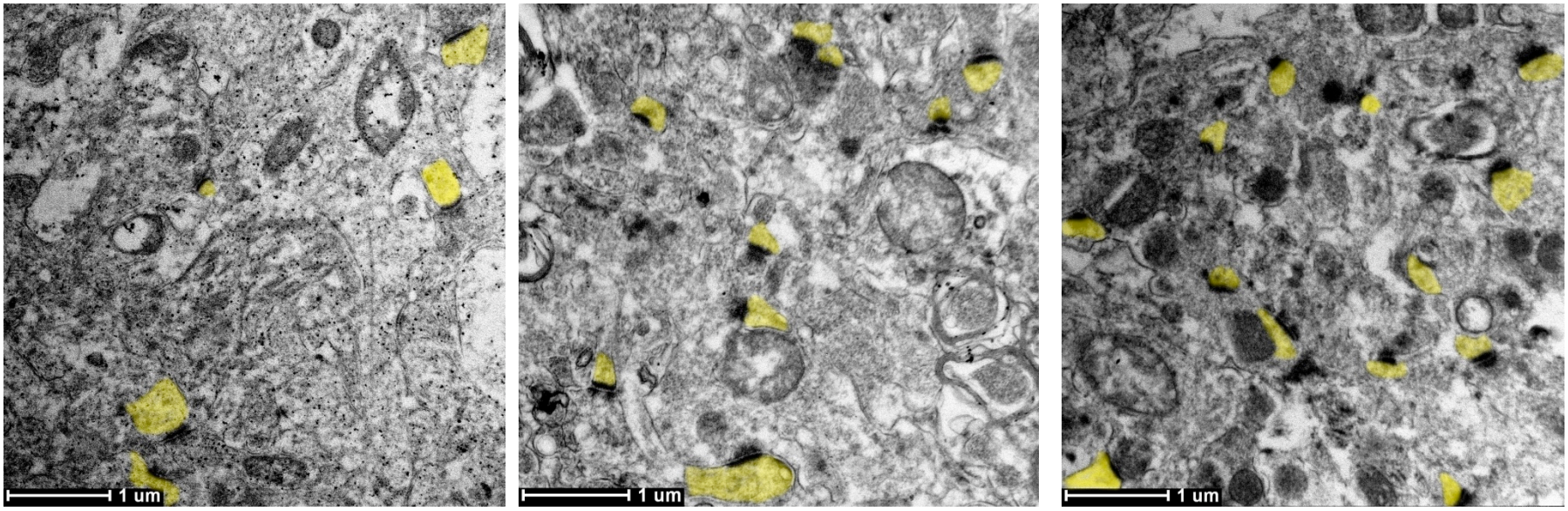


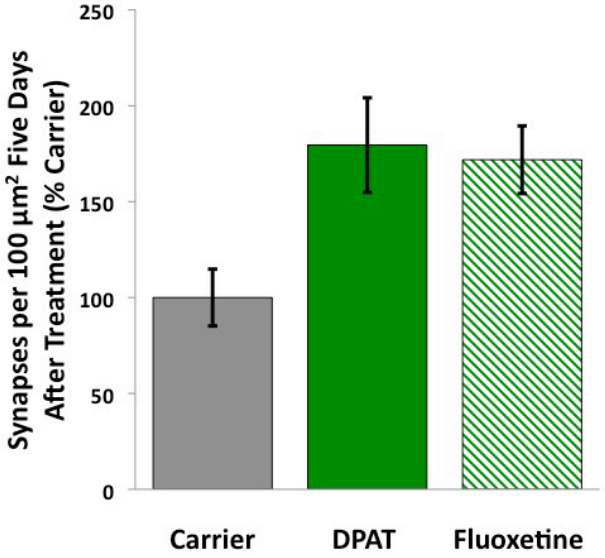
(a) Wild Type (C57BL6) **(b) 5-HT$_{1A}$-R(-/-) (C57BL6)**



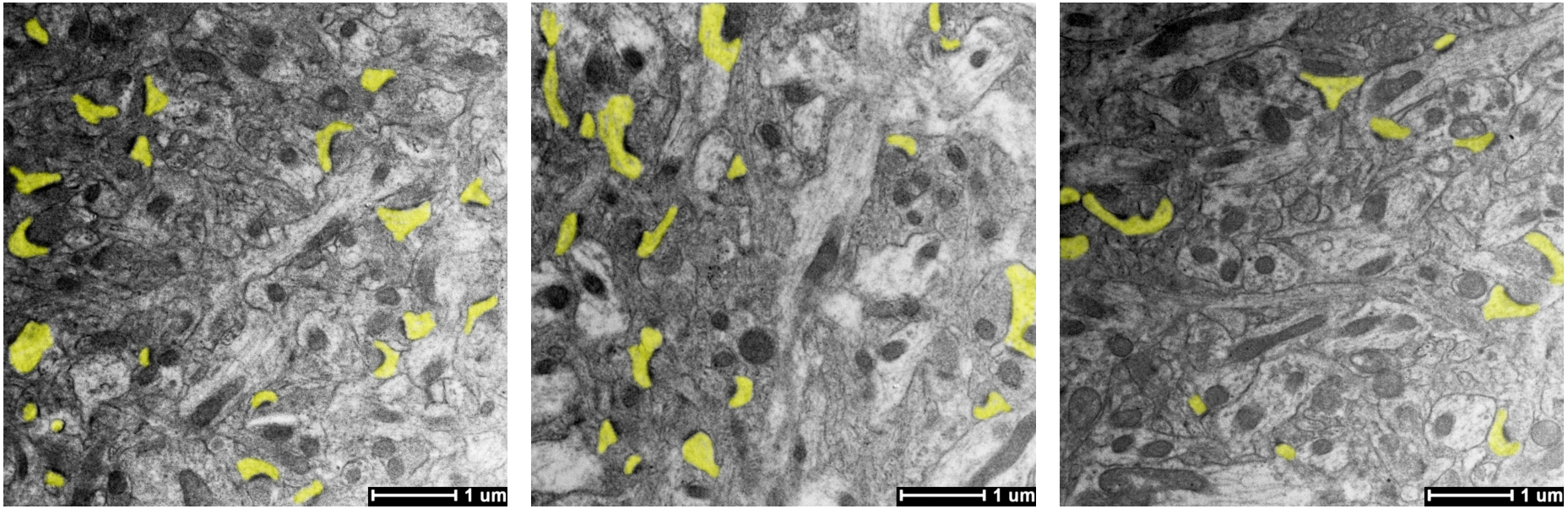
(c)

Supplementary Figure 4. The 5-HT$_{1A}$-R(-/-) mice have less hippocampal synapses at P15. Measurement of the basal levels of excitatory synapses in the CA1 region of the wild type and 5-HT$_{1A}$-R(-/-) C57BL6 mice show a significant decrease in synaptic connections in the knockout mice ($P < 0.0001$; $n = 3$).

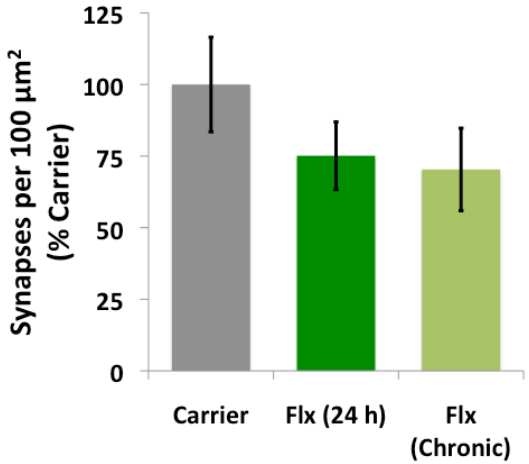




Supplementary Figure 5. Higher synaptic density persists in the CA1 region five days after intrahippocampal 8-OH-DPAT or Fluoxetine treatment at P15. Significantly higher number synapses were observed five days after the infusion of DPAT or Flx ($n = 3$, $P < 0.001$ for both DPAT and Flx, compared to carrier-treated; $n = 3$).



(a) Saline (b) Flx (24 h) (c) Flx (Chronic)



(b)

Supplementary Figure 6. Systemic administration of fluoxetine into P15 mice does not cause an increase in synaptogenesis. Three C57BL6 mice in each group were given i.p. injections of (a) PBS (carrier) once daily for five days (b) a single treatment of Flx or (c) daily treatment of Flx for five days. EM analysis of the hippocampal sections (eight per mouse) revealed a decrease in synaptogenesis in both Flx-treated groups compared to the carrier-treated mice ($P < 0.001$; $n = 3$; Flx dosage = 18 mg/kg body weight).

Supplementary Methods

Reagents: Bryostatin, 8-OH-DPAT, WAY100635, and Ro 31-8820 were purchased from Sigma Chemical Company, St. Louis, MO. Bisindolyl maleimide I (GFX) and U0126 were procured from Calbiochem, San Diego, CA.

Hippocampal Slice Culture:

As described in out earlier report (12), mouse pups of specific ages were anesthetized with ketamine (100 mg/kg) and decapitated. Under sterile conditions, the brains were isolated and then cut at 60^{o} from the longitudinal fissure at the top using a hippocampus-dissecting tool to expose the hippocampus. The hemispheres containing the hippocampi were then placed in modified Gey's balanced salt solution (mGBSS) at 4 °C for 30-40 minutes, while bubbling a mixture of 95% O_2 and 5% CO_2. Individual hippocampi were isolated using dissection tool and then 400 μM thick transverse slices were prepared using a tissue chopper (Stoelting, Wood Dale, IL, USA). The slices were placed in ice-cold mGBSS and inspected using a dissection microscope for the presence of uninterrupted bright transparent neuronal layers characteristic of the hippocampal structure. Only such slices were placed on Millicell CM filters (Millipore, Bedford, MA, USA). The filters were placed in a six well dish with 1ml of medium in each well. The slices were incubated at 32 °C in a 5% CO_2 atmosphere on high K^+ culture medium (25% horse serum, 50% Basal Essential Media- Eagles, 25% Eagle's Balanced Salt Solution (EBSS), 25 mM Na-HEPES, 1mM Glutamine, 28 mM Glucose, pH 7.2) for the first two days. Next, the culture medium was changed to physiological K^+ slice culture medium (20% horse serum, 5% Basal Essential Media-Eagles, and EBSS modified to adjust the K^+ concentration to 2.66 mM). After incubation for two days, the slices were placed in 5% horse serum medium for two days. This was followed by treatment with inhibitors and antagonists for 30 min (overnight for WAY) followed by treatment with agonist for the specific time periods (15 to 60 min for the regulation of phosphorylation and 16 to 24 hours for PSD95 expression and synaptogenesis). The slices were placed in serum free medium for 1 hour before the short-term drug treatment (15 – 60 min) with agonist, The long-term treatments were performed in fresh medium with 5% horse serum. In our high performance liquid chromatography analysis we could not detect serotonin in the heat-inactivated horse serum used in the culture. After drug treatment, the slices were either fixed for the immunohistochemistry or were lysed for western blot analysis.

mGBSS composition (in mM) : $CaCl_2$ (1.5), KCl (4.9), KH_2PO_4 (.02), $MgCl_2$ (11.0), NaCl (138), $NaHCO_3$ (2.7), Na_2HPO_4 (0.8), Na-HEPES (25), glucose 6% (w/v), pH 7.2. The slices were routinely treated with drugs on the sixth day of culture.

Western Blotting:

The drug treated slices were lysed in 1 ml RIPA buffer (PBS containing 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM Na_3VO_4 plus freshly added protease inhibitor cocktail; Roche Diagnostic GmbH, Mannheim, Germany), lysates resolved on a 10% acrylamide gel, protein bands transferred to nitrocellulose membrane, membrane blocked in 5% solution of dry milk dissolved in 0.1% Tween in TBS (20 mM Tris-HCL, pH 7.4, 0.8% NaCl). The membranes were then probed with a PSD-95 antibody (N-18) (Santa Cruz Biotechnology, Santa Cruz, CA) (1:1000) followed by treatment with HRP linked goat anti-mouse IgG (1:40,000). Additionally, antibodies to P-PKCα/ßII ($T^{638/641}$) (#9375) (rabbit polyclonal, Cell Signaling, Danver, MA) and P-p44/42 (P-Erk1/2) (mouse monoclonal) (#9102) (Cell Signaling, Danver, MA) were each used at 1:1000 dilution. HRP-linked goat anti-rabbit secondary antibody was used at 1:50,000 dilution. A mouse monoclonal ß-actin antibody (Sigma, St. Louis, MO) was used at 1:10,000 dilution and the HRP-linked antimouse antibody was used at 50,000 dilution. The immunoreactive bands are visualized using the Supersignal West Pico luminol kit (Thermo Scientific, Rockford, IL) and incubation with an X-ray film. The β-actin bands were used to normalize for protein loading.

Intra-hippocampal Injections: Mouse pups at P15 were anesthetized using a mixture of ketamine (100 mg/Kg) and xylazine (10 mg/Kg) in the ratio recommended by an IACUC protocol. The heads were

shaved, cleaned with 70% ethanol and then, using a sterile scalpel, a midline incision was made and subcutaneous muscle and fascia were retracted to expose the skull. Injections were made at stereotaxic coordinates corresponding to Bregma: anterioposterior (AP) = −1.8 mm, mediolateral (ML) = −1.5 mm, dorsoventral (DV) = −1.8 mm. This corresponds to a site in the dorsal hippocampus in the apical dendritic zones of the CA1 region near the hippocampal fissure (16). Small holes were made in the skull on the right side of pups used for immunohistochemistry or electron microscopy. As the mouse pups were too small for a dental drill, holes were carefully made using a 27-gauge needle. A 10-µl Hamilton syringe was used to deliver the drugs into the hippocampi of the pups (17) at the rate of 1 µl per minute using a stereotaxic set-up (KDS Model 310 plus infusion-withdrawal syringe pump). Before injection, the mouse was ensured to be in deep anesthesia by checking for regular, relaxed respiration and the lack of response to tail/toe pinch. After the injection, the holes in the skull were sealed with sterile bone wax and the skin and muscle were replaced with appropriate 7-mm stainless steel clips (Reflex Skin closure system; Cellpoint Scientific). For wound healing, a topical antibiotic (Triple Antibiotic, containing Polymyxin B, Bacitracin, and Neomycin) were applied and for post-operative care the animal were kept in a warm blanket at 35-37 °C taking precautions to avoid thermal injury. After injection, pups were kept under constant supervision until they came back from anesthesia.

Drug Concentrations and infusion strategy: Drug concentrations were made for the observed hippocampal volume of 10 µl at P15. One-microliter injection was administered for each drug and sham (0.1 M PBS). Stock solutions were made as follows: 8-OH-DPAT: 1 µM stock solution in PBS for the final concentration of 100 nM in the hippocampus. WAY 100635: Infusion of 1 µl of a 100-µM stock solution in PBS 30 min before the injection of 1 µM 8-OH-DPAT (1 µl). This yields the final concentration of 10 µM of WAY and 100 nM 8-OH-DPAT in the hippocampus. U0126: 1 µl of a 1-mM stock solution in DMSO was diluted with 9 µl of PBS to obtain a 100-µM solution. One microliter of this solution was injected into the hippocampus 30 min before the infusion of 1-µM 8-OH-DPAT (1 µl). This yields the final concentrations of 10 µM for U0126 and 100 nM for 8-OH-DPAT in the hippocampus. Ro 31-8220: Infusion of 1 µl of a 100-nM stock solution in PBS 30 min before injection of 1 µl of a 1-µM solution of 8-OH-DPAT (1 µM). This yields the final concentrations of 10 nM for Ro 31-8820 and 100 nM for 8-OH-DPAT in the hippocampus. Bryostatin: Infusion of 1 µl of a 200 nM stock solution in PBS yields the final concentration of 20 nM for bryostatin in the hippocampus. Fluoxetine: 18 mg/Kg, as described earlier (58). Since the density of brain tissue is 1.05 g/ml, which is quite close to that of water, we approximated this value to 18 mg/L, which was about 52 µM. A 520-µM solution in PBS (1 µl) was injected per P15 hippocampus to achieve the final concentration of 52 µM in the whole hippocampus. In the experiment including U0126 treatment, the stock solution of every inhibitor and the carrier (PBS+10% DMSO) contained 10% DMSO. In this experiment, when the stock solution of 8-OH-DPAT was injected alone, it also contained 10% DMSO.

Immunohistochemistry: The drug-treated cultured hippocampal slices were washed quickly with chilled 10 mM Phosphate buffer (PB) and then fixed overnight at 4 °C in 4% PFA. The sections were then removed from the membrane with a brush and placed in a 48-well plate in 0.1 M PBS (PBS). This was followed by 2-3 washes of 15 minutes each with PBS at room temperature. The sections were then treated overnight with blocking buffer (0.1% Triton X-100- 10% serum from the animal used to raise the 2° antibody-in 0.1 M PBS) at 4 °C. This was followed by treatment with a monoclonal PSD95 antibody (N-18) at 1: 200 dilution (Santa Cruz Biotech, Santa Cruz, CA) in 0.1% Triton X-100- 2% serum-in 0.1 M PBS for 48 hours at 4 $^{\circ}$C with gentle rocking. The samples were washed 3 times for 30 minutes each in PBS at room temperature and then treated with fluorescent anti-mouse 2° antibody covalently linked to Alexa Fluor 568 (Red) (1:400) for 24 h. Next, slices were washed 3 times (30 min each) in PBS, blocked again, and then treated with a mouse monoclonal NeuN antibody (MAB377; Millipore, Billerica, MA) (1:150) for 48 h, followed by three washes and treatment with anti-mouse 2° antibody linked to Alexa Fluor 488 (Green)(1:200) for 24 h at 4 °C. Next, the sections were rinsed in PBS (3x) at room temperature and then mounted on slides with ProLong antifade reagent (Molecular Probes, Eugene, OR, USA) for visualization and photography using a laser confocal microscope. In the *in vivo*

studies, the cryo-sectioned slices were washed with PBS three times for 30 min each before blocking over-night and then were treated as explained above.

Confocal Microscopy of the immunostained slices, fluorescence quantification, counting of spine numbers and statistical analysis: The cultured and cryo-sectioned slices were viewed under Nikon C1-LU3 laser scanning confocal system (Nikon Instruments Inc., 1300 Walt Whitman Road, Melville, NY, U.S.A.). We used 488 nm exciting wavelength for NeuN and 568 nm for PSD95, the slices were viewed at different magnification with objective lenses 20x and 40x. All the pictures were taken at 1024x1024 resolution with the frame average of 4. The Nikon EZ-C1-system software was used to determine the total thickness of each slice after adjusting channels to obtain pictures from each exciting wavelength separately. Subsequently, the superimposed images were created from the individual colored images. Quantification of pixel intensity was performed from quadruplicate snapshots acquired at one fixed magnification using "ImageJ" for the measurement of fluorescence pixel intensity of anti-PSD95 staining (18, 23, 59-62). Appropriate secondary antibody controls were used to verify specific staining by each primary antibody. In addition to images taken at 4x and 20x, Z-stacks were also acquired at 40x and such volume-rendered representative images were used in the figures shown in this report. To quantify spine number and the fluorescent intensity of PSD95 immunostaining in each spine (puncta intensity), spines were counted manually and the number per unit length of the dendritic length was calculated. Puncta intensity was obtained by drawing contours around the puncta and then measuring the fluorescence pixel intensity in the enclosed area using ImageJ. All the values were then converted to percent "Carrier-treated". Statistical analysis was carried out using ANOVA with Bonferroni post hoc test.

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