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Targeted disruption of serine racemase affects glutamatergic neurotransmission and behavior

Alo C. Basu[1,2], **Guochuan E. Tsai**[3], **Chun-Lei Ma**[4], **Jeffrey T. Ehmsen**[5], **Asif K. Mustafa**[5], **Liqun Han**[1,2], **Zhichun I. Jiang**[3], **Michael A. Benneyworth**[1,2], **Michael P. Froimowitz**[1,6], **Nicholas Lange**[1,6], **Solomon H. Snyder**[5], **Richard Bergeron**[4], and **Joseph T. Coyle**[1,2]

[1]Department of Psychiatry, Harvard Medical School

[2]Laboratory for Psychiatric and Molecular Neuroscience, McLean Hospital

[3]Harbor-UCLA Medical Center

[4]Ottawa Health Research Institute

[5]Solomon H. Snyder Department of Neuroscience, Johns Hopkins University

[6]Neurostatistics Laboratory, McLean Hospital

Abstract

A subset of glutamate receptors that are specifically sensitive to the glutamate analog N-methyl-D-aspartate (NMDA) are molecular coincidence detectors, necessary for activity-dependent processes of neurodevelopment and in sensory and cognitive functions. The activity of these receptors is modulated by the endogenous amino acid D-serine, but the extent to which D-serine is necessary for the normal development and function of the mammalian nervous system was previously unknown. Decreased signaling at NMDA receptors has been implicated in the pathophysiology of schizophrenia based on pharmacological evidence, and several human genes related to D-serine metabolism and glutamatergic neurotransmission have been implicated in the etiology of schizophrenia. Here we show that genetically modified mice lacking the ability to produce D-serine endogenously have profoundly altered glutamatergic neurotransmission, and relatively subtle but significant behavioral abnormalities that reflect hyperactivity and impaired spatial memory, and that are consistent with elevated anxiety.

N-methyl-D-aspartate receptor (NMDAR)-mediated neuronal signaling is critical in the development, function, and plasticity of the mammalian nervous system. Activation of NMDARs requires postsynaptic depolarization, binding of glutamate, and binding of either glycine or D-serine at the glycine modulatory site (GMS)1,2. D-serine is more efficacious than glycine at potentiating the activity of NMDARs than glycine3,4, and acute depletion of endogenous D-serine has been shown to greatly attenuate NMDAR-mediated currents5. Functional importance of D-serine has been recently demonstrated in the vertebrate retina

Corresponding author: Joseph T. Coyle Eben S. Draper Professor of Neuroscience and Psychiatry, Harvard Medical School McLean Hospital, 115 Mill Street, Belmont, Massachusetts, 02478, U.S.A. Tel. No. 617 855 2101 Fax No. 617 855 2705 joseph_coyle@hms.harvard.edu.

and the supraoptic nucleus of the hypothalamus6,7. D-serine is notably enriched in corticolimbic regions of the brain, where its localization closely parallels that of NMDARs, and is highest in the CA1 region of the hippocampus8,9. Endogenous levels of D-serine are determined by its synthetic enzyme, serine racemase (SR), and its degradatory enzyme, D-amino acid oxidase (DAAO)9-11.

Hypofunction of NMDAR-mediated signaling pathways, and specifically D-serine deficiency, have been implicated in the pathophysiology of schizophrenia12. Genetic linkage and association studies have implicated serine racemase, DAAO, and G72, a putative activator of DAAO, as risk genes for schizophrenia13,14. DAAO and G72/G30 have been implicated in bipolar disorder as well15, though recent meta-analyses have highlighted the difficulty of coming to firm conclusions regarding their involvement due to inconsistency of associated variants across studies16,17.

The finding that D-serine is decreased in cerebrospinal fluid and serum samples from individuals with schizophrenia18,19, although not direct evidence of a specific difference in regulation of D-serine, is consistent with the hypothesis that lower D-serine may be part of the pathophysiology of the disorder. Furthermore, clinical trials of D-serine added to atypical anitpsychotics other than clozapine in the treatment of schizophrenia have reported modest improvement in negative and cognitive symptoms20-22, suggesting that intervention at this level may be of therapeutic value. Adding D-serine to clozapine treatment resulted in no clinical change in subjects with schizophrenia, suggesting that clozapine may act through the NMDA receptor pathway, or that patients for whom clozapine treatment is indicated are unresponsive to D-serine due to other factors that differentiate them as a clinical subgroup23.

We have created a murine model of constitutive D-serine deficiency to investigate its effects on brain function. Here, we report that mutant mice lacking the ability to endogenously produce D-serine have altered glutamatergic neurotransmission, attenuated synaptic plasticity, a spatial memory deficit, and subtle behavioral abnormalities consistent with elevated anxiety.

Materials and Methods

All animal procedures were approved by the Institutional Animal Care and Use Committees.

Model generation

To obtain mouse SR genomic DNA, we screened the lambda FIXII 129SvJ mouse genomic library (Stratagene, La Jolla, CA, USA) with mouse SR-specific primers. We identified three positive genomic clones. We selected the 18 kb SR genomic fragment containing the whole coding region from exon 1 to exon 7, and subcloned it into pBluescriptIISK(-) vector. We inserted loxP-flanked neomycin phosphotransferase gene into the BstE2 site in intron 1 and another loxP sequence 5.1(Kas I site) −4.3 kb (Eco47 III site) 5′ upstream to exon 1. The final targeting vector included 3.9 kb of upstream and 7.0 Kb of downstream sequence to enable homologous recombination. R1 mouse embryonic stem (ES) cells were electroporated (10^7 cells using Bio-Rad gene pulser set at 240V and 500F) with 40 g of

SmaI-linearized construct. G418-resistant colonies were screened for homologous recombination using primers *c* (for exon 1), *e* (for the neomycin cassette) and nested primers *g* and *h*, which were external to the targeting construct. Desired recombination generated an extra band of 8 kb using the primers e and g/h in addition to a band of 9 kb generated using primers *c* and *g/h*. The three loxP sequences were confirmed by amplifying the loxP-containing regions using primers *a* and *b* for loxP1, primers *c* and *d* for loxP2, and primers *e* and *f* for loxP3. ES cells carrying the correct recombination were injected into C57BL/6 blastocytes and transferred to pseudopregnant females. Chimeric mice were bred with 129S6/SvEv Tac mice. The F1 mice with germ line transmission of the targeted sequence were crossed with Cre Recombinase-expressing mice. Effective selection to delete the neomycin cassette by Cre recombinase was confirmed by sequencing the amplicon resulting from use of primers c and f. Genotype of the recombinant mice was determined by PCR applying the following primer combinations: for loxP2/loxP3 recombination, primers *c* and *f*; for loxP1/loxP2 recombination, primers *a* and *d*; for loxP1/loxP3 recombination, primers *a* and *f*. loxP1/loxP3 recombinant mice are knockout mice, while loxP2/loxP3 recombinant mice are exon 1 “floxed” mice. We obtained all the possible recombinant mice in the F1 generation. The F1 mice with germ line transmission of the SR exon 1 knockout mutation as determined by PCR were bred to C57/BL6 mice to generate F2. The strain was maintained on a C57/BL6 background. RT-PCR of SR mRNA was used to confirm absence of SR message containing exon 1 sequence in the brains of serine racemase exon 1 knockout (SR−/−) mice (data not shown). Primer sequences: *a*, 5′-GAATTTCCTCCTGTTAAGTGAATCTTCC-3′; *b*, 5′-TGTGGACCAGGCTGGCCTCGAACTCAG-3′; *c*, 5′-ATGTGAGCTCTTCCAGAAAACTGGG-3′; *d*, 5′-CTACCGGTGGATGTGGAATG-3′; *e*, 5′-CTGATGGCATTAAGGGCACCTCGA-3′; *f*, 5′-CTGAAGGCTCTTTACTATTG-3′; *g*, 5′-GGCTACATTCCCTGCCCT-3′; *h*, 5′-TCTCACCCAAGCTGCTATCA-3′

Biochemistry

Western Blotting and immunohistochemistry24, HPLC25, and biotin switch assay26 were performed as previously described.

Electrophysiology

Mouse brains were removed and placed in oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) at 4°C. A vibrating microtome (VT 1000S, Leica, Bannockburn, IL) was used to obtain coronal sections (300 μm) containing the hippocampus. Acute brain slices were stored for 1 hour in an oxygenated chamber at room temperature before recording. Voltage–clamp recordings of CA1 pyramidal cells were obtained with a Multiclamp 700A amplifier (Axon Instruments, Foster City, CA) under visual control using differential interference contrast and infrared video microscopy (IR-DIC) (DMLFSA, Leica, Germany). Excitatory postsynaptic currents (EPSC) were evoked by electrical stimulation of the Schaffer collaterals with a bipolar microelectrode positioned in the *stratum radiatum* with 100 μs current pulses (0.1–1 mA, 0.3–0.01 Hz), which were adjusted to evoke a current amplitude in the range of 60–120 pA at *V*m = −70 mV.

The recordings were first obtained in normal ACSF, and then the NMDAR component was isolated pharmacologically by taking the average EPSC at −40 mV in ACSF containing picrotoxin (50 μM), CGP 52432 (10 μM), and strychnine (0.5 μM) in the absence and presence of 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline (NBQX) (20 μM). The average response in the presence of NBQX (NMDAR only) was subtracted from that measured in its absence. To record NMDAR currents, lidocaine N-ethyl bromide (QX-314, 5 mM) was added to the intracellular solution and cesium-BAPTA (10 mM) was used instead of EGTA. pH of the intracellular solutions was adjusted to 7.2 and osmolarity to 280-290 mOsm. The pipettes had a resistance of 3-7 MΩ when filled with these solutions.

The LTP voltage-clamp experiments were performed with a solution containing (in mM) 130 Cs^+-methanesulphonate, to further minimize current attenuation, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 10 KCl, 2 $MgCl_2$, 0.2 ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetracetic acid (EGTA), 2 ATP-Mg and 0.2 GTP-tris(hydroxymethil) aminomethane. The recordings for the experiments using the pairing protocol to induce LTP were obtained in ACSF in the presence of picrotoxin (50μM). The pairing protocol was composed of 3 brief high frequency tetani (50 pulses at 100 Hz, 4 s intervals) given at the end of a long depolarization (3 min at 0 mV)27. This protocol induced an increase of the synaptic responses lasting for more than 40 min. Data were collected by using PCLAMP 9 software (Molecular Devices, Sunnyvale, CA, USA). Analyses were performed using IGOR software (WaveMetrics, Lake Oswego, OR).

Behavioral testing

The SR+/− construct was bred to a C57BL/6 background for at least 7 generations before SR+/− parents were bred to produce SR−/− and WT offspring. The ratio of SR−/−, SR+/− and WT offspring resulting from heterozygote crosses did not deviate significantly from the expected 1:2:1 ratio. SR−/− and SR+/− animals were grossly normal, with normal body weight, appearance, grooming, and neurological reflexes. Animals were maintained on a 12h:12h light/dark cycle and provided with food and water *ad libitum*. Fifteen male and fifteen female animals of the SR−/− and WT genotypes were tested in behavioral assays at 7-12 weeks of age in the following sequence: locomotor activity, rotarod, PPI, water maze. The experimenter (ACB) was blind to genotype when conducting the experiments.

Locomotor activity assay

Spontaneous locomotor activity and habituation to a novel open field environment were assessed using clear Plexiglas® chambers fitted with three 16-beam I/R arrays and placed inside sound-attenuating cubicles (ENV-510 system, Med Associates, Inc., St. Albans, VT, USA). Photobeam breaks were recorded and translated into locomotor activity using open field activity software (Activity Monitor 5.0, Med Associates, Inc.) The center zone was defined using the coordinates (4.5,12; 12,12; 4.5,4.5; 12,4.5).

Rotarod task

The rotarod task of motor coordination and learning was conducted using an accelerating Rota-Rod for Rats (Model 7750, Ugo Basile, Italy) on the "acceleration" setting.

Prepulse Inhibition (PPI) protocol

The PPI phenomenon was observed using a non-restraining cylindrical enclosure attached to a platform fitted with a motion transducer contained within a sound-attenuating chamber. Stimuli were delivered from a PC and the motion signal from the animal in the 65 ms following each stimulus presentation was converted to a voltage output and analyzed (SR-LAB™ Startle Response System, San Diego Instruments, Inc.). Each mouse was tested in a single session consisting of 72 trials. Animals were first acclimatized to 5 minutes of white noise at 70 dB. Trials were presented at randomized intervals of 10-20 seconds with an average of 15 sec. The first 6 and last 6 trials of the session consisted of startle stimulus alone (120dB white noise, 20 msec). The intervening 60 trials consisted of no startle stimulus, no prepulse prior to the startle stimulus, or a prepulse (3, 6, or 12 dB white noise over background for 20 msec) prior to the startle stimulus (50 msec interstimulus interval). 70 dB of background white noise was presented throughout the session.

Water Maze testing

A water maze tank (San Diego Instruments, mouse tank) was filled with 30 cm of water made barely opaque by the addition of powered milk and kept at ~21°C. A 10 cm-diameter circular escape platform was placed in the bath at ~1 cm below the surface. Four distinct visual cues (colorful geometric shapes on a square background) were placed 6 inches above the water surface placed at even intervals on the inside of the tank. On each trial, the test subject was placed in the water in one of four equally-spaced starting locations. The order of these starting locations was the same for every mouse in the test group, but pseudo-randomized among training days. Each trial lasted a maximum of 90 sec. For days 1-3, mice were given 15 min between trials to dry off and recover in a cage under a warming lamp. For days 4-12, the intertrial interval was 10 min. The path of the mouse was collected by video camera and movements were analyzed using EthoVisionXT (Noldus Information Technology, Wageningen, Netherlands).

Statistical analysis

Behavioral data were analyzed by fitting standard least squares mixed models stratified by sex with subject as a random effect, except where stated otherwise. Day, trial, prepulse amplitude, block, and genotype were included as fixed effects as appropriate for the experiment. All effects were assessed by F tests based on the fitted regression models.

Results

The serine racemase exon 1 knockout (SR−/−) mouse model

The only known endogenous source of D-serine in the mammalian brain is the conversion of L-serine to D-serine by the pyridoxal 5′-phosphate-dependent enzyme serine racemase (SR). We have generated a mouse genetic model of D-serine deficiency by targeted deletion of exon 1 of the SR gene, which encodes the pyridoxal 5′-phosphate domain of the SR enzyme, using the Cre/loxP system. This strategy resulted in a SR exon 1 knockout line, studies of which are reported in this manuscript, as well as a SR exon 1 conditional knockout line, in which exon 1 of the SR gene is flanked by loxP sequences (Fig. 1a). Absence of SR protein

was confirmed by Western blot of whole brain protein sample from SR exon 1 homozygous knockout (SR−/−) mice, SR heterozygous (SR+/−) mice, and wildtype (WT) mice (Fig. 1b). The antibody that we used has been shown to recognize SR protein in neurons and in astrocytes25. Immunohistochemistry using this antibody revealed that SR is undetectable above background in SR−/− animals. In brains obtained from adult WT littermates, strong immunostaining was present in cortex, striatum and hippocampus, but not in cerebellum, as previously described (Fig. 1c). In contrast, brains from SR−/− animals showed no staining in these areas (Fig. 1d). Detection of cortical D-serine levels by HPLC revealed that levels in SR−/− mice are approximately 10% of WT and levels in SR+/− mice are approximately 70% of WT (Table 1). Exogenous sources of brain D-serine in SR−/− mice may include the laboratory diet and gastrointestinal bacteria. Our measurement of free serine in the laboratory diet (Purina Lab Diet 5P76 irradiate ProLab IsoPro RMH 3000) revealed equal concentrations of L-serine and D-serine of approximately 1.5μg per gram protein (data not shown). There was no effect of sex on D-serine levels (2-way ANOVA, main effect of genotype $p<0.0001$).

NMDAR-mediated neurotransmission is altered in SR−/− mice

Whole-cell patch-clamp recordings of hippocampal CA1 pyramidal cells and stimulation of Schaffer collaterals in acute slices from P21-P28 mice show that the decay kinetics of NMDAR-mediated excitatory postsynaptic currents (EPSCs) are significantly slower in mutant compared to WT mice (Fig. 2a,b). This shift toward slower decay kinetics suggests an increased contribution of NR2B-containing NMDARs in SR−/− animals. This change is in contrast to a shift toward faster decay kinetics of the NMDAR-mediated EPSC previously described in glycine transporter 1 (GlyT1) heterozygous knockout mice28.

We investigated the level of occupancy of the GMS on NR1 in SR−/− and SR+/− animals by asking to what degree NMDAR-mediated EPSCs could be enhanced by providing D-serine in the bath. Perfusion of the slice with D-serine (10 μM) induced an 81% increase in the amplitude of the pharmacologically isolated NMDAR current in SR+/− mice and a 93% increase in SR−/− mice compared to a 41.8% increase in WT mice, consistent with lower occupancy of the GMS in the SR mutant mice (Fig. 2c,d). Moreover, the application of the inhibitor of glycine transporter 1, NFPS (100 nM), which results in elevated synaptic glycine29, had a significantly more pronounced effect in mutant compared to WT mice.

We performed a biotin-switch assay to measure the global level of NMDAR-mediated neurotransmission in SR−/− brains. Because nitric oxide synthase is a calmodulin-requiring enzyme30, NMDAR-mediated neurotransmission activates nitric oxide (NO) formation, which results in S-nitrosylation of target proteins31. In whole brain homogenates from SR−/− mice, we observed a ~70% reduction in basal S-nitrosylation of beta-tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), indicating substantially decreased NMDAR-mediated neurotransmission (Fig. 2e).

We then investigated the effect of the SR−/− and SR+/− genotypes on hippocampal plasticity. Using a pairing paradigm to induce long-term potentiation (LTP) at the Schaffer collateral-CA1 pyramidal neuron synapse, we observed no significant LTP in SR−/− mice, while the same paradigm induced a 90% increase in EPSC amplitude in WT mice. In the

presence of bath-applied D-serine, it was possible to induce LTP in SR−/− hippocampal slices with this protocol (Fig. 2f).

Motor coordination, locomotor activity, and acoustic startle response in SR−/− mice

Because of the putative importance of D-serine in cerebellar development32, motor coordination and learning were assessed using the rotarod task. Mice were placed on an accelerating rotarod for 3 trials per session, with an intertrial interval of 10 minutes, on 3 consecutive days. Male and female SR−/− mice performed and learned the rotarod task normally (Fig. 3a). (males: main effects of day, $p<0.0001$ and trial, $p = 0.0449$; females: main effect of day, $p<0.0001$; no significant effects of genotype). Locomotor activity was assessed in three 30-minute sessions over three consecutive days. Male SR−/− mice showed relatively high levels of activity when first introduced into the activity monitoring chambers compared to WT mice (Fig. 3b), and remained hyperactive throughout each session (main effects of day $p< 0.0001$, bin $p<0.0001$, and genotype $p=0.0136$). Male SR−/− mice also showed more vertical behavior (counts of rearing and jumping) in this assay compared to WT males (Fig. 3c) (main effects of day $p<0.0001$ and genotype $p<0.0001$). Female SR−/− mice were not different from WT in distance traveled or vertical behavior but spent significantly less time in the center zone of the activity monitoring chambers during the first session (Fig. 3d) (Mann-Whitney U test, $p=0.0381$).

Prepulse inhibition (PPI) of the acoustic startle response (ASR), a sensory gating phenomenon in which the presentation of a sub-startle threshold prepulse inhibits the response to a startle-inducing stimulus, has been shown to be impaired in a subset of schizophrenia patients and their clinically unaffected relatives33. Robust PPI of the ASR was observed in SR−/− and WT mice (Fig. 3e). Thus, the deficits in NMDAR-mediated neurotransmission we observed in our electrophysiological and biochemical experiments do not correlate with PPI deficits in these animals (males and females, main effects of prepulse amplitude, $p<0.0001$, no effects of genotype). The ASR of male and female SR−/− mice showed habituation during the PPI testing session. However, we observed that the ASR itself is elevated in SR−/− females (males: main effect of block, $p<0.0001$, no effect of genotype; females: main effects of block, $p=0.0016$, and genotype, $p=0.0069$). Although the ASR of SR−/− females did habituate over the course of the session, it did not decrease to WT levels.

SR−/− males have spatial memory deficits

We trained SR−/− and WT animals to navigate a water bath to find a hidden platform using visual cues (Fig. 4a). The spatial memory acquisition phase consisted of eight consecutive days of training, with four trials per day, using a fixed platform location. For the first 3 days the intertrial interval was 15 minutes. For days 4-8 the intertrial interval was 10 minutes. Acquisition of spatial memory appeared normal in SR−/− males and females when mean latency to platform was used as a measure of performance (Fig. 4b). On the eighth day, the mice were held in the testing room for 3 hours following the end of the training session and then subjected to a 60-second probe trial. WT animals and SR−/− females showed normal spatial memory in this assay, but SR−/− males showed impairment. While male and female WT mice and female SR−/− mice spent significantly more time in the target quadrant of the

water maze (which had previously contained the hidden platform) than in any other quadrant of the maze, male SR−/− mice did not, indicating that they lacked spatial reference memory (Fig. 4c) (one-way repeated measures ANOVA p= 0.017; Dunnet's post hoc test comparing adjacent and opposite quadrant time to target quadrant time revealed that time spent in an adjacent quadrant was not significantly different from time spent in target quadrant). On days 9-12, the platform was moved to a new position each day to test reversal learning and within-session improvement based on information gained from previous trials; the intertrial interval was 10 minutes. There was no significant difference between SR−/− and WT mice in this phase of the task (Fig. 4d).

Discussion

The critical role of the NMDAR GMS in glutamatergic neurotransmission has been previously established. We have demonstrated that genetic depletion of D-serine perturbs physiology and behavior. The decay kinetics of evoked NMDAR-mediated EPSCs are slower in hippocampal slices from SR−/− mice than in WT, suggesting that these synapses contain a greater proportion of NR2B-containing NMDARs. This shift in kinetics and molecular composition of the NMDAR may constitute a compensatory response to the overall decrease in NMDAR-mediated neurotransmission as demonstrated by lower S-nitrosylation of intracellular proteins. The occupancy of the GMS of NR1 is lower in SR−/− animals, suggesting that a basal increase in synaptic glycine to compensate for low D-serine levels is unlikely. The marked attenuation in LTP of the Schaffer collateral-CA1 synapse in SR−/− mice is a striking correlate to the impairment in spatial memory shown by SR−/− males in the water maze probe trial.

The SR−/− mouse exhibits mild hyperactivity, a feature common to many pharmacologic and genetic models of NMDAR hypofunction, and elevated startle reactivity. Sexual dimorphism in the ASR has been widely reproduced, and it has been reported that males of inbred mouse strains have higher baseline ASR than females34. The unconditioned ASR can be considered a measure of baseline anxiety35. SR−/− females were less likely to explore the center zone of the assay chamber under conditions that were not designed to serve as an anxiogenic open field test. We believe that this experiment has allowed us to observe behavior consistent with elevated anxiety in the female SR−/− animals, whereas more anxiogenic conditions may have occluded such an effect relative to WT. The nature of the effects of the SR−/− genotype on anxiety and fear is of particular interest given the demonstrated role of the GMS in extinction of conditioned fear36.

The intact PPI response in the SR−/− mouse may reflect either that D-serine is not necessary for the function of the circuitry underlying this phenomenon in C57BL6 mice, or that compensations in the constitutive knockout occlude its importance. The possibility that this animal model has differential vulnerability to pharmacologic manipulations that disrupt PPI has yet to be addressed. The locomotor activity, anxiety-like, and spatial memory aspects of the SR−/− behavioral phenotype are sexually dimorphic. The differential appearance of phenotypic features arises from a complex interaction between sex, genotype, and the nature of the behavioral tasks. As these tasks are performed differently by males and females, it is not surprising that one sex might display a deficit more readily than the other. Based on the

absence of any effect of sex in the analysis of our HPLC data, we conclude that the dimorphisms are not due to a differential effect of our mutation on D-serine levels. Our genetic model may be useful in understanding sex differences in the manifestations of NMDAR hypofunction.

Given the fact that schizophrenia appears to be a disorder of complex genetics with a strong environmental component, manipulating one genetic pathway implicated in the disorder would not be expected to replicate the entire phenotype but rather components thereof; in other words, an endophenotype. Marked reduction of endogenous D-serine thus appears to reproduce the subtle cognitive impairment associated with the schizophrenia endophenotype but not the disruption in PPI. In this regard, the SR−/− mouse shares phenotypic features with a DISC1 mutant mouse, in which another schizophrenia risk gene has been manipulated37. Further investigations of these animals are expected to yield new insights into the biological basis of psychiatric illness and aid in the development of novel therapeutic strategies.

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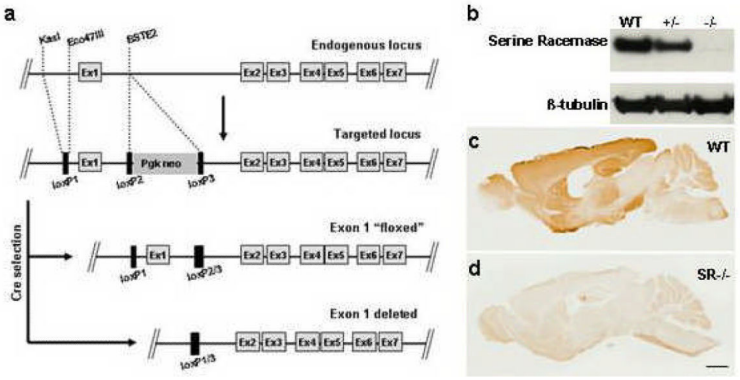

Figure 1.

The serine racemase exon 1 knockout (SR−/−) mouse model. (a) Exon 1 of the SR gene was targeted for deletion by insertion of a loxP sequence and a loxP-flanked PGK-Neo neomycin resistance sequence at upstream and downstream restriction sites respectively. Breeding of animals carrying the targeted locus with animals that constitutively expressed Cre protein produced offspring carrying desired constructs. (b) Western blot of whole brain protein extracts revealed a decrease in SR protein in SR+/− animals and an absence of SR protein in SR−/− animals. (c) SR protein expression was evident throughout the cortex, striatum, and hippocampus of WT mice. (d) SR protein expression was undetectable in SR−/− mice by immunohistochemical analysis. Scale bar represents 2 mm.

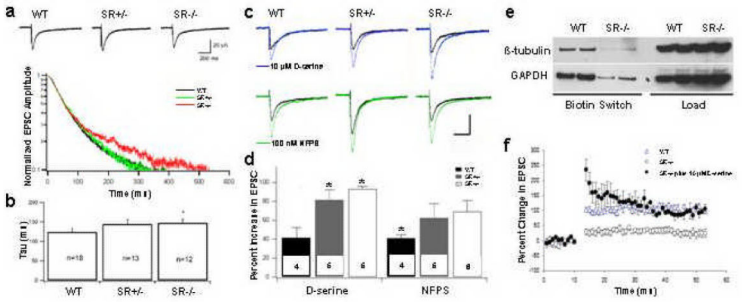

Figure 2.

Changes in NMDAR-mediated physiology in SR mutant mice. Values are shown and stated as Mean ± SEM. P-values less than 0.05, indicated by asterisks, are of comparisons to wild type using one-tailed Student's t test. (a) NMDAR EPSCs take longer to decay in SR mutants. (b) Time constant of decay (tau) of NMDAR EPSCs is higher in SR−/− mice (WT: 124.32 ± 8.72 ms, n=18; SR+/− : 144.31 ± 11.01 ms, n=16, $p < 0.05$; SR−/− : 157.38 ± 8.67 ms, n=16, $p < 0.05$). (c) Baseline amplitudes (black) of NMDAR EPSCs of WT, SR+/− and SR−/− were enhanced by application of 10 μM D-serine (blue) or 100 nM NFPS (green). (d) Amplitude increase of NMDAR EPSCs induced by 10 μM D-serine was higher in SR +/− and SR −/− compared to WT mice (WT: 41.29 ± 10.93% , n=4; SR+/− : 81.03 ±11.21% , n=5, $p < 0.05$; SR−/− : 93.19 ± 2.24%, n=5, $p < 0.05$).) Amplitude increase of NMDAR EPSCs induced by 100 nM NFPS was higher in SR −/− compared to WT mice (WT: 40.68 ± 4.04 %, n=4; SR+/−: 62.11 ± 15.57%, n=5; SR−/− : 69.19 ±11.73%, n=6, $p < 0.05$. (e) Biotin-switch assay showed that SR−/− mice have ~70% reduced S-nitrosylation of proteins (n=4 p<0.001) (f) Using a pairing protocol to induce LTP, no significant changes were observed in the amplitude of the EPSC in SR−/− (○, 23.72 ± 11.75%, n = 10) while the same protocol induced an increase in the amplitude of EPSC in WT mice (△, 104.96 ± 22.5%, n = 5). 10 μM D-serine in the bath solution 10 min prior to the application of the protocol restored LTP in slices from SR−/− mice (●, 93.71 + 6.83%, n = 5).

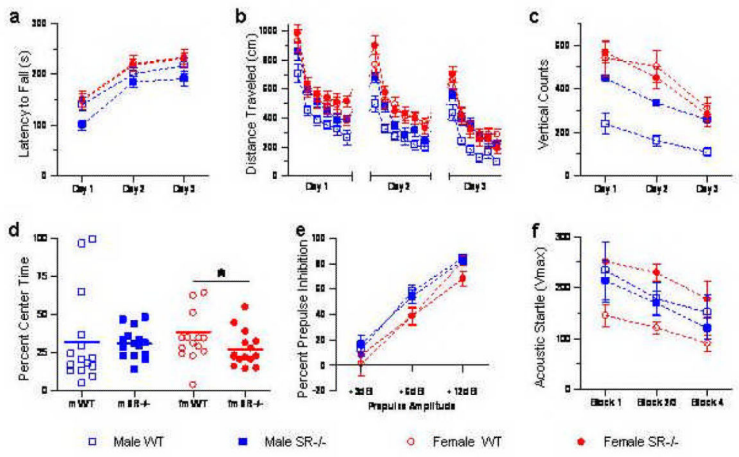

Figure 3.

Motor activity and startle in SR−/− mice. (a) SR−/− mice showed no significant deficit in motor coordination or learning in a three-day rotarod task. (b) SR−/− male mice traversed more distance and (c) exhibited more vertical behavior (rearing and jumping) than WT controls in a three-day locomotor activity assay. (d) SR−/− female mice spent less time in the center zone of the activity chamber on the first day of the activity assay. (e) SR−/− showed no deficit in prepulse inhibition (PPI) of the acoustic startle response (ASR). (f) All experimental groups showed habituation of the ASR, but SR−/− female mice showed elevated startle reactivity. Values are shown as Mean ± SEM. Male WT: blue open squares; male SR−/−: blue closed squares; female WT: red open circles; female SR−/−: red closed circles; n= 15 per group.

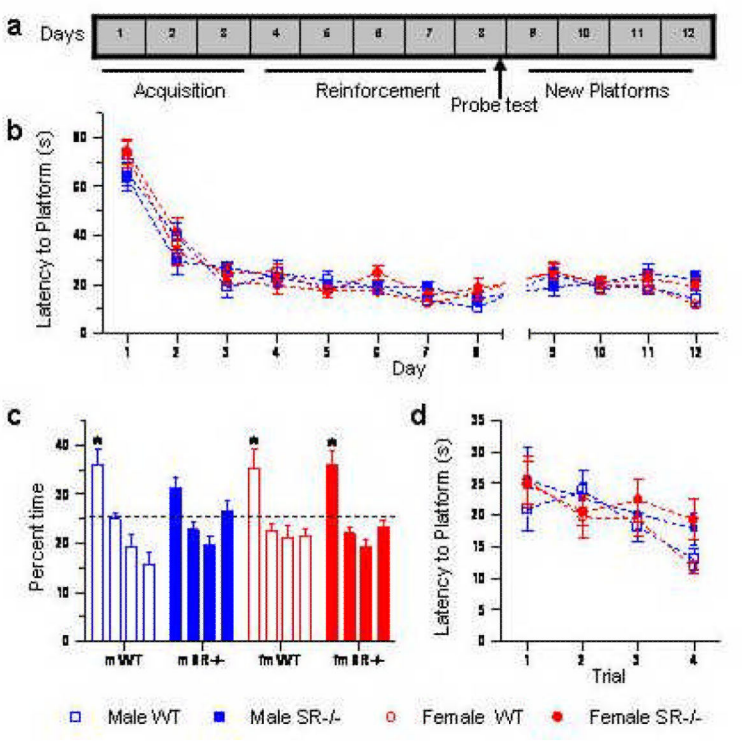

Figure 4.

Spatial learning and memory in SR−/− mice. (a) Mice were trained to learn the location of a hidden platform for eight days, subjected to a probe test on the eighth day, and then tested with new platform positions on each day for four days. (b) SR−/− mice matched WT performance during as assessed by latency to find the hidden platform. (c) The probe test revealed a deficit in spatial reference memory in SR−/− males. For each experimental group, the bars shown represent the percent time spent in the target quadrant (formerly containing the platform), an adjacent quadrant, the opposite quadrant, and the other adjacent quadrant of the maze. (d) There was no significant difference between SR−/− and WT mice in the trial-to-trial learning of new platform positions as assessed by latency to platform. Values shown are mean latencies for trials 1-4 from days 9-12. Values are shown as Mean ± SEM. Male WT: blue open squares; male SR−/−: blue closed squares; female WT: red open circles; female SR−/−: red closed circles; n= 15 per group.

Table 1

Levels of D-serine and L-serine in cortex.

Genotype	Sex	N	D-serine	L-serine	D/L ratio
WT	M	14	3.36 ± 0.52	8.54 ± 1.17	0.385 ± 0.010
	F	11	3.79 ± 0.32	10.72 ± 0.86	0.356 ± 0.014
SR+/−	M	12	2.49 ± 0.56	9.89 ± 1.47	0.235 ± 0.022***
	F	11	2.69 ± 0.39*	9.60 ± 1.12	0.275 ± 0.011***
SR−/−	M	11	0.37 ± 0.11**	10.67 ± 2.29	0.030 ± 0.003***
	F	11	0.41 ± 0.10**	10.78 ± 1.54	0.037 ± 0.007***

D-serine and L-serine levels in cortex dissected from SR−/−, SR+/− and WT animals were analyzed by HPLC. Values reported are in μmoles per gram protein (Mean ± SEM). D-serine in SR−/− animals was significantly lower than in WT animals, and D-serine/L-serine ratio was significantly lower in SR+/− and SR−/− animals than in WT animals (2-way ANOVAs revealed significant main effects of genotyope $p<0.0001$ for D-serine levels and D/L ratios and no significant effects of sex). Asterisks indicate statistically significant differences from WT of the same sex reveled by post-hoc Bonferroni multiple comparison tests

* $p<0.05$

** $p<0.01$

*** $p<0.001$

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