Serine Racemase and D-Serine in the Amygdala Are Dynamically Involved in Fear Learning

Supplemental Information

SUPPLEMENTAL METHODS AND MATERIALS

Immunohistochemistry and Immunofluorescence

The rabbit anti-D-serine antibody (Abcam; ab6472) was used at a dilution of 1:30,000 and incubated with 7.5mM (final concentration) L-serine-BSA blocking conjugate for 2 nights at 4°C. Sections were then washed and incubated for 60 minutes at room temperature with the ImmPRESS Polymerized Reporter Enzyme Staining System (Anti-rabbit IgG peroxidase; Vector Laboratories; RRID: AB_2336529). Sections were developed using the ImmPACT DAB peroxidase substrate kit (Vector Laboratories; RRID: AB_2336520) according to manufacturer's instructions. WT and SR-/- tissue sections were incubated in the same wells for all steps, including during DAB exposure.

Mice were transcardially perfused with 4% paraformaldehyde in PBS (pH7.4), their brains removed and post-fixed overnight, and then cryoprotected in 30% sucrose. Immunofluorescence was performed on 40μm free-floating sections. For SR/NeuN (neuronal nuclei) and SR/GAD67 staining, sections were blocked with 5% NGS and incubated with primary antibodies (mouse anti-NeuN: Millipore, RRID: AB_2298772, 1:1000; rabbit anti-SR (1), 1:1000; mouse anti-GAD67: Millipore, RRID: AB_2278725) overnight at 4°C, followed by incubation with goat anti-mouse AlexaFluor488 IgG (H+L) (1:500) and goat anti-rabbit AlexaFluor555 IgG (H+L).

For human *post-mortem* studies, we utilized two-week formalin-fixed amygdala brain tissue from 2 human control cases (3877: age = 62, PMI = 15.3, male, cause of death: heart attack; 4656: age = 67, PMI = 26.9, female, cause of death: unknown). Brain tissue was cut at a thickness of 40 μm on a freezing microtome and sections were stored in cryoprotectant at -20°C. All solutions were made in 0.1 M PBS with 0.5% TritonX-100 (washing solution) except where noted. The tissue sections were washed 3x for 5 min between all steps. Tissue sections were washed and then incubated in a citrate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4 made in water) antigen unmasking solution. The sections were placed into citrate buffer that was heated to 80°C and then allowed to cool for 30 min with agitation. Sections were washed, incubated in washing solution containing 0.3% H_20_2 and 10% methanol, and washed again. The tissue was blocked at room temperature in 2% BSA for 1 hour and then placed in blocking solution containing the primary antibodies (Rabbit anti-SR, Santa Cruz, RRID: AB_2195009, 1:100); Mouse anti-parvalbumin (Sigma Aldrich, RRID: AB_477329, 1:5000; Rat anti-somatostatin, Millipore, RRID: AB_2255365, 1:250) for 2 nights at 4°C. Sections were washed and placed in a 1% BSA solution containing biotinylated goat anti-rabbit secondary antibody for 2 hours at room temperature. After washing, the sections were placed in a 1% BSA solution containing a streptavidin-AlexaFluor488 conjugated antibody and a species appropriate fluorophore conjugated secondary antibodies (Goat anti-mouse AlexaFluor647 and Goat anti-rat AlexaFluor555) for 4 hours at room temperature. Lipofuchsin autofluorescence was quenched by placing the tissue in a cupric sulfate (0.5 M sodium acetate, 0.001 M cupric III sulfate in water) solution for 10 min at room temperature. After washing in 0.1 M PBS, sections were mounted on slides, allowed to dry, and cover-slipped using ProLong Gold Antifade Reagent (Life Technologies).

Brain fixation and immunofluorescent staining for D-serine were performed as previously described (2), with the following modification. The rabbit anti-D-serine antibody (Abcam) was used at a dilution of 1:7,500 and incubated with 7.5mM (final concentration) L-serine-BSA blocking conjugate for 2 nights at 4°C. For D-serine colocalization, sections were blocked with 10% NGS and incubated with primary antibodies (mouse anti-NeuN: Millipore, 1:1000 or mouse anti-GAD67, Millipore, 1:1000, RRID: AB_2278725 and rabbit anti-D-serine) for 48h at 4°C.

Immunofluorescence was visualized on a Zeiss Axio ImagerM2 equipped with an Axiocam MRm camera and Apotome 2.0. Immunofluorescent images appearing in figures were obtained using a Zeiss Axio ImagerM2 at 20x and 40x. Pseudocoloring was performed in StereoInvestigator. In PowerPoint 2011, the images were adjusted for contrast/brightness (same settings applied to all panels in each figure) and were cropped to improve the display of the regions of interest.

Stereological Estimation of Colocalization

Contours were drawn outlining either the BLA or CeA on both hemispheres for 8-10 brain sections per animal (2 mice per colocalization experiment). Every 6^{th} brain section was sampled as to include the entirety of each amygdala subdivision in the counting session. The following parameters were used for SR/NeuN sampling: square counting frame (45µm), dissector height (Z; 25µm), sampling grid (BLA- X: 177µm, Y: 253µm; CeA- X: 107µm, Y: 101µm). The following parameters were used for SR/GAD67 and D-serine/GAD67 sampling: square counting frame (60µm), dissector height (Z; 15µm), sampling grid (BLA- X: 141µm, Y: 253µm). The following parameters were used for D-serine/NeuN sampling: square counting

frame (45μm), dissector height (Z; 15μm), sampling grid (BLA- X: 150μm, Y: 200μm; CeA- X: 68μm, Y: 65μm)

Western Blot Analysis

Nitrocellulose membranes were incubated overnight at 4°C with primary antibody (mouse anti-Arc: Santa Cruz, 1:800, RRID: AB_626696; goat anti-serine racemase: Santa Cruz, RRID: AB_2195015, 1:100). Membranes were then incubated with species appropriate horseradish peroxidase-conjugated secondary antibodies (1:5,000). Chemiluminescent values of the protein of interest were divided by its corresponding β-actin chemiluminescent values. The ratio of each experimental sample was divided by the average of all the control sample values in each gel and multiplied by 100. The average of the normalized control values from each gel was 100**%** ± SEM. The normalized values were then averaged and used for statistical analysis.

Trace Fear Conditioning and Extinction

To assess fear-conditioned learning and extinction, there were four experimental groups: 1) naïve, 2) trace-fear conditioned, which were sacrificed 60min after training, 3) trace-fear conditioned, which were re-exposed to the training context the next day (no tones) and sacrificed 30min later, and 4) trace-fear conditioned, which were re-exposed to only the training context for 12min (days 2-5) and sacrificed 30min later. On Day 1, each conditioning session consisted of a 3min acclimation period followed by 5 trials of the following structure: a 20s tone at 75dB, followed by a 20s trace period, followed by a foot shock (duration 2s, amplitude 0.7mA). Trials were roughly 4min apart. The house light in the chamber was illuminated during all sessions. We also included additional sham control groups that went through the same conditioning

procedures, but did not receive foot shocks. For testing the effect of D-serine on extinction, all mice received one intraperitoneal (i.p.; injection volume = 10ml/kg) injection of sterile saline for three days prior to conditioning (day 1). On day 2, mice were injected with either saline or D-serine (300mg/kg; i.p.) 30min prior to being placed back in the chamber. The context of the chamber was changed [plastic flooring, roof, peppermint (diluted 1:1000)] from day 1. After 180s, mice were presented with the same 20s tones as day 1 (15x; 45s inter-tone interval) for three consecutive days and freezing was analyzed during and the 20s after (trace period) the tone presentations.

qPCR

RNA was isolated from the tissue samples using the miRvana miRNA isolation kit (Ambion; Austin, TX). cDNA for each RNA sample (2 μg input) was generated using the High Capacity cDNA Reverse transcription kit (Applied Biosystems; Foster City, CA). qPCR for SR (Mm01246014_m1) and GAPDH (Mm99999915_g1) were performed using TaqMan gene expression assays (Applied Biosystems; Foster City, CA). Data was collected using a 96-well thermal cycler (BioRad; Hercules, CA). Each sample was assayed in triplicate.

HPLC Analysis of Amino Acids

Brain tissue samples were flash frozen on dry ice following dissection. The samples were deproteinized by precipitation with TCA 5% and centrifugation. After extraction of TCA with water-saturated ether, the samples were analyzed in a Merck-Hitachi LaChrom liquid chromatograph equipped with an autosampler (L-7250), a quaternary gradient pump (L-7100), degasser unit (L-7614), a fluorescent detector (FL-7485), and a column oven adjusted to 30°C

(L-7350). After derivatization with o-phtalodialdehyde and Boc-L-cysteine (3), the enantiomers were separated using a Spheri-5C18 column (220 mm 4.6 mm i.d., 5mm particle size) from Alltech (Deerfield), fitted with precolumn NewGuard RP-18, 7 mm, 15 mm, 3.2 mm by Grace (Deerfield). Glycine was detected using the same chromatographic conditions for the detection of D-serine (4), except that o-phtalodialdehyde and beta-mercaptoethanol were used as derivatization agents, as previously described (5).

Human Genetics Analyses

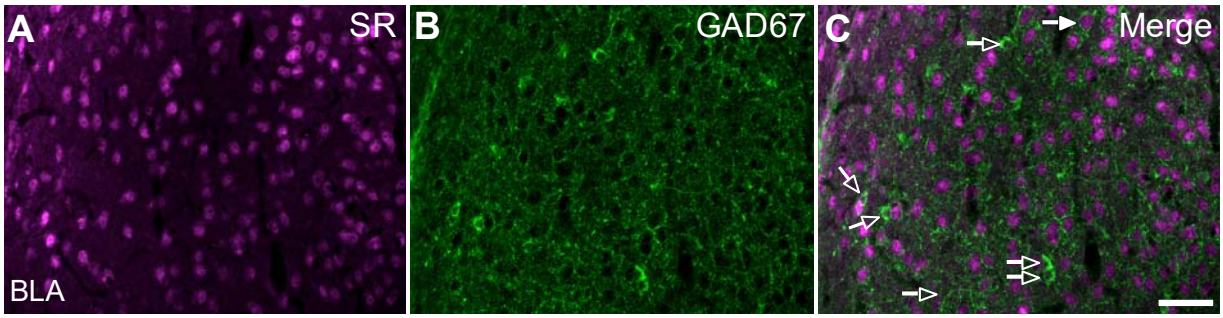
The Grady Trauma Project is an investigation into the genetic and environmental factors that predict the response to stressful life events in a predominantly African American, urban population of low socioeconomic status. Lifetime traumatic life events were assessed via a semi-structured interview using the Traumatic Events Inventory (TEI), as previously described (6). Current PTSD was assessed using the modified PTSD Symptom Scale (PSS), an 18-item self-report scale with excellent internal consistency, high test-retest reliability and concurrent validity to diagnose PTSD consistent with DSM-IV criteria (7). The PTSD cases in our analysis had current PTSD symptoms while the controls did not. Both the cases and controls had been exposed to at least one traumatic life experience. Since the mPSS assesses current PTSD symptoms, we cannot rule out that some of the controls might have had PTSD in the past.

For initial quality control, samples were removed if they had very low call rates and outside acceptable levels of heterozygosity ($-0.25<\text{Fhet}>0.25$), with the remaining samples re-called in GenomeStudio (8). Further quality control excluded SNPs that had a call rate <98%, a minor allele frequency (MAF) <0.01 or significant deviation from Hardy–Weinberg proportions ($P<1 \times 10^{-6}$ in controls and $P<1 \times 10^{-10}$ in PTSD cases), and excluding samples with >2%

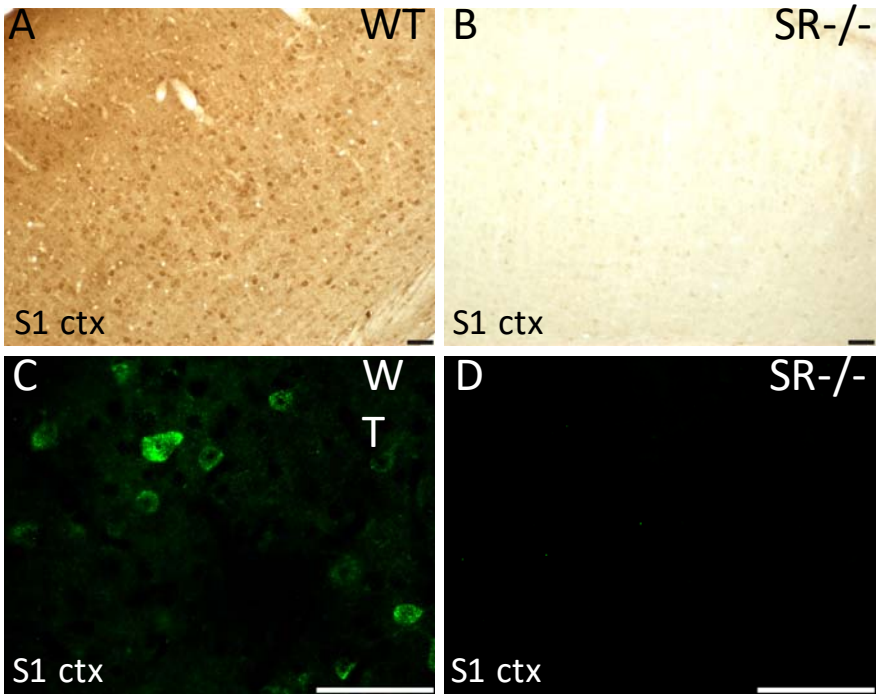
missing data. Related individuals were identified and removed by using PLINK to estimate the proportion of identity by descent (IBD) for each pair of individuals as previously described (8).

Main effects of the continuous variables (PTSD total symptoms, hyper-arousal, avoidance, and intrusive symptoms) were examined with one-way ANOVA with rs4523957 genotype as the independent variable. In different analyses, univariate ANOVA was used to determine the effects of genotype as an independent variable on PTSD continuous symptoms as outcome variables, with race, sex, education, childhood trauma, total trauma, and prior schizophrenia diagnosis as covariates. Determination of rs4523957 as a potential eQTL for SRR mRNA expression levels was performed with the GTEx database (http://gtexportal.org) based on healthy human brain tissue.

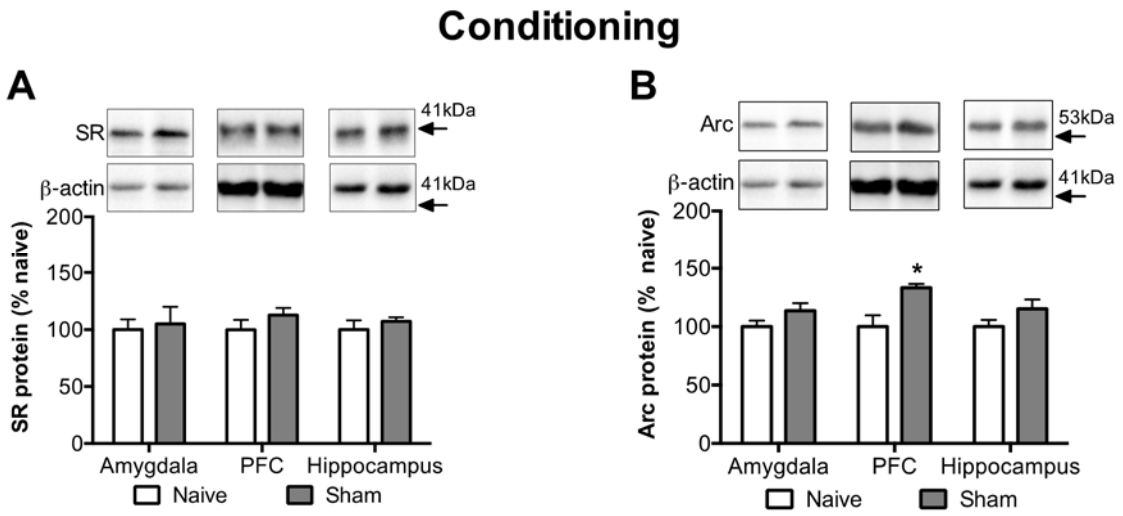
SUPPLEMENTAL FIGURES



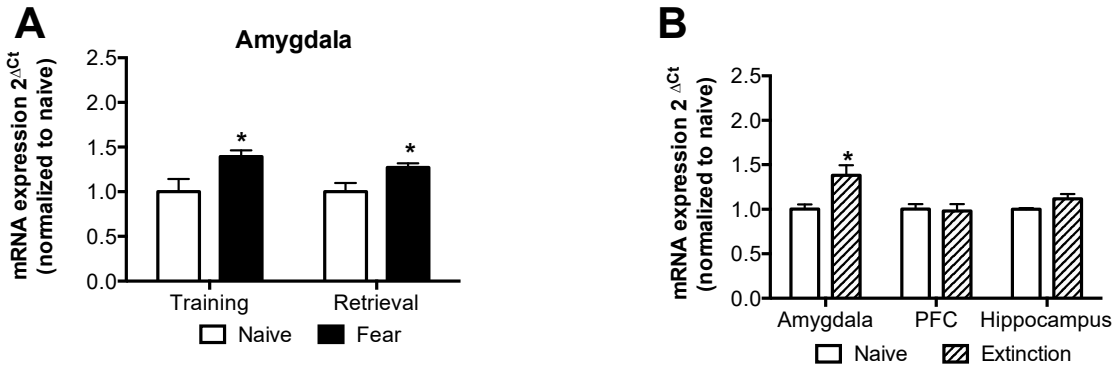
Supplemental Figure S1. Serine racemase is very sparsely colocalized with GAD67 in the BLA. Using antibodies against serine racemase (**A**; SR; magenta) and glutamic acid decarboxylase 67kDa (**B**; GAD67; green), we show that SR shows minimal colocalization with GAD67 inhibitory neurons in the BLA (**C**; open arrows = GAD67; white arrow = SR/GAD67). Scale bar represents 50μm.



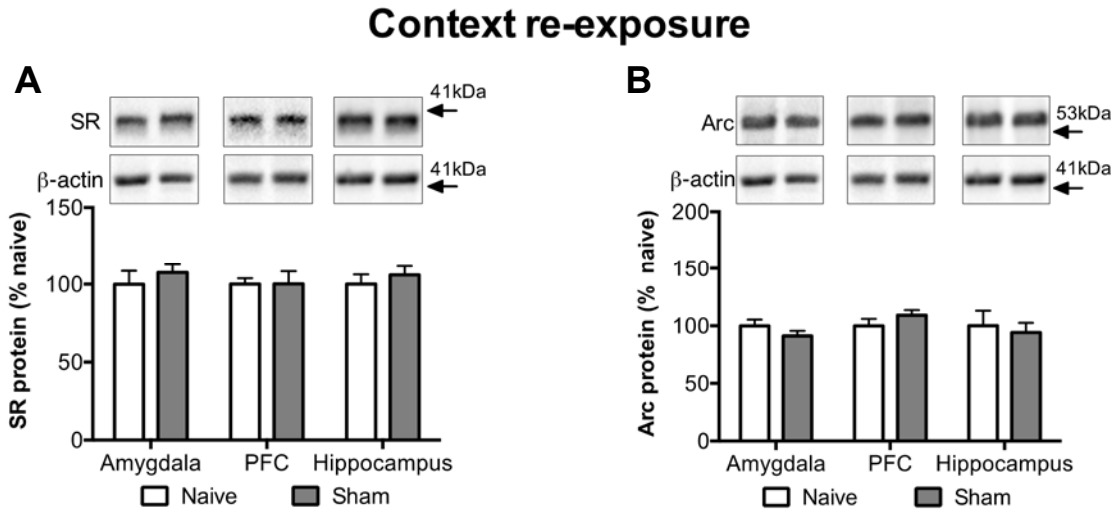
Supplemental Figure S2. Demonstration of D-serine antibody specificity. Using SR deficient (SR-/-; **B** and **D**) mice, which have ~90% less D-serine than wild-type (WT; **A**, **C**) mice, we demonstrate an almost complete absence of D-serine immunoreactivity in SR-/- tissue (S1 ctx: primary somatosensory cortex) for both immunohistochemistry (**A-B**) and immunofluorescence (**C-D**). Scale bars represent 50μm.



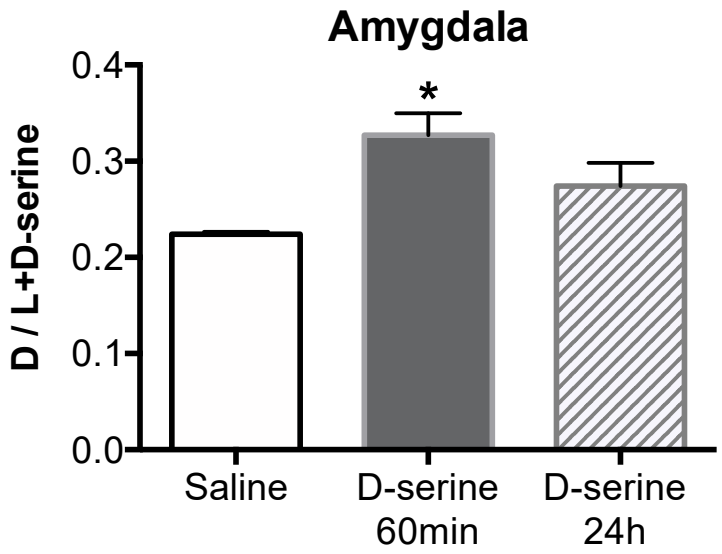
Supplemental Figure S3. Effects of sham conditioning on serine racemase and Arc protein expression. Mice were naïve (white bars; n = 7) or subjected to a sham trace fear-conditioning paradigm (gray bars; n = 6; tones with no foot shock). (**A-B**) Animals were sacrificed 60 minutes after training on day 1. Protein levels of serine racemase (SR; **A**) and Arc (**B**) were measured in the amygdala, prefrontal cortex (PFC), and hippocampus. Asterisk (*) indicates significant differences from naïve mice ($P < 0.05$). All values represent the mean ± SEM.



Supplemental Figure S4. Fear learning and extinction increase serine racemase mRNA expression in the amygdala. Mice (n = 7/group) were naïve (white bars) or subjected to a trace fear-conditioning paradigm (black bars). (**A**) Animals were sacrificed 60 minutes after training, 30 minutes after re-exposure to the training context on day 2, or (**B**) after re-exposure to the training context for four days (extinction; hatched bars). mRNA levels for serine racemase (SR) were measured in the amygdala, prefrontal cortex (PFC), and hippocampus. Asterisk (*) indicates significant differences from naïve mice ($P < 0.05$). All values represent the mean ± SEM.



Supplemental Figure S5. Sham conditioning and context re-exposure does not increase serine racemase or Arc protein expression. Mice were naïve (white bars; n = 7) or subjected to a sham trace fear-conditioning paradigm (gray bars; n = 6; tones with no foot shock). Animals were sacrificed 30 minutes after re-exposure to the training context on day 2 (**A-B**). Protein levels of serine racemase (SR; **A**) and Arc (**B**) were measured in the amygdala, prefrontal cortex (PFC), and hippocampus. All values represent the mean ± SEM.



Supplemental Figure S6. Acute peripheral D-serine administration increases amygdalar D-serine levels. D-serine content was measured in amygdala tissue from mice (n = 4/group) that received a single intraperitoneal injection of saline (white bar) or D-serine (300 mg/kg; gray bar: sacrificed 60 minutes after injection; striped bar: sacrificed 24 hours after injection). Asterisk (*) indicates significant difference from the saline group ($P < 0.05$; Tukey's multiple comparisons test). All values represent the mean ± SEM.

SUPPLEMENTAL REFERENCES

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