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

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ABSTRACT

BACKGROUND: The amygdala is a central component of the neural circuitry that underlies fear learning. *N*-methyl-D-aspartate receptor–dependent plasticity in the amygdala is required for pavlovian fear conditioning and extinction. *N*-methyl-D-aspartate receptor activation requires the binding of a coagonist, D-serine, which is synthesized from L-serine by the neuronal enzyme serine racemase (SR). However, little is known about SR and D-serine function in the amygdala.

METHODS: We used immunohistochemical methods to characterize the cellular localization of SR and D-serine in the mouse and human amygdala. Using biochemical and molecular techniques, we determined whether trace fear conditioning and extinction engages the SR/D-serine system in the brain. D-serine was administered systemically to mice to evaluate its effect on fear extinction. Finally, we investigated whether the functional single nucleotide polymorphism rs4523957, which is an expression quantitative trait locus of the human serine racemase (*SRR*) gene, was associated with fear-related phenotypes in a highly traumatized human cohort.

RESULTS: We demonstrate that approximately half of the neurons in the amygdala express SR, including both excitatory and inhibitory neurons. We find that the acquisition and extinction of fear memory engages the SR/D-serine system in the mouse amygdala and that D-serine administration facilitates fear extinction. We also demonstrate that the *SRR* single nucleotide polymorphism, rs4523957, is associated with posttraumatic stress disorder in humans, consistent with the facilitatory effect of D-serine on fear extinction.

CONCLUSIONS: These new findings have important implications for understanding D-serine–mediated *N*-methyl-D-aspartate receptor plasticity in the amygdala and how this system could contribute to disorders with maladaptive fear circuitry.

Keywords: Amygdala, D-serine, Fear conditioning, NMDA receptor, Posttraumatic stress disorder, Serine racemase

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N-methyl-D-aspartate receptors (NMDARs) belong to the class of ionotropic glutamate receptors that are essential mediators of synaptic plasticity, learning, and memory (1). These receptors are unique in that their activation requires the concomitant binding of glutamate and a coagonist, glycine or D-serine (2,3). D-serine is synthesized from L-serine by the enzyme serine racemase (SR) (4). Both SR and D-serine are enriched in corticolimbic regions of the brain and are localized to the same areas as NMDARs (5). Although the endogenous coagonists glycine and D-serine are present in the extracellular space (3), the glycine modulatory site is not saturated in vivo (6). In the adult hippocampus, D-serine is important for proper NMDAR function, NMDAR-dependent long-term potentiation (LTP), and it is believed to be the primary coagonist for synaptic, but not extrasynaptic, NMDARs (7–11). From studies with mice with a constitutive deletion in SR ($SR^{-/-}$) mice, D-serine was shown to also be important for maintaining glutamatergic dendritic integrity in the adult cortex and hippocampus (8,12,13). However, outside of these cortical regions, little is known about the function and cellular localization of SR and D-serine. Thus, we investigated the distribution of SR and D-serine in the murine and human amygdala.

The amygdala is a central hub in the emotional learning circuit, integrating sensory information from both cortical and subcortical brain regions related to the conditioning experience (14). LTP in the amygdala is NMDAR dependent (15,16). Furthermore, NMDAR activation in the amygdala is necessary for fear conditioning and fear extinction (17). Using $SR^{-/-}$ mice, and enzymatic degradation of D-serine with D-amino acid oxidase in brain slices from control mice, we found that the induction of NMDAR-dependent LTP at thalamo-lateral amygdala synapses is dependent on D-serine (11). Moreover, we demonstrated that the magnitude of LTP in thalamic inputs is directly determined by the level of NMDAR activation (11).

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Pavlovian fear conditioning is one of the most widely used models for studying emotional memory and associative learning in rodents (18). In this form of conditioning, a neutral stimulus (conditioned stimulus) acquires predictive value by pairing it with an aversive unconditioned stimulus (foot shock) that has an intrinsic value to the subject. After training, exposure of the animal to the conditioned stimulus or context alone elicits conditioned fear responses such as freezing. Using $SR^{-/-}$ mice, we previously demonstrated that SR and D-serine are important for fear learning (19). Therefore, in the current study, we examined whether the D-serine system is dynamically involved in fear-conditioned learning, specifically within the amygdala, and other brain regions known to be critical for this behavior.

There is abundant evidence that the amygdala is also dysfunctional in posttraumatic stress disorder (PTSD) and related anxiety disorders (20). Thus, we next demonstrated that the previously examined single nucleotide polymorphism (SNP) rs4523957, within the human serine racemase (*SRR*) gene previously associated with other disorders (21–23), is a functional expression quantitative trait locus (eQTL) at the level of regulating SR messenger RNA (mRNA) expression in postmortem human brain. Finally, we found that this functional *SRR* SNP was associated with PTSD in a highly traumatized population (24,25).

METHODS AND MATERIALS

Animals

Adult male mice (3–5 months old) were used for all the experiments. Animals were group housed in polycarbonate cages and maintained on a 12-hour light/dark cycle in a temperature (22°C)- and humidity-controlled vivarium. Animals were given access to food and water ad libitum. All animal procedures were approved by the McLean Hospital Institutional Animal Care and Use Committee.

Immunohistochemistry

Brain fixation and immunohistochemistry/immunofluorescence in mouse and human brain tissue were performed as previously described (26), with modifications described in the Supplement.

Stereological Estimation of Colocalization

Brain sections were visualized on a Zeiss Axio Imager M2 (Zeiss, Oberkochen, Germany) equipped with Stereo Investigator software (MBF Bioscience, Williston, VT). The Optical Fractionator Workflow in Stereo Investigator was used to stereologically sample neurons to estimate the percentage of neurons (neuronal nuclei [NeuN]) in the basolateral amygdala (BLA) and central amygdala (CeA) that express SR or contain D-serine as well as the number of inhibitory neurons that express SR or contain D-serine in the BLA.

Trace Fear Conditioning and Extinction

Behavioral procedures are described in the Supplement. All testing was performed using the Near Infrared Fear Conditioning System (Med Associates, St. Albans, VT). Freezing behavior was quantified using Video Freeze software.

Western Blot Analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting were performed and analyzed as previously described (27).

Quantitative Polymerase Chain Reaction

For relative quantification of mRNA expression (n = 6–8/group), geometric means were calculated using the comparative $2^{\Delta Ct}$ (where Ct is cycle threshold) method, with the housekeeping gene *GAPDH* used as the endogenous reference as previously described (12).

High-Pressure Liquid Chromatography Analysis of Amino Acids

D-serine levels in brain tissue were determined by high-pressure liquid chromatography analysis as previously described (28).

Human Genetic Analyses

The human subjects in this cohort analyzed for *SRR* genetic association with PTSD were part of the Grady Trauma Project (25). All procedures were approved by the Institutional Review Board of the Emory University School of Medicine and the Grady Health Systems Research Oversight Committee. Genotyping was performed on DNA derived from saliva or blood using the Omni-Quad 1M or the Omni Express BeadChip (Illumina, San Diego, CA), and genotypes were called in GenomeStudio (Illumina). Quality control measures were performed using the toolset PLINK (29). Previously, one SNP (rs4523957) within the *SRR* gene had been associated as a potential functional variant, with multiple disorders related to NMDAR and D-serine function (21–23). The genotype calls for rs4523957 were determined from the Illumina genome-wide association study platform to address whether this variant was associated with PTSD. Association with categorical PTSD diagnosis based on DSM-IV criteria from the modified PTSD Symptom Scale was performed with chi-square analyses based on rs4523957 GG, GT, or TT genotype.

Statistical Analyses

Unpaired t tests were used to analyze Western blot and high-pressure liquid chromatography results when appropriate. Type I (fixed effect) one-way analyses of variance were used to analyze Western blot, high-pressure liquid chromatography, and fear conditioning results. Significant one-way analysis of variance results were followed up with Tukey's multiple comparisons test. Two-way repeated-measures analysis of variance was used to analyze the D-serine extinction results. Values of $p < .05$ were considered statistically significant.

RESULTS

Cellular Characterization of SR in the Amygdala

Although the distribution and expression of SR in neurons of the murine hippocampus and cortex are well established (26,30–32), there has been little research done to characterize SR expression in the amygdala. We found that SR protein is widely expressed in neurons, but not in astrocytes, throughout the amygdala, including the BLA complex and the CeA