Roles of p75^{NTR}, long-term depression and cholinergic transmission in anxiety and acute stress coping

Supplemental Information

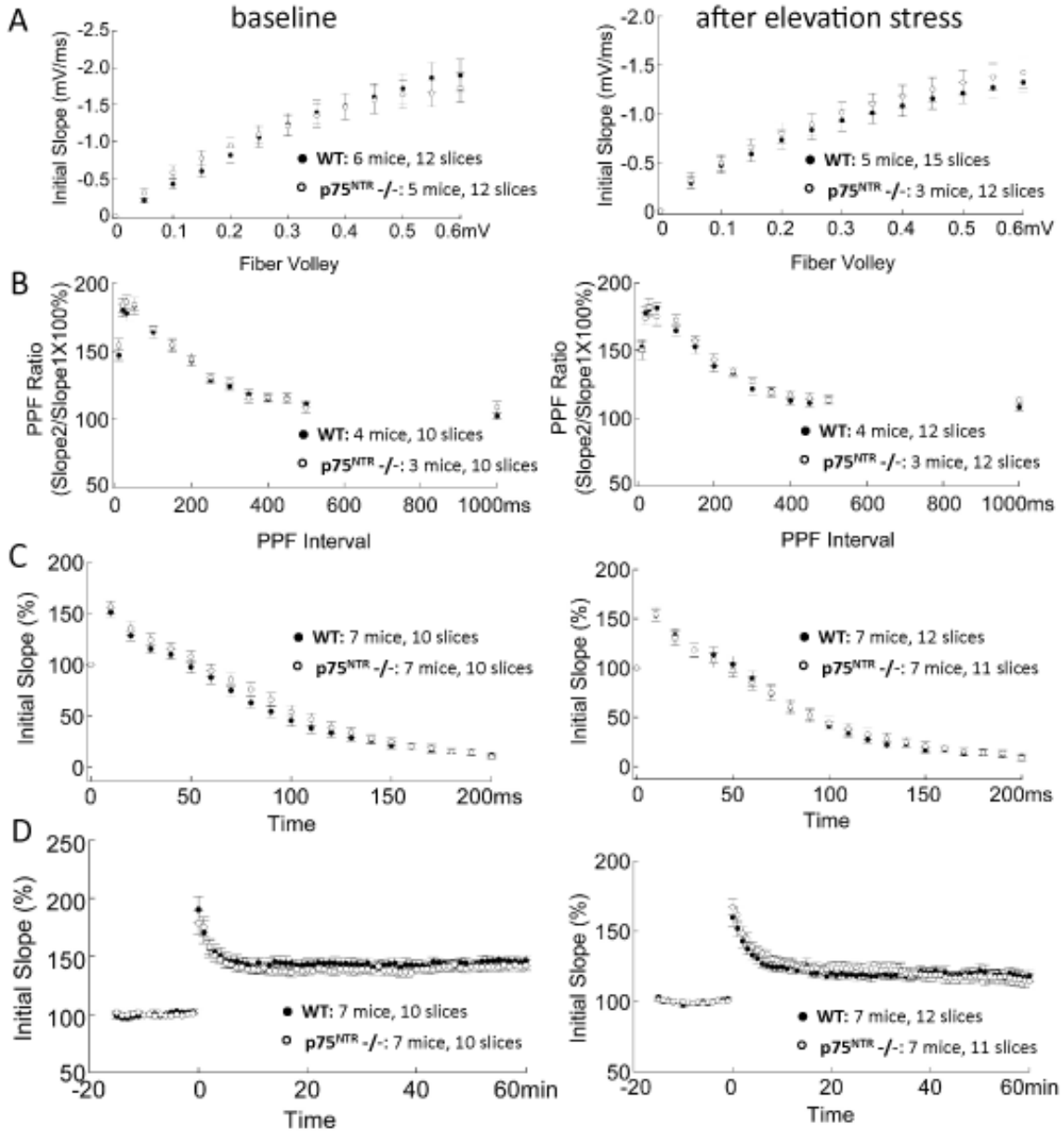

Figure S1. Normal long-term potentiation (LTP) and other synaptic properties in p75^{NTR} -/- mice at baseline and after acute stress. **(A)** Input-output (I-O) curves. Postsynaptic responses (initial slope of field excitatory postsynaptic responses) were plotted as a function of the presynaptic fiber volley amplitude. No difference was found between wild-type (WT) and p75^{NTR} -/- mice at

baseline or following exposure to elevation stress. **(B)** Paired pulse facilitation (PPF). PPF is virtually identical for WT and $p75^{NTR}$ -/- mice over the whole range of inter-stimuli intervals at baseline and following exposure to elevation stress. **(C)** Synaptic fatigue. Synaptic response to a train of 100 Hz stimulation is comparable between WT and $p75^{NTR}$ -/- mice at and after exposure to elevation stress. **(D)** LTP. A standard protocol (1s 100 Hz) was used to induce LTP. Normal LTP was observed in $p75^{NTR}$ -/- mice (142 ± 4% compared to 146 ± 4% in WT) at baseline and after stress (116 ± 5% compared to 118 ± 4% in WT).

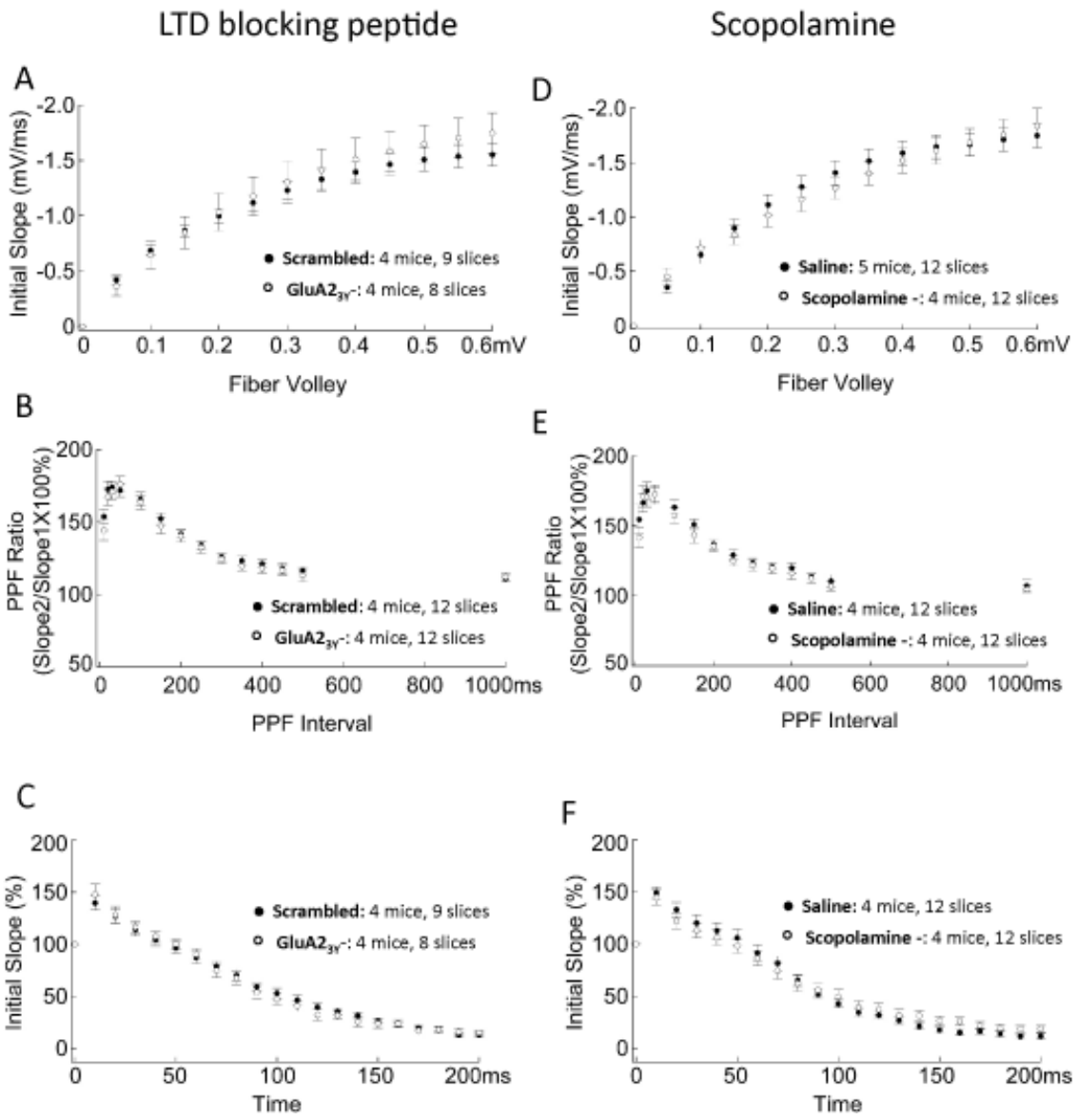

Figure S2. Normal basal synaptic transmission in mice exposed to acute behavioral stress and administered the GluA2$_{3Y}$-Tat peptide or scopolamine. **(A)** Input-output (I-O) curves in mice injected with the long-term depression (LTD)-blocking peptide. No differences in I-O curves were found between mice injected with GluA2$_{3Y}$ versus scrambled peptides. **(B)** Paired pulse facilitation (PPF). PPF is virtually identical for mice injected with GluA2$_{3Y}$ and scrambled peptides over the whole range of interstimulus intervals. **(C)** Synaptic fatigue. Synaptic response to high-frequency stimulation (HFS) is comparable between mice injected with GluA2$_{3Y}$ and scrambled peptides. **(D)** I-O curves in mice injected with scopolamine. No differences in I-O curves were found between mice injected with saline versus scopolamine. **(E)** PPF. PPF is virtually identical for mice injected with scopolamine and saline over the whole range of

interstimulus intervals. **(F)** Synaptic fatigue. Synaptic response to HFS is comparable between mice injected with scopolamine and saline.

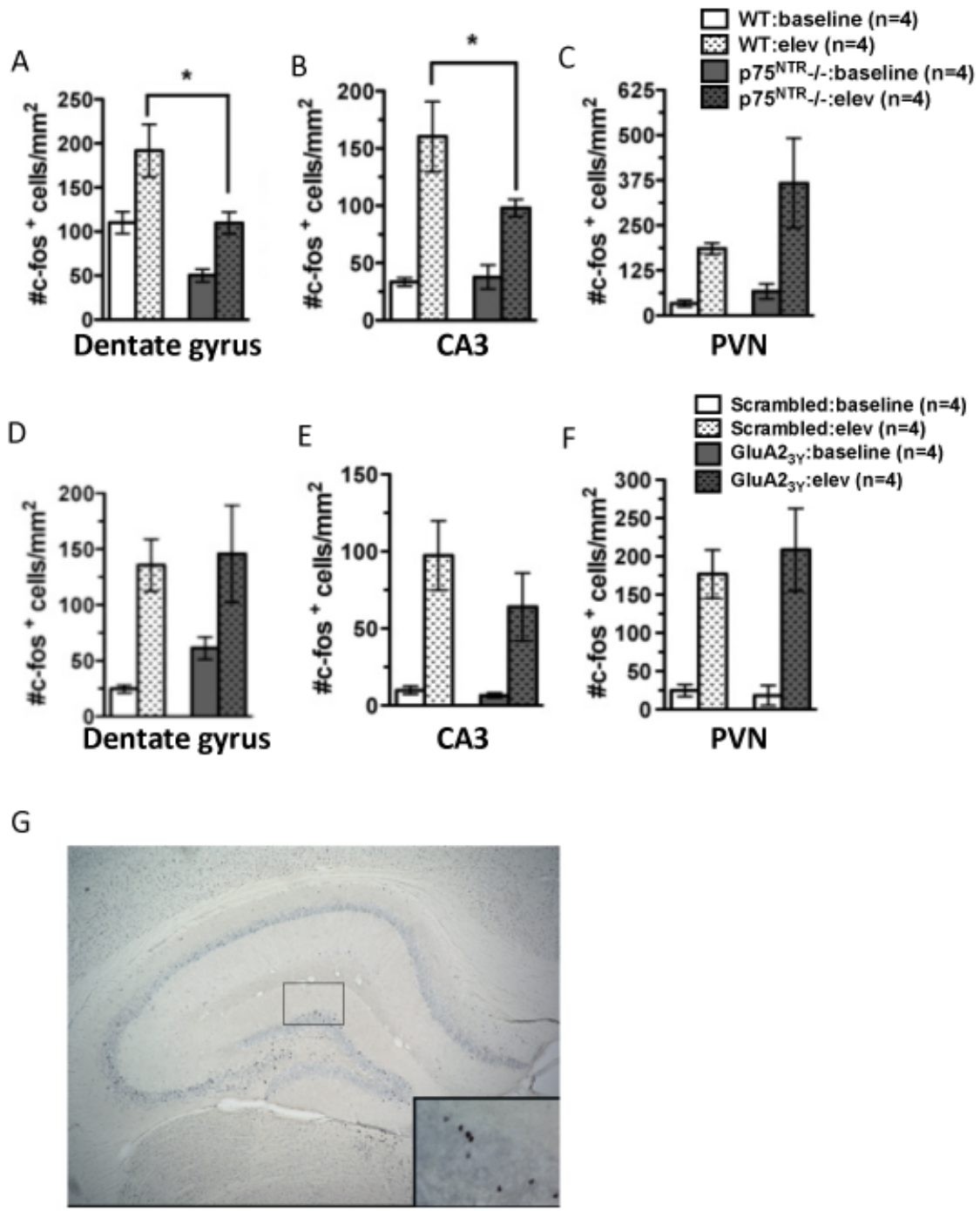

Figure S3. No difference in extent of baseline or acute stress-induced neuronal activation between wild-type (WT) and p75^{NTR}-/- or scrambled and GluA2$_{3Y}$ injected animals. **(A)** The extent of stress-induced neuronal activation is attenuated in p75^{NTR} -/- dentate gyrus. There is a significant increase in labeling of c-fos positive cells in both genotypes following stress exposure, but this effect is attenuated in p75^{NTR} -/-. **(B)** Compared to WT animals, p75^{NTR}-/- show less neuronal activation in hippocampal CA3 following acute stress. **(C)** WT and p75^{NTR} -/- animals

show comparable neuronal activation in the paraventricular nucleus (PVN) of the hypothalamus following acute stress. **(D)** Extent of neuronal activation in the dentate gyrus of animals injected with the $GluA2_{3Y}$ peptide does not differ from those injected with the scrambled peptide following acute stress. **(E)** Extent of neuronal activation in CA3 of animals injected with the $GluA2_{3Y}$ peptide does not differ from those injected with the scrambled peptide following acute stress. **(F)** Animals injected with the $GluA2_{3Y}$ peptide show no difference in neuronal activation in the PVN following acute stress as compared to WT animals. **(G)** Representative c-fos immunohistochemistry in the hippocampus of a WT animal shows elevated levels of c-fos immunolabeled cells after exposures to acute stress.

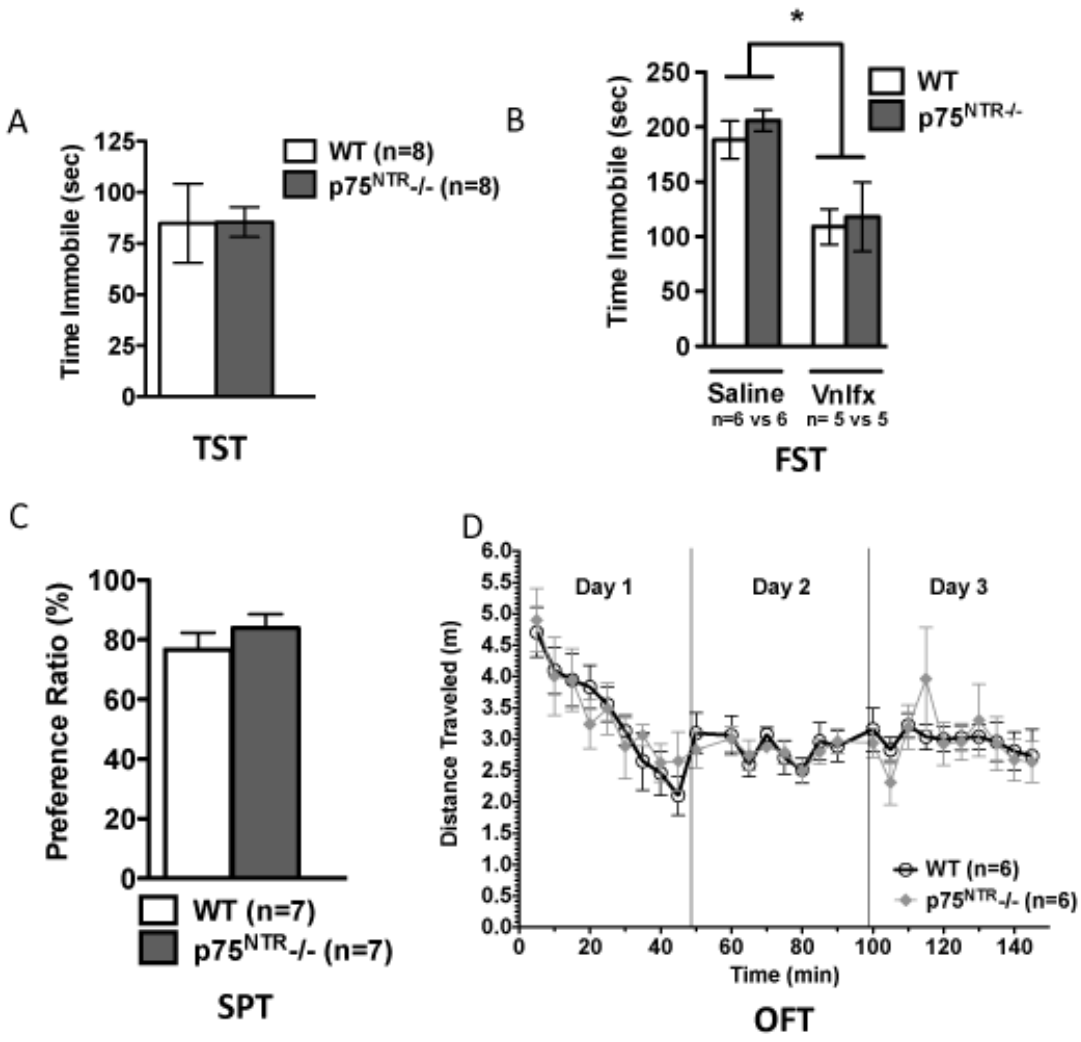

Figure S4. Selective impairment in anxiety-like but not depressive-like behaviors. **(A)** Similar immobile times in the tail suspension test (TST) between wild-type (WT) and p75^{NTR} -/- animals. Animals were hung from their tails with 6 in of laboratory tape for 6 min and the total time immobile was scored (p = 0.975). **(B)** Similar immobile times in the forced swimming test (FST) between WT and p75^{NTR} -/- animals before and after antidepressant administration. WT and p75^{NTR} -/- mice were injected with either saline or venlafaxine (Vnlfx, 16 mg/kg, intraperitoneal) and subjected to the FST (p < 0.004 for Vnlfx treatment, but 0.4987 for genotype). **(C)** Indistinguishable hedonic response between WT and p75^{NTR} -/- animals. WT and p75^{NTR}-/- animals show comparable responses in the sucrose preference test (SPT) (p = 0.3436). **(D)** Both WT and p75^{NTR}-/- animals habituate to an arena in the open field test (OFT) during 45 min of testing on day 1 and show comparable levels of locomotion on two subsequent days of testing (d1: p < 0.0001 for variation over time, p = 0.9348 for variation between genotypes, d2: p

= 0.2213 for variation over time, p = 0.6721 for variation between genotypes, d3: p = 0.063 for variation over time, p = 0.9496 for variation between genotypes), indicating normal locomotor behavior.

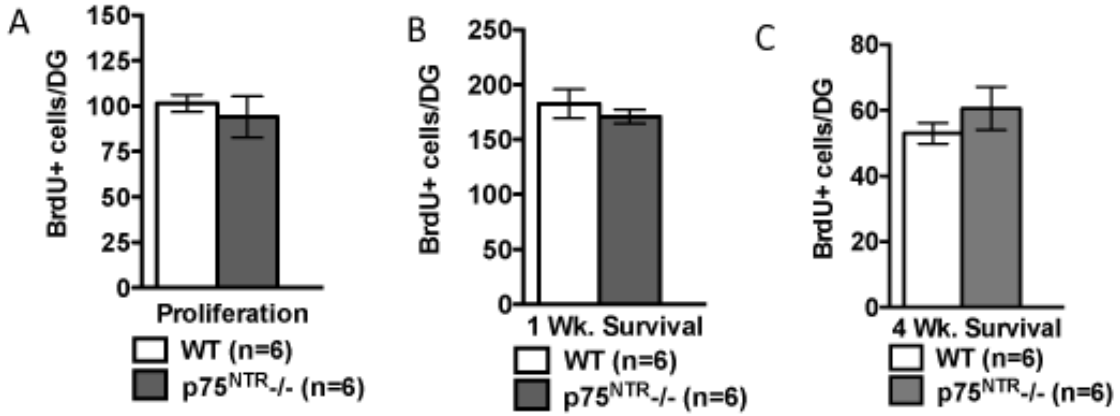

Figure S5. Similar rates of hippocampal neurogenesis in wild-type (WT) and p75^{NTR}-/- animals. WT and p75^{NTR} -/- animals received a single pulse of bromodeoxyuridine (BrdU) before performing stereological cell counting in the dentate gyrus (DG) to determine any difference in levels of neurogenesis. Animals were sacrificed after 2 h to monitor proliferation **(A)**, 1 wk to monitor short-term survival **(B)** and after 4 wks to monitor long-term survival **(C)**. Note no differences in the rate of adult hippocampal neurogenesis between WT and p75^{NTR} -/- animals at any time point (proliferation: p = 0.5531; 1 wk survival: p = 0.4761; 4 wk survival: p = 0.3002).

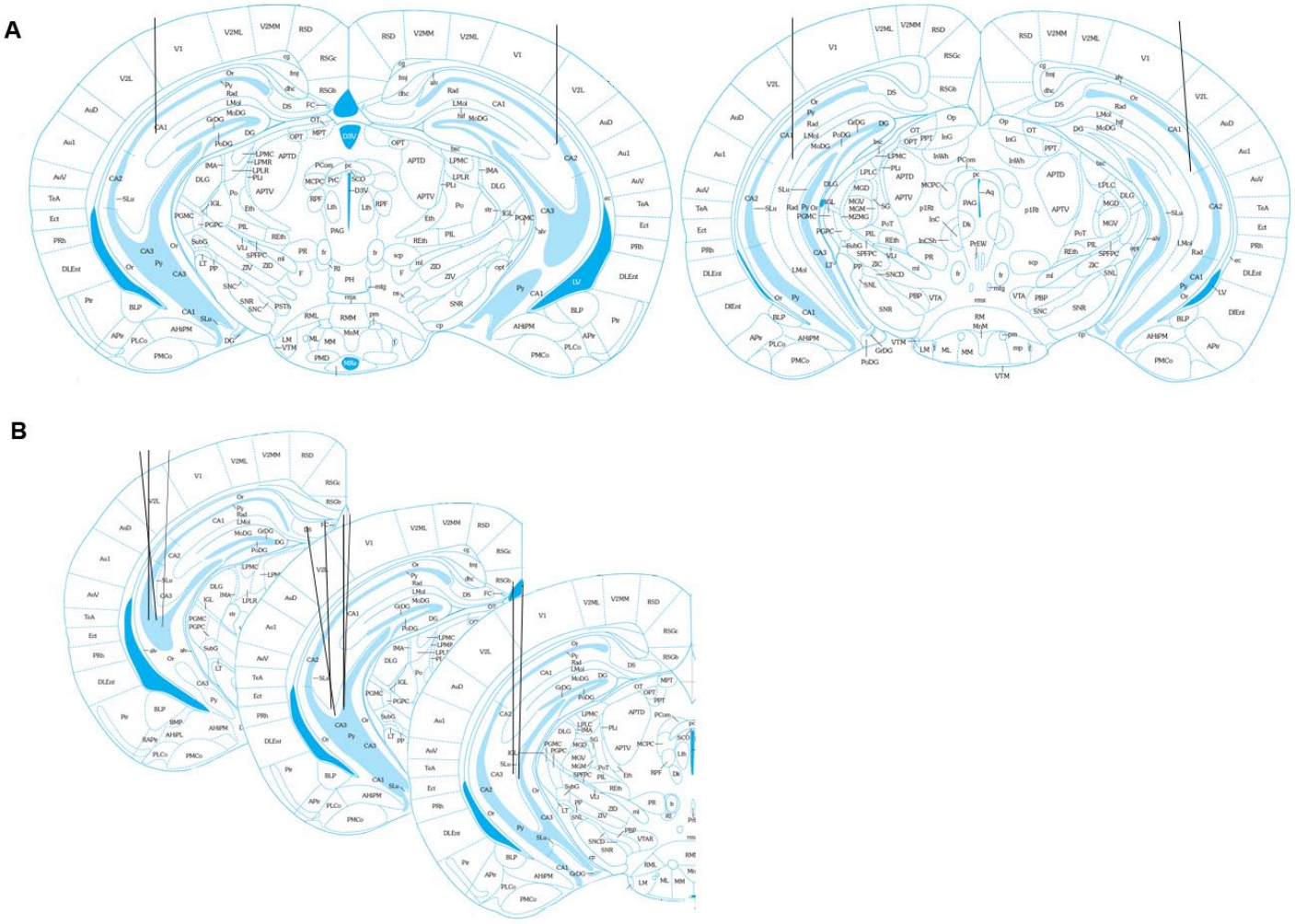

Figure S6. Representative placement of CA1 infusion cannulae and CA3 microdialysis cannulae. **(A)** Drawings depicting where placement of CA1 infusion cannulae were found on Nissl stained sections through the hippocampus of animals with bilateral placement of CA1 infusion cannulae. **(B)** Drawings depicting where placement of CA3 microdialysis cannulae were found by assessment of Nissl stained sections through the hippocampus of animals with unilateral placement of CA3 microdialysis cannulae. Reprinted with permission from Elsevier (Franklin KBJ, Paxinos G (2007): *The Mouse Brain in Stereotaxic Coordinates, 3rd ed.* San Diego, CA: Academic Press).

Supplemental Methods & Materials

Forced Swimming Test

Transparent, Plexiglas cylinders, 25 cm tall X 12 cm diameter, were filled with 30°C water to ~21 cm. Animals were injected (intraperitoneal (i.p.)) with either saline or venlafaxine (gift of Wyeth Pharmaceuticals, Madison, NJ): 16 mg/kg in 0.9% saline, i.p.

30 min prior to testing. Individual mice were placed in the water for a 6 min session, and their behavior was videotaped and later analyzed with Clever Systems Forced Swim Test Scan (CleverSys Inc., Reston, VA). At the end of each session, mice were dried with a paper towel and returned to their home cage. Water was replaced in between each trial.

Tail Suspension Test

Mice were suspended by their tails for 6 min using a 15 cm piece of lab tape wrapped around the tip of the tail. Sessions were videotaped and later scored by an observer blind to genotype and treatment. Any significant movement of the body or the limbs was considered as mobility.

Saccharin Preference Test

Mice were submitted to a 2-bottle choice assay (tap water and a sweetened solution of water containing 50 mg/L of saccharine (Sigma, St. Louis, MO). Bottles were left in the cages for 3 d and their position was switched daily. To ensure that bottles were not leaking, double ball sipper tubes (AnaCare, Potomac, MD) were used. The amount of fluid consumed was measured and the preference for the sweetened solution was calculated.

Social Conflict (SC)

Aggressor CD-1 male mice were single-housed in a large (24.0 cm x 46.0 cm x 15.5 cm) polycarbonate cage (Lab Products Inc., Seaford, DE) for 2–4 wk with bedding incompletely refreshed 1X/wk prior to experiment start. Male wild-type (WT) and $p75^{NTR}$-/- mice as well as mice injected 1.5 h prior to each daily conflict situation with either the scrambled or the $GluR2_{3Y}$ peptide were placed into the resident CD-1 mouse's home cage into which a 1/8-inch thick perforated transparent polycarbonate partition had been placed to separate the pair. Testing commenced after a 2 d accommodation period. The partition was removed for 5 min/d (between 1600 h and 1800 h) to allow for agonistic encounters. After each interaction period, the partition was replaced with the aggressor male returned to his initial compartment. Interaction periods

were videotaped under red light and later analyzed by an unbiased observer for aggressive, submissive, and exploratory behaviors using computer assisted behavioral scoring software (AnnoStar Suite, CleverSys Inc.).

Tissue Preparation

Animals were anaesthetized under isofluorane and transcardially perfused with 50 mL of 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). Brains were removed from the skull and postfixed overnight at 4°C in 4% PFA/PBS, then transferred to 30% sucrose in PBS for 72 h for cryopreservation. Brains were mounted on a freezing stage (Model BFS-MP30, Physitemp Instruments, Inc., Clifton, NJ) set to -25°C and coronal sections (50 μm) were cut using a sliding microtome (Leica, Wetzlar, Germany) and collected in PBS containing 0.015 M sodium azide.

Immunohistochemistry

Every 6th (frontal cortex) or 12th (hypothalamus/hippocampus) section was rinsed free-floating in PBS containing 0.5% Tween-20. Non-specific binding was blocked by incubating tissue for 30 min with 3% normal goat serum. Sections were then incubated with an anti-c-fos antibody (PC38, Calbiochem, San Diego, CA) for 24 h at 4°C. Sections were then rinsed in PBS/0.5% Tween-20, incubated for 2 h at room temperature with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA), and rinsed again with PBS. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide for 30 min. The HRP-DAB reaction was carried out using an avidin/biotin peroxidase complex (VectaStain ABC Kit, Vector Laboratories). Sections were incubated in ABC for 1 h and DAB-cobalt (Sigma Aldrich) for 3 min. They were then mounted on SuperFrost-plus treated slides (Fisher Scientific, Pittsburgh, PA), air-dried, Nissl stained, dehydrated with alcohol rinses, cleared with CitriSolv, and finally coverslipped with Permount.

Adult Hippocampal Neurogenesis

Immunohistochemistry and stereological cell counting were performed to examine adult hippocampal neurogenesis in p75^{NTR}-/- mice. Eight wk old animals received a single injection of the thymidine analogue Bromodeoxyuridine (BrdU, 200 mg/kg, Sigma) and were perfused either 2 h, 1 wk or 4 wks thereafter. Seven-8 sections 480 μm apart through the whole hippocampus were stained with an antibody against BrdU using immunohistochemical methods described

below and BrdU positive cell nuclei in the hippocampal dentate gyrus were counted by an observer blind to the genotype.

In Vitro Slice Preparation and Electrophysiological Recording

Brains were rapidly removed and immersed in ice-cold slicing buffer (in mM: 127 NaCl, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.9 KCl, 1.1 $CaCl_2$, 2 $MgSO_4$, 10 D-Glucose) bubbled with 95% O_2 and 5% CO_2. Transverse slices (350 μM) were prepared with a vibrating microtome (NVSLM1, World Precision Instruments Inc., Sarasota, FL), kept in oxygenated artificial cerebrospinal fluid (ACSF, in mM: 127 NaCl, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.9 KCl, 2.2 $CaCl_2$, 1 $MgSO_4$, 10 D-Glucose) for 30 min at 34°C and another 30 min at 22°C, and transferred to a submersion recording chamber continually perfused with 32°C oxygenated ACSF (rate: 2 ml/min). For long-term depression (LTD) recordings only bicuculline methiodide (10 uM) was added to the recording chamber to block $GABA_A$ receptor-mediated inhibitory synaptic currents. Slices were equilibrated for at least 15 min before recording. ACSF-filled glass electrodes (resistance <1 MΩ) were positioned in the stratum radiatum of CA1 for extracellular recording. Synaptic responses were evoked by stimulating Schaffer collaterals with 0.2 ms pulses with a bipolar tungsten electrode (World Precision Instruments Inc.) once every 15 s. The stimulation intensity was systematically increased to determine the maximal field excitatory postsynaptic responses (fEPSP) slope and then adjusted to yield 40-60% of the maximal fEPSP slope. Experiments with maximal fEPSPs of less than 0.5 mV or with substantial changes in the fiber volley were rejected. After recording of a stable baseline for 15-20 min, long-term potentiation (LTP) was induced by one 1 s/100Hz stimulus train, and LTD was induced by 900 pulses delivered with a frequency of 1 Hz.

Field EPSPs were recorded (AxoClamp 2B amplifier, Axon Instruments, Ontario, Canada), filtered at 1 kHz, digitized at 10 kHz (Axon Digidata 1322A), and stored for off-line analysis (Clampfit 9). Initial slopes of fEPSPs were expressed as percentages of baseline averages. In summary graphs, each point represents the average of 4 consecutive responses. The time-matched, normalized data were averaged across experiments and expressed as means ± SEM.

Stereotaxic Surgeries and Microinjection

Animals were anesthetized with isoflurane in oxygen (2%) and placed in a stereotactic instrument. For CA1 microinfusions, a single 3 mm long guide shaft made of 28 gauge stainless-steel tubing was implanted directly above the targeted CA1 area. The guide shaft was

attached to the skull by Jeweler's screws and cemented with dental acrylic. Coordinates for implantation were AP -2.92, ML ±3.37, DV -1.43 with reference to bregma, midsaggital line and ventral to level brain surface were used to implant the guide cannula for bilateral microinjection into ventral CA1. A dummy cannula (28 gauge, 3.0 mm in length) was inserted into the guide cannula at the time of surgery to prevent occlusion. Surgeries for microdialysis were conducted similarly to those for microinjections with the exception of implanting a single unilateral 5 mm long guide shaft made of 22 gauge stainless-steel tubing with a dummy cannula, 22 gauge fit to guide with 0.5 mm projection into the right ventral CA3. The coordinates for implantation were AP -2.70, ML 3.25, DV -0.5. Postoperative analgesia consisting of 0.5 ml ketoprofen and a topical antibiotic were provided for 3 days for all surgeries. All mice were allowed at least 2 weeks of postoperative recovery before manipulation occurred. A 3.5 mm injection cannula was used to complete the DV coordinates to -1.93 at time of infusion. Each animal was lightly anesthetized prior to insertion of the internal cannula and connected to an injection unit with isoflurane in oxygen (1.5%). The microinjection unit was attached to a 25 μl Hamilton microsyringe via polyethylene tubing (PE-10, 22Q) and silicone tubing connectors (0.015 in I.D. x 10 mm). Lyophilized peptides were dissolved in 0.9% saline solution on the same day prior to injection and maintain on 4°C until loaded into microinjection unit. Injections were given 5 h into light cycle. Simultaneous bilateral administration of either peptide was controlled by an infusion pump at 0.04 μl/min for a total delivered volume of 0.5 μl. An additional 5 minutes with injection unit in situ post injection was allowed to ensure appropriate diffusion and to avoid reflux. All surgical animals were euthanized and perfused in 4% paraformaldehyde, further post-fixed in 4% paraformaldehyde for 24 h and then cyroprotected in 30% sucrose. Brains were then sectioned at 50 μm and Nissl stained to evaluate injection cannula and microdialysis probe placement. All animals with placements outside of target areas were removed from analysis.

Microdialysis and Acetylcholine (ACh) Assay

Microdialysis probes were purchased from Plastics One (Roanoke, VA; part number HMD-1/CS, membrane molecular weigh cut-off 13 KD, 2 mm long membrane). Probes were inserted in place 12 h prior to the first collection by briefly anesthetizing the mice with isoflurane. Probes were perfused with aCSF made of 142 mM NaCl, 3.9 mM KCl, 1.2 mM CaCl2, 1.0 mM MgCl2, 1.35 mM Na2HPO4 and 0.3 mM NaH2PO4 with pH 7.4. The flow rate was set at 0.2 μl/min overnight and 1.0 μl/min starting 3 h before and during sample collection. Samples were collected every 15 min into a refrigerated collection tubes, four consecutives samples were collected to determine basal levels of ACh before placing the mice on the elevated platform

(stressor) for 30 min while collections continued at 15 min intervals. ACh in dialysates was determined by microbore high-performance liquid chromatography (HPLC) using a column (530 × 1 mm I.D.) (MF-8904, BAS, West Lafayette, IN). A post-column IMER (50 x 1 mm I.D.) (BAS MF-8903) was used to convert ACh to hydrogen peroxide. The hydrogen peroxide was detected in a wired enzyme electrode (MF-2095, BAS), a carbon electrode set at +100 mV vs Ag/AgCl was used. The HPLC mobile phase consisted of 50 mM of Na2HPO4 at pH 8.4. The mobile phase flow rate was 140 µl/min. Injection volume of 10 µl yielded a detection limit of 15 fmol/10 uL. Choline and acetylcholine were quantified using external standards. The assay was linear from 10 to 1000 fmol. The quantification was done using BAS ChromGraph software. Only experimental data from animals with correctly located needles/probes were used for analysis.

Additional Behavioral Apparatus and Procedures

Elevated Plus Maze was carried out as reported using a Plexiglas plus-shaped maze containing 2 closed arms (30 cm X 5 cm X 50 cm H) and 2 equally-sized open arms. After a 1 h habituation period, mice were placed in the center of the maze and tracked for 5 min. Behavior was scored using TopScan Suite (CleverSys Inc.).

Light Dark Box: After 1 h habituating to a dimly lit room (30 lux) mice were placed in the light part of a chamber composed of a light (30 x 15 cm) and a dark (10 x 15 cm) compartment for 10 min. Behavior was scored using TopScan Suite (CleverSys Inc.).

Open Field Test: A 120 cm X 120 cm arena was used. Mice were placed in the center of the arena and behavior was recorded and analyzed with Ethovision Tracking System (Noldus, Leesburg, VA). Center zone defined as inner 60 cm X 60 cm.

Novelty Induced Hypophagia: Animals were trained for 30 min/d/3 d to drink 1:3 diluted sweetened condensed milk. On d4 animals were placed into an empty cage (no bedding, wire rack, food or water) in a brightly lit area in a different room than their animal holding room and the latency to drink was recorded.

Stress Induced Hyperthermia: Three d prior to testing a temperature sensitive implantable electronic ID transponder (BMDS, Seaford, DE) was implanted under the skin. On d4, baseline temperatures were recorded in the home cage with a smart probe (BMDS). Temperatures were recorded in 5 min intervals for stressor duration. Animals were returned to their home cages and lights were dimmed to promote recovery. Temperatures were recorded every 5 min for 60 min post-stress.