

Serine racemase deletion disrupts memory for order and alters cortical dendritic morphology

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There is substantial evidence implicating *N*-methyl-D-aspartate receptors (NMDARs) in memory and cognition. It has also been suggested that NMDAR hypofunction might underlie the cognitive deficits observed in schizophrenia as morphological changes, including alterations in the dendritic architecture of pyramidal neurons in the prefrontal cortex (PFC), have been reported in the schizophrenic brain *post mortem*. Here, we used a genetic model of NMDAR hypofunction, a serine racemase knockout (SR^{-/-}) mouse in which the first coding exon of the mouse SR gene has been deleted, to explore the role of D-serine in regulating cognitive functions as well as dendritic architecture. SR^{-/-} mice exhibited a significantly disrupted representation of the order of events in distinct experiences as showed by object recognition and odor sequence tests; however, SR^{-/-} animals were unimpaired in the detection of novel objects and in spatial displacement, and showed intact relational memory in a test of transitive inference. In addition, SR^{-/-} mice exhibited normal sociability and preference for social novelty. Neurons in the medial PFC of SR^{-/-} mice displayed reductions in the complexity, total length and spine density of apical dendrites. These findings show that D-serine is important for specific aspects of cognition, as well as in regulating dendritic morphology of pyramidal neurons in the medial PFC (mPFC). Moreover, they suggest that NMDAR hypofunction might, in part, be responsible for the cognitive deficits and synaptic changes associated with schizophrenia, and highlight this signaling pathway as a potential target for therapeutic intervention.

Keywords: Dendritic morphology, D-serine, NMDA receptors, schizophrenia, sequence memory, temporal order memory

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Substantial evidence has shown that *N*-methyl-D-aspartate receptors (NMDARs), a type of ionotropic glutamate receptor, are essential for multiple aspects of episodic memory and cognition (Nakazawa *et al.* 2003; Rajji *et al.* 2006; Tsien *et al.* 1996). NMDARs are considered molecular 'coincidence detectors', as activation requires postsynaptic depolarization, and the binding of two agonists, glutamate, and either glycine or D-serine, at the glycine modulatory site (GMS; Tsien 2000). D-serine is enriched in corticolimbic regions of the brain, where its localization closely parallels that of NMDARs (Schell *et al.* 1995). Reduced levels of D-serine in serum and in cerebrospinal fluid (CSF), as well as alterations in the level of NMDAR expression, have been associated with cognitive impairment (Potier *et al.* 2010; Turpin *et al.* in press; Yang *et al.* 2005), as well as schizophrenia (Akbarian *et al.* 1996; Bendikov *et al.* 2007; Hashimoto *et al.* 2003; Morita *et al.* 2007).

Patients suffering from schizophrenia exhibit significant impairments in memory, problem solving, and executive functions (Mesholam-Gately *et al.* 2009), which are associated with both structural and functional abnormalities within the hippocampus and prefrontal cortex (PFC). In particular, the complexity of dendritic branching, total dendritic length, and dendritic spine density of pyramidal neurons is reduced in the PFC of patients with schizophrenia (Garey *et al.* 1998; Glantz & Lewis 2000; Kalus *et al.* 2000; Rajkowska *et al.* 1998). Disruptions in dendritic morphology alter the cortical and/or thalamic circuitry, which in turn might be the neurobiological substrate underlying the cognitive deficits observed in patients with schizophrenia (Lewis & Gonzalez-Burgos 2008).

Animals lacking serine racemase (SR^{-/-}), the enzyme that synthesizes D-serine from L-serine, provide an important tool for exploring the contribution of D-serine to memory and cognitive function (Morita *et al.* 2007; Verrall *et al.* 2007). A prior assessment of SR^{-/-} mice, an animal with reduced NMDAR-dependent glutamatergic transmission, has showed that these animals exhibit disruptions in anxiety as well as impairment in spatial memory (Basu *et al.* 2009). In order to expand upon these findings, here, we assessed several domains of higher-level cognitive function, including episodic memories, which are known to engage the hippocampus and PFC, and examined dendritic plasticity of pyramidal neurons in the PFC as a biological marker of neurotransmission. We report here that D-serine plays a critical and selective role in memory for order and is important for proper dendritic

patterning and maintenance of spine density on excitatory neurons.

Materials and methods

Animals

Serine racemase heterozygous (Het; SR+/-) mice were originally generated at McLean Hospital in Belmont, MA, as previously described (Basu *et al.* 2009). A colony of SR+/- mice is maintained at McLean Hospital by continuous backcrossing of each generation to a C57BL/6J background. Subjects of this study were bred from SR+/- mice of F10 or higher backcross generation. C57BL/6J breeders were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and bred in-house at McLean Hospital. New C57BL/6J breeders were imported every five generations. Mice on which *post mortem* analyses were performed were bred and housed at McLean Hospital as previously described (Basu *et al.* 2009). Briefly, non-sibling heterozygous (SR+/-) offspring of backcrosses were mated to each other to produce homozygous (KO; SR-/-) and wildtype (WT; SR+/+) experimental subjects. The SR+/- strain was re-derived by embryo transfer to pseudopregnant C57BL/6J surrogate mothers at the Jackson Laboratory (Bar Harbor, ME, USA) before transfer to Boston University. Homozygous (KO; SR-/-) and WT (SR+/+) mutants tested in behavioral experiments were generated from heterozygous breeding pairs at Boston University. At weaning, tissue samples were sent to the Charles River Laboratory for genotyping.

All animals at Boston University were maintained on a reverse 12-h light/dark cycle (0900 off; 2100 on), while those housed at McLean Hospital were maintained on a regular 12-h light/dark cycle. Animals were given *ad libitum* access to food and water, unless otherwise specified in behavioral methods. Only age-matched, littermate males were used in experiments reported here. Animals were used for more than one behavioral experiment in order to reduce the overall number of animals used in these studies. The group sizes were as follows for each behavioral experiment: object recognition: 21 WT and 17 SR-/- mice; object displacement: 12 WT and 12 SR-/- mice; object order: 13 WT and 11 SR-/- mice; odor sequence memory: 12 WT and 11 SR-/- mice; transitive inference: 8 WT and 10 SR-/- mice; sociability: 20 WT and 16 SR-/- mice; social novelty: 19 WT and 14 SR-/- mice. The group sizes for Golgi-staining are: 5 WT and 5 SR-/- mice. The IACUCs of Boston University and McLean Hospital approved the treatment and use of the animals in these experiments.

Memory for objects: object recognition, object displacement and object order memory

Episodic memory is commonly characterized as the ability to remember specific events in the place and time that they occurred (Clayton & Dickinson 1998). We assessed these components of episodic memory by using a series of tests of memory for objects, their spatial locations, and the order in which they were experienced (Barker *et al.* 2007). Animals were first habituated to a rectangular Plexiglas apparatus with proximal visual cues on the walls for a 5-min period (40 cm length \times 20 cm width \times 37 cm height). Exploratory behavior was recorded via a WebCam (Gigaware®, Norwich, UK) positioned above the testing arena and later scored for investigation time of each object in the sample sessions and the test session. Investigation was measured as the time over a 5-min test in which the animal directly explored each object by sniffing or whisking, when the animal's nose was facing and within 2 cm of the object. Investigation also included rearing or climbing onto the object. Two researchers blind to the subject's genotype coded each video for investigation of each object using a stopwatch. Any animal showing a significant spatial bias, as defined by the greatest amount of exploration occurring at the same location relative to all other locations in successive trials, was removed from the analysis. Only one WT mouse was removed from analysis of the object recognition test for exhibiting a significant spatial bias. Discrimination ratios based on exploration of the objects were used for measures of each type of memory.

Object recognition

In the object recognition assay, mice were presented with a set of two identical objects A (small glass beaker; Corning, Lowell, MA, USA), to which they had not been exposed previously, in a rectangular Plexiglas apparatus (40 cm length \times 20 cm width \times 37 cm height; Fig. 1a, left panel). The objects were presented on the east and west walls of the testing apparatus, mid-way between the north and south corners. Mice were allowed 5 min to explore both objects. After this exploration period, they were briefly returned to their home cages for sufficient time to clean the apparatus and replace the objects (approximately 1 min) and then placed back into the testing apparatus. In the test phase, one object was replaced by a copy of object A (left-right position was counter-balanced) and the other object was replaced in the same location by a different object B (plastic cylinder; Nalgene, Rochester, NY, USA). The animals were then given another 5-min exploration period and a discrimination ratio was calculated as the difference between the exploration time for object B and the exploration time for object A, divided by the sum of those times. Pilot studies ensured that there were no innate biases for the objects used in this study ($t_{(14)} = 0.024$, $P = 0.981$).

Object displacement

In the object displacement assay, animals were presented with two identical objects C (small plastic container; Nalgene, Rochester, NY, USA), different from those they experienced in the object recognition task (Fig. 1b, left panel). The objects were presented on the northeast and northwest walls of the same testing apparatus used in the object recognition task, and animals were allowed 5 min to explore both objects. After this exploration period, they were briefly returned to their home cages for sufficient time to clean the apparatus and replace the objects (approximately 1 min), and then placed back into the testing apparatus. In the test phase, one object (left-right position was counter-balanced) was replaced by a copy of object C in a different location, while the other object was replaced by another copy of object C in its original location. Then the animals were allowed to explore the objects for 5 min and the discrimination ratio was calculated as the difference between the exploration time for the relocated object C and the exploration time for the stationary object C, divided by the sum of those times.

Object order memory

The object order assay was conducted in two phases (Fig. 1c, top panel). In Sample 1, animals were allowed 5 min to explore two identical objects D (glass container; VWR, West Chester, PA, USA) in the east and west corners of the same testing apparatus used in the previously described tests. After a 50-min delay, in Sample 2, animals were allowed 5 min to explore two new identical objects E (small plastic bottle; VWR, West Chester, PA, USA) presented in the same locations. After another 50-min delay, in the test phase, animals were presented with a copy of object D and a copy of object E in the same location (left-right position was counter-balanced) and allowed to explore the objects for 5 min. The discrimination ratio was calculated as the difference between the exploration time for the less recent object D and the more recent object E, divided by the sum of those times. Pilot studies ensured that there were no innate biases for the objects used in this study ($t_{(14)} = -0.987$, $P = 0.340$).

Memory for the order of events in distinct sequences

Mice were tested on a sequence memory task that assessed the ability to remember the order of specific odors presented within two different series (DeVito & Eichenbaum, under review). Animals were placed on food restriction and maintained at 85% of free feeding weight. Over a 3-day period, animals were introduced to Plexiglas cages (26 cm length \times 15 cm width \times 13 cm height) in which they were allowed to dig for chocolate sprinkle rewards buried in small plastic cups filled with sand. Once all subjects exhibited reliable digging behavior, the animals were exposed to two different odor sequences each day over a 5-day period. All odors were prepared at 1% concentration by weight in sand.

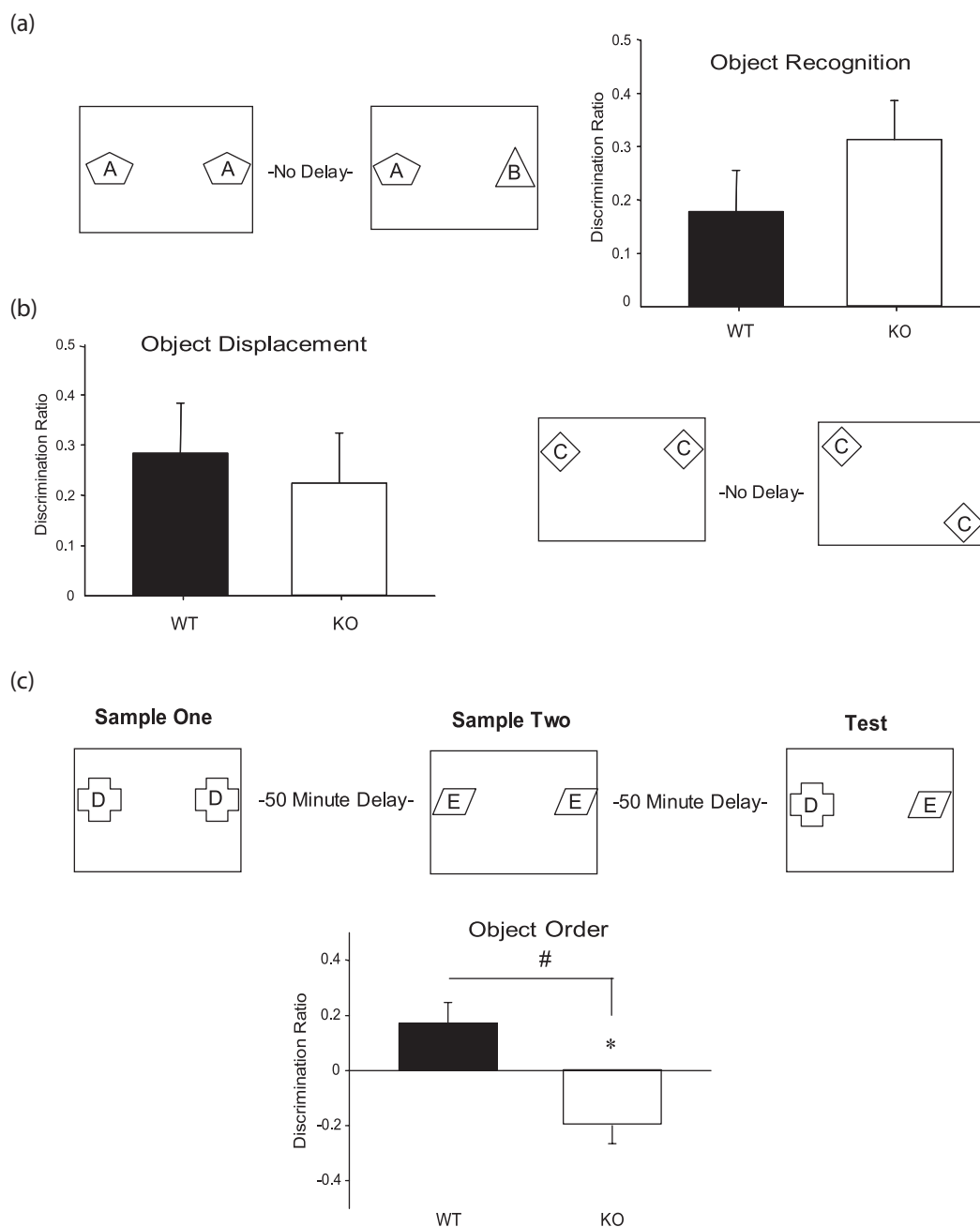


Figure 1: Performance of the WT mice and SR^{-/-} mice on object recognition, object displacement and object order.

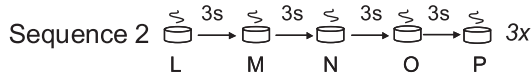
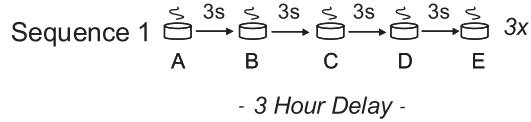
(a) Arrangement of stimuli in the object recognition test. A discrimination ratio was used to measure preference for a novel vs. a familiar object. (b) Arrangement of stimuli in the object location test. A discrimination ratio was used to measure preference for a displaced vs. a stationary object. (c) Arrangement of stimuli in the object order test. A discrimination ratio was used to measure preference for an older vs. a more recent object. * indicates $P < 0.05$ discrimination ratio less than zero; # indicates $P < 0.05$ difference in discrimination ratio between groups.

Sequence 1, with the odors referred to as A, B, C, D, E, consisted of the following odors: A = cilantro (Spice Islands Trading Company, San Francisco, CA, USA), B = marjoram (Frontier Natural Products Co-op, Norway, IA, USA), C = dill (Frontier Natural Products, Norway, IA, USA), D = parsley (Frontier Natural Products Co-op,

Norway, IA, USA) and E = allspice (Spice Islands Trading Company, San Francisco, CA, USA). Sequence 2, referred to as L, M, N, O, P, consisted of the following odors: L = lemon, (McCormick, Hunt Valley, MD, USA), M = cinnamon (McCormick, Hunt Valley, MD, USA), N = tarragon [(Frontier Natural Products Co-op, Norway, IA,

(a)

Days 1-5: Sequence Presentation Days



Days 6-9: Probe Test Days

Within Sequence		Between Sequence	Novelty	
Day 6	Day 7	Day 8	Day 9	
L vs. P	A vs. D	A vs. N	L vs. X	A vs. X
C vs. D	N vs. P	E vs. O	C vs. X	N vs. X
M vs. O	B vs. C	B vs. L	M vs. X	B vs. X



(b)

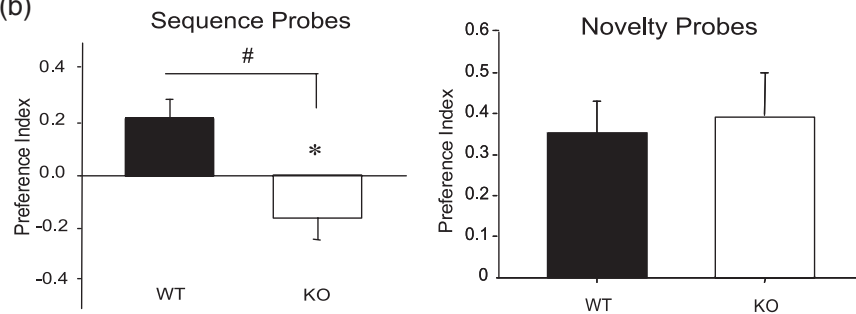


Figure 2: Performance of the WT mice and SR^{-/-} mice on odor sequence memory. (a) Schematic of the apparatus and odors used to test the animals on sequence memory. (b) A PI was used to assess the amount of time spent digging in the item occurring earlier in the sequence compared to an item occurring later in the sequence. * indicates $P < 0.05$ discrimination ratio less than zero; # indicates $P < 0.05$ difference in discrimination ratio between groups.

USA), O = anise (McCormick, Hunt Valley, MD, USA) and P = orange (McCormick, Hunt Valley, MD, USA). These odors were selected as stimuli for which mice show no strong natural attraction or aversion.

In the first session each day, animals were presented with Sequence 1. At the beginning of each sequence, the animal was presented with the first odor, which contained a buried chocolate sprinkle that the mouse could obtain by digging in the sand (Fig. 2a). This process was repeated for the remaining four odors in that sequence until the animal had been exposed to all five odors successively with a 3-second inter-stimulus interval. Each animal was presented with Sequence 1 three times with a 3-min inter-trial interval. Three hours after the first session was completed, the animals were exposed to Sequence 2 in the same manner. Each odor was presented in one of several locations within the testing apparatus in order to prevent the development of spatial biases. This procedure for sequence presentation was repeated for 5 days. After these 5 days, the animals were not subsequently exposed to the odor sequences.

Beginning the next day, the animals were administered probe tests that assessed memory for the odors within and between each of the sequences. On each of these probe tests, two odors were selected, and both were presented without buried rewards. We measured the amount of time the animals spent digging in each cup and the two digging times were used to calculate a preference index (PI; Bunsey

& Eichenbaum 1996); $PI = (\text{Time spent digging in the odor that occurred earlier in the sequence} - \text{time spent digging in the odor that occurred later in the sequence}) / (\text{Total time digging in both odors})$. In order to prevent digging behavior from extinguishing on these probe trials, the animal was presented with a cup of unscented sand baited with a chocolate sprinkle on the surface of the cup immediately after each probe trial.

On the first two days of probe testing (days 6 and 7), the animals were presented with odors selected from the same sequence with which they were presented earlier. These were considered 'within sequence' probe tests as they evaluated the ability of the animals to remember the order of odors that were presented within one of the specific sequences. On the first day of probe testing (day 6), the animals were presented with L vs. P, C vs. D, and M vs. O. On the second day of probe testing (day 7), the animals were presented with A vs. D, N vs. P and B vs. C.

On the third day of probe testing (day 8), the animals were presented with pairs consisting of one odor from Sequence 1 and one odor from Sequence 2. These were considered 'between sequence' probe tests because they evaluated the ability of the animals to compare the times at which the odors were presented over the course of each day, i.e. earlier in Sequence 1 vs. 3 h later in Sequence 2. The animals were presented with A vs. N, E vs. O and B vs. L.

Finally, on the fourth day (day 9), the animals were presented with an odor from either sequence against a novel odor they had never experienced. These were considered 'novelty' probe tests because they evaluated the ability of the animals to recognize novel as compared to familiar odors. The animals were presented with L vs. caraway (McCormick, Hunt Valley, MD, USA), C vs. mace (McCormick, Hunt Valley, MD, USA) and M vs. sage (McCormick, Hunt Valley, MD, USA), N vs. onion (McCormick, Hunt Valley, MD, USA) and B vs. strawberry (Nestle, Glendale, CA, USA).

We did not counterbalance for the assignment of the order of odor presentations during training because there would be a very large number of permutations of the order of five items in each sequence. And we did not counterbalance the order of probe tests on within-sequence and between-sequence preferences. However, our previous study showed that animals that are not trained on these sequences do not exhibit within-sequence or between-sequence order preferences, and the pattern of performance on the two types of probe tests does not depend upon the order of their presentation (DeVito & Eichenbaum, under review).

Relational memory

The transitive inference task assesses the ability of animals to learn relations among stimuli formulated as a series of overlapping discrimination problems that share common elements (Dusek & Eichenbaum 1997). In the classic test for relational memory, subjects

are initially trained on a series of premise problems (e.g. choose A over B, then chose B over C, then chose C over D, then chose D over E), then tested for the capacity to infer the transitive relation between items that had never appeared together (B vs. D). The choice of B over D indicates the existence of a relational hierarchy (A over B over C over D over E).

Mice were trained on the premise problems and probe tests for transitive inference in a series of stages. Initially, animals were placed on food restriction and maintained at 85% of free feeding weight. Over a 3-day period they were trained to dig for chocolate sprinkle rewards buried in sand that filled small plastic cups placed in Plexiglas cages (26 cm length \times 15 cm width \times 13 cm height). Once all subjects exhibited reliable digging and reward retrieval behavior, they were trained on a preliminary olfactory discrimination. All odors were prepared at a concentration of 1% by weight. The reward-baited odor cup was presented at one of several locations randomly selected on each trial, in order to prevent the development of spatial biases.

Following the preliminary discrimination, animals were trained on a series of premise problems formulated as overlapping discriminations [A+ vs. B-, B+ vs. C-, C+ vs. D- and D+ vs. E-, where A = paprika (CVS, Woonsocket, RI, USA), B = coffee (Folger's, Cincinnati, OH, USA), C = basil (McCormick, Hunt Valley, MD, USA), D = cumin (McCormick, Hunt Valley, MD, USA) and E = cocoa (Hershey's, Hershey, PA, USA); + and - refer to rewarded and non-rewarded odors, respectively; Fig. 3a]. Animals were trained in two eight-trial sessions per day across four training stages that began with large blocks of trials of the same type and involved progressively including more of the trial types. Training continued until animals reached a

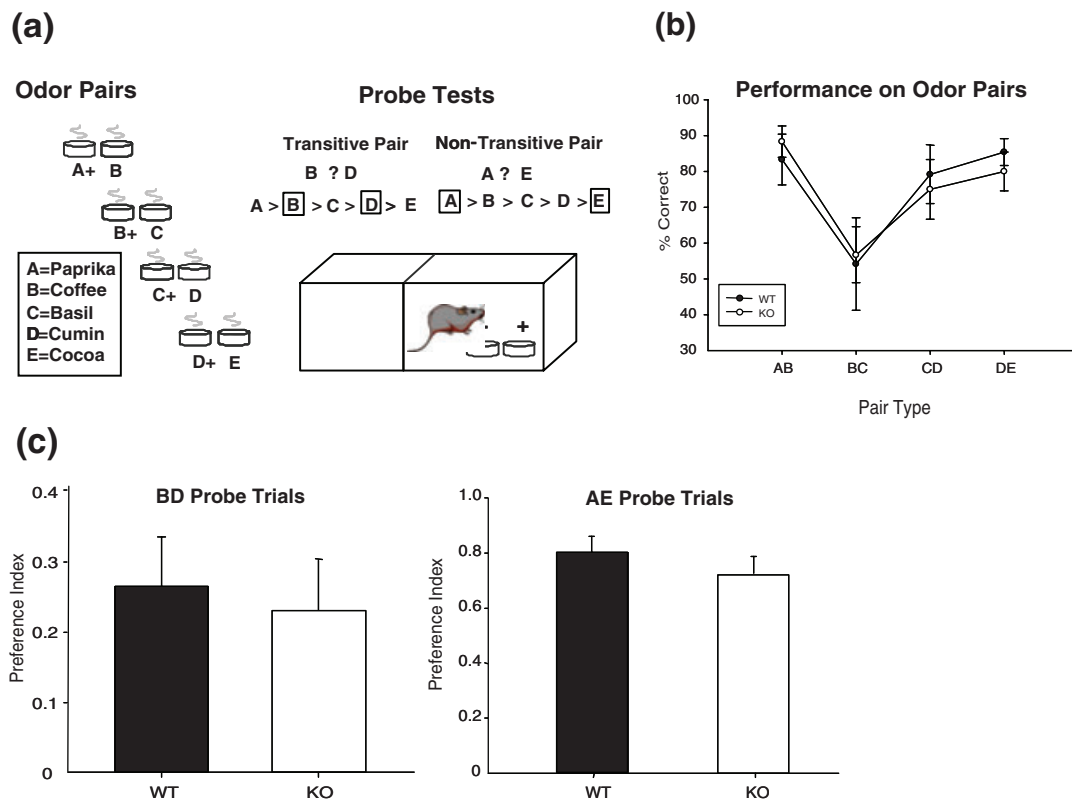


Figure 3: Performance of WT mice and SR^{-/-} mice on transitive inference. (a) Schematic of the apparatus and odors used to train and test the animals on transitive inference. (b) The number of days to reach criterion across the four stages of training was used to assess acquisition of the odor pairs. Percentage correct was used to assess performance on the training pairs during the transitive inference test. (c) A PI was used to assess the amount of time spent digging in B vs. D ('transitive pair') and A vs. E ('non-transitive pair') on the probe tests.

criterion of 75% accuracy (6/8 trials) across two consecutive days at each stage of training.

The day after reaching criterion on the last training stage, animals were given probe tests for transitive (B vs. D) as well as non-transitive (A vs. E) judgments, composed as novel discriminations between items that had not previously been presented together. Four BD and four AE probe tests were intermixed within the training pairs over a 2-day period, given at trials 3, 6, 11 and 14 within the regular 16 trials. A correct judgment (choosing B over D) on the BD pair required that animals had linked the odor premises into an integrated hierarchy so that they could make the inference across the missing overlapping element C. In contrast, a correct judgment of A over E could be made without reference to the structure of the odor premises because odor A was always rewarded and odor E was never rewarded; the AE pair served as a control for the ability to make a judgment about a novel pairing in which performance did not depend on existence of the hierarchical representation. Neither cup was baited during probe trials and we measured the amount of time the animals spent digging in each cup. These digging times were used to calculate a PI (Bunsey & Eichenbaum 1996). For the B vs. D test, $PI = (B - D)/(B + D)$; for A vs. E, $PI = (A - E)/(A + E)$.

Social recognition: sociability and preference for social novelty

A social recognition paradigm was adapted from Moy *et al.* (2004). The main difference between the Moy *et al.* (2004) protocol and the methods described here is the use of littermates as stimulus animals as compared to animals from different litters. Our version of this paradigm was used in a previous study and showed that this protocol is robust at detecting differences in sociability and preferences for social novelty (DeVito *et al.* 2009). The apparatus was a rectangular Plexiglas apparatus (40 cm length x 20 cm width x 37 cm height), divided into three areas of equal width by a thin plastic mesh (20 cm height x 20 cm length) that allowed the animals to explore one another but prevented aggressive interactions. A stimulus animal, an age-matched, naive male SR+/- mouse from a different litter, was placed in one of the outer compartments (left and right placement

was counter-balanced). The test animal was then placed in the center compartment and allowed to investigate both outer compartments. The apparatus was cleaned with 70% ethanol after each test.

Sociability

In this test animals were exposed to one compartment containing a familiar littermate while the other compartment was empty (Fig. 4a, left panel). A stimulus animal, an age-matched, naive male SR+/- mouse from a different litter, was placed in one compartment and the other compartment was empty. Investigation was measured as the time over a 5-min test in which the animal directly explored each compartment by sniffing, when the animal's nose was facing and within 2 cm of the compartment, and climbing the mesh divider. Each animal's behavior was recorded following a 1-min period of habituation to the testing apparatus. An animal expressing normal sociability will spend a greater amount of time investigating the compartment containing the familiar littermate as compared to the empty compartment. The score for sociability was calculated as the difference between the exploration time for the compartment containing the familiar littermate and that for the empty compartment, divided by the sum of those times.

Preference for social novelty

The day following sociability testing, the animals were assessed on preference for a novel conspecific animal (Fig. 4b, left panel). A stimulus animal, an age-matched, naive male SR+/- mouse from a different litter, was placed in one compartment and a littermate (different from the animal used in the sociability test) was placed in the other compartment. As in the test for sociability, investigation of each compartment was defined as direct exploration by sniffing and climbing the mesh divider. Each animal's behavior was recorded following a 1-min period of habituation to the testing apparatus. An animal expressing a normal preference for social novelty will spend a greater amount of time investigating the compartment containing the animal from a different litter (novel) as compared to the compartment containing the littermate (familiar). The score for social novelty was

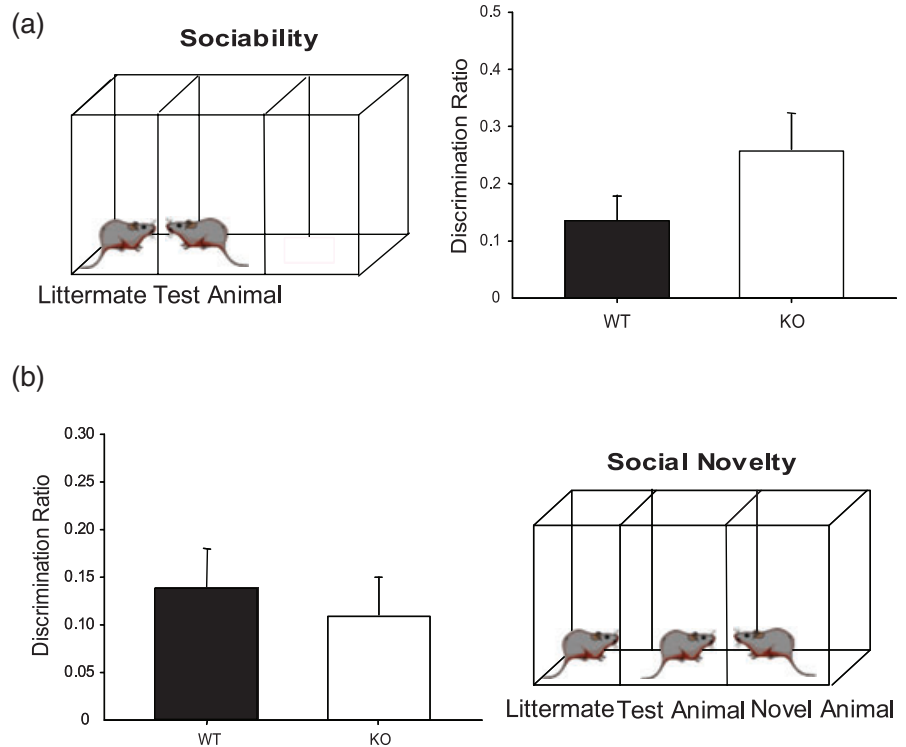


Figure 4: Performance of WT mice and SR-/- mice on sociability and social novelty. (a) Apparatus used to assess sociability. A discrimination ratio was used to assess preference for a compartment containing a littermate as compared to an empty compartment. (b) Apparatus used to assess social novelty. A discrimination ratio was used to compare the amount of time spent investigating a familiar vs. novel animal.

calculated as the difference between the exploration times for the compartment containing the novel animal and that for the familiar littermate divided by the sum of those times.

Golgi staining

Golgi staining was performed on adult, male WT ($n = 5$) and SR $-/-$ ($n = 5$) mice using the FD Rapid GolgiStain Kit (FD NeuroTechnologies, Ellicott City, MD, USA) according to the manufacturer's instructions. Brains were removed, immersed in fixative (5 ml; equal volume of Solutions A/B) for 40 h, and then transferred into Solution C and stored at 4°C until slicing. The fixed brains were sliced on a freezing microtome (American Optical 860, Buffalo, NY, USA) at a thickness of 120 μm . Sections were mounted with Solution C on 3% gelatin-coated slides (75 \times 25 mm; FD NeuroTechnologies, Ellicott City, MD, USA). Excess Solution C was immediately removed from the slide using a Pasteur pipette and a fine paintbrush. Sections were kept at room temperature overnight, protected from light. Slides were then stained, dehydrated, and cleared with xylenes according to the manufacturer's protocol, without counterstaining.

Quantification of dendritic morphology

Pyramidal neurons in the mPFC chosen for reconstruction were located approximately 2.22 mm–1.54 mm anterior to bregma and included the cingulate, prelimbic and infralimbic cortices (Paxinos & Franklin 2001). Neurons selected for reconstruction were located in the middle third of the section and were not obscured by neighboring neurons and glia. Within each region examined, a minimum of three neurons was reconstructed for each mouse. Neurons were drawn at 40 \times on a Zeiss Axioskop40, and the morphology of apical and basilar arbors was quantified in three dimensions using Neurolucida (MBF Bioscience, Williston, VT, USA), with the experimenter blind to genotype. Sholl analysis was performed using the Neurolucida Explorer software version 8 to assess dendritic complexity (concentric circles increased in diameter by 10 μm increments).

Spine density analysis

Spines were counted on dendritic branches from a minimum of three neurons per region in each animal. For apical dendrites, spines were counted on the apical trunk (last 40 μm) and oblique segments separately. Neurons were visualized at 63 \times (oil-immersion) using a Leitz Laborlux12 microscope. One to two branches at each order were selected and spines were counted using BioQuant Nova Prime (BioQuant Image Analysis Corp., Nashville, TN, USA). Spines were counted on branches from the same neurons that were reconstructed at 40 \times for dendritic analysis.

Statistical analysis of morphometric data

The effect of genotype on total dendritic length and segment analyses was analyzed using two-tailed, unpaired Student's t -test. Dendritic complexity was analyzed using two-factor analysis of variance (ANOVA) with repeated measures, while spine density and maturity were compared using two-factor ANOVA. Significant F tests were followed up with Student's t -test comparisons. $P < 0.05$ was considered statistically significant.

Results

Memory for objects: object recognition, object displacement and object order memory

A two-way ANOVA comparing performance across the three tests showed a significant effect of test type and group \times test type interaction (group $F_{1,85} = 2.096$, $P = 0.152$; test type $F_{2,85} = 6.043$, $P = 0.004$; group \times test type interaction $F_{2,85} = 4.76$, $P = 0.011$). *Post hoc* tests showed that there was a significant difference between groups on the object

order task only ($t_{(22)} = 3.70$, $P = 0.001$; Fig. 1c, bottom panel). Further analyses showed that WT mice exhibited a significant preference for an earlier experienced object over a more recently experienced object ($t_{(24)} = 2.346$, $P = 0.028$). In striking contrast, SR $-/-$ mice showed the opposite preference, that is, greater exploration of the more recently experienced object than the less recently experienced object ($t_{(20)} = -3.016$, $P = 0.007$). Therefore, although the SR $-/-$ mice can discriminate objects previously experienced at different times, which clearly reflects some form of memory for the order of object presentations, the expression of that order memory is abnormal.

Both WT and SR $-/-$ mice exhibited a significant preference for the novel object as compared to the familiar object (WT mice: $t_{(40)} = 2.32$, $P = 0.025$; SR $-/-$ mice: $t_{(32)} = 4.31$, $P < 0.001$) and there was no significant difference in performance between the two groups ($t_{(36)} = -1.23$, $P = 0.225$; Fig. 1a, right panel). In addition, both WT and SR $-/-$ mice also exhibited a significant preference for the displaced object over the stationary object (WT mice: $t_{(22)} = 2.88$, $P = 0.009$; SR $-/-$ mice: $t_{(22)} = 2.25$, $P = 0.034$) and there was no significant difference in performance between the two groups ($t_{(22)} = 0.441$, $P = 0.663$; Fig. 1b, left panel). Therefore, the SR $-/-$ mice showed intact memory for object identity and object location.

Analysis of total exploration time during both the training sessions and test sessions showed that there was a significant difference in exploration time between the WT and SR $-/-$ mice on the training session of object recognition, such that the SR $-/-$ mice explored the objects for a greater period of time than did the WT mice ($t_{(74)} = 8.198$; $P = 0.005$); however, there were no differences between exploration in the test phase of the object recognition test ($t_{(74)} = 0.378$, $P = -0.888$). There were also no differences in exploration between the WT and SR $-/-$ mice at any other phase of the object displacement (training session: $t_{(46)} = 0.189$, $P = 0.851$; test session: $t_{(46)} = -1.07$, $P = 0.290$) and object order tasks (training session 1: $t_{(48)} = -1.354$, $P = 0.182$; training session 2: $t_{(48)} = -1.002$, $P = 0.321$; test session: $t_{(48)} = -1.097$, $P = 0.278$).

Memory for the order of events in distinct sequences

WT mice significantly preferred odors presented earlier within a sequence over odors presented later in the same sequence ($t_{(22)} = 3.205$, $P = 0.004$). In contrast, SR $-/-$ mice exhibited the reverse preference, preferring late-presented odors over earlier-presented odors in the same sequence ($t_{(20)} = -2.086$, $P = 0.05$). Correspondingly, the PI differed between the groups ($t_{(21)} = -3.69$, $P = 0.001$; Fig. 2b, left panel). These findings indicate that SR $-/-$ mice exhibit some form of memory for the order of experienced odors, in that they discriminate between odors previously experienced in different ordinal positions, but they express this memory abnormally.

Neither the WT ($t_{(22)} = -0.617$, $P = 0.544$) nor the SR $-/-$ mice ($t_{(20)} = 1.882$, $P = 0.074$) exhibited a significant preference for items that had been experienced in different sequences. The PI for the WT mice was -0.07 ± 0.11 and the PI for the SR $-/-$ mice was 0.21 ± 0.11 ; thus, the

SR^{-/-} mice showed a *marginal* preference for items occurring in Sequence 1 compared to Sequence 2 ($t_{(20)} = 1.882$, $P = 0.074$, as noted above). However, the difference in group mean preference scores failed to reach statistical significance ($t_{(21)} = -1.769$, $P = 0.091$).

We also tested whether animals could recognize the previously experienced odors using recognition tests in which the animals were given a choice between a novel odor and odors they had experienced in the trained sequences. Both groups performed at levels that were significantly different from chance (WT mice: $t_{(22)} = 4.546$, $P < 0.001$; SR^{-/-} mice: $t_{(20)} = 3.807$, $P = 0.001$), and there was no difference in performance between the groups ($t_{(21)} = -0.288$, $P = 0.776$; Fig. 2b, right panel). Finally, there was a significant difference across groups in the total amount of time spent digging during probe tests for items within each sequence ($t_{(284)} = 2.851$, $P = 0.005$), such that the WT mice spent a greater time digging in the probe trials; however, there was no difference in the total amount of time spent digging during probe trials for items between each sequence ($t_{(136)} = 1.311$, $P = 0.192$) or during novelty judgments ($t_{(274)} = -0.414$, $P = 0.679$).

Relational memory

SR^{-/-} and WT mice learned the four odor pair discriminations (premise problems) successfully and there was no difference between the two groups in the number of days required to reach criterion across the four stages of training (two-way ANOVA: group $F_{1,71} = 0.19$, $P = 0.659$; stage $F_{3,71} = 0.77$, $P = 0.514$; group \times stage interaction $F_{3,71} = 0.08$, $P = 0.967$). This indicates that SR^{-/-} mice have an intact ability to learn a series of overlapping odor discriminations.

Both groups also performed similarly on the premises when tested along with the probe pairs BD and AE (two-way ANOVA: group $F_{1,71} = 0.54$, $P = 0.923$; pair type $F_{3,71} = 6.50$, $P = 0.001$; group \times pair type interaction $F_{3,71} = 0.22$; $P = 0.881$; Fig. 3b). Although there were significant differences in performance on different premise pairs, there was no difference in performance between the two groups on performance of any of the premise pairs (AB: $t_{(16)} = -0.630$, $P = 0.537$; BC: $t_{(16)} = -0.172$, $P = 0.865$; CD: $t_{(16)} = 0.351$, $P = 0.730$; DE: $t_{(16)} = 0.776$, $P = 0.449$). Therefore, in addition to performing similarly on each odor pair, WT and SR^{-/-} mice also exhibited a similar pattern of performance across the pair types.

In the probe tests (Fig. 3c), both WT and SR^{-/-} mice exhibited a significant preference for B over D (WT: $t_{(14)} = 3.822$, $P = 0.002$; SR^{-/-} mice: $t_{(18)} = 3.344$, $P = 0.004$) and A over E (WT: $t_{(14)} = 13.858$, $P < 0.001$; SR^{-/-} mice: $t_{(18)} = 12.246$, $P < 0.001$) at levels that are significantly different from chance. Correspondingly, there was no difference in performance between the two groups on the BD ($t_{(16)} = 0.321$, $P = 0.752$) or AE probe tests ($t_{(16)} = 0.910$, $P = 0.376$). In addition, the total amount of time spent digging in all probe cups was comparable, indicating that both the WT mice and SR^{-/-} mice were equally motivated and displayed no differences in activity levels ($t_{(284)} = -0.676$, $P = 0.499$).

Social recognition: sociability and preference for social novelty

The sociability test assesses the animal's level of social motivation and interest in social interaction. WT and SR^{-/-} mice spent more time investigating the compartment containing the littermate vs. the empty compartment, and there was no significant difference between the group scores ($t_{(34)} = -1.679$, $P = 0.102$; Fig. 4a, right panel). The social preference of WT mice was significantly different from zero ($t_{(38)} = 3.209$, $P = 0.003$), as was the social preference of the SR^{-/-} mice ($t_{(30)} = 4.204$, $P < 0.001$).

The social novelty test assesses the animal's social memory, reflected by a preference for investigating a novel conspecific over a familiar one. WT and SR^{-/-} mice spent considerably more time investigating the compartment containing the novel animal vs. the compartment containing the familiar littermate, and there was no difference between groups ($t_{(31)} = 0.486$, $P = 0.630$; Fig. 4b, right panel). Both the WT mice ($t_{(36)} = 3.449$, $P = 0.001$) and the SR^{-/-} mice ($t_{(26)} = 2.830$, $P = 0.009$) exhibited preference scores that were significantly greater than zero.

Dendritic morphology of pyramidal neurons in the medial PFC

Multiple parameters were compared between WT and SR^{-/-} mice across the apical and basilar dendritic systems of pyramidal neurons. Sholl analysis showed that the neurons in SR^{-/-} had less complex branching of their apical dendrites (distance from soma: $F_{13,40} = 28.80$, $P < 0.0001$; genotype: $F_{1,40} = 5.01$, $P = 0.03$; distance \times genotype: $F_{9,40} = 1.57$, $P = 0.09$; Fig. 5a,b). The neurons of SR^{-/-} mice also had significantly less total apical dendritic length than WT mice ($t_{(40)} = 2.45$, $P = 0.02$; Fig. 5c); however, their basal dendrites did not show significant reductions in complexity (distance from soma: $F_{9,40} = 107.7$, $P < 0.0001$; genotype: $F_{1,40} = 1.641$, $P = 0.21$; distance \times genotype: $F_{9,40} = 0.59$, $P = 0.81$; Fig. 5d) or total dendritic length ($t_{(40)} = 1.51$, $P = 0.14$; Fig. 5e).

Segment analysis was performed to determine whether there were certain areas of the apical dendritic tree where these perturbations were concentrated in the neurons of SR^{-/-} mice (Table 1). The total length ($t_{(40)} = 2.45$, $P = 0.02$) and number of fifth-order branches ($t_{(40)} = 2.50$, $P = 0.02$) were significantly lower in SR^{-/-} mice, while the average length of fourth-order branches ($t_{(40)} = 2.07$, $P = 0.04$) was also reduced in the SR^{-/-} animals. Although there were no overall differences in basilar dendritic morphology, segment analysis showed that pyramidal neurons of SR^{-/-} mice had significant reductions specifically in the total length ($t_{(40)} = 2.18$, $P = 0.03$) and number ($t_{(40)} = 2.39$, $P = 0.02$) of fourth-order basal dendrites (Table 1).

Spine density on pyramidal neurons was compared across genotypes on the last 40 μm of the primary apical dendrite and on oblique apical dendrites, as well as on basal dendrites. SR^{-/-} mice had reduced spine density on both primary and oblique branches, with the distal primary dendrite more densely occupied with spines than oblique branches in both genotypes (branch type: $F_{1,32} = 11.19$, $P = 0.002$;

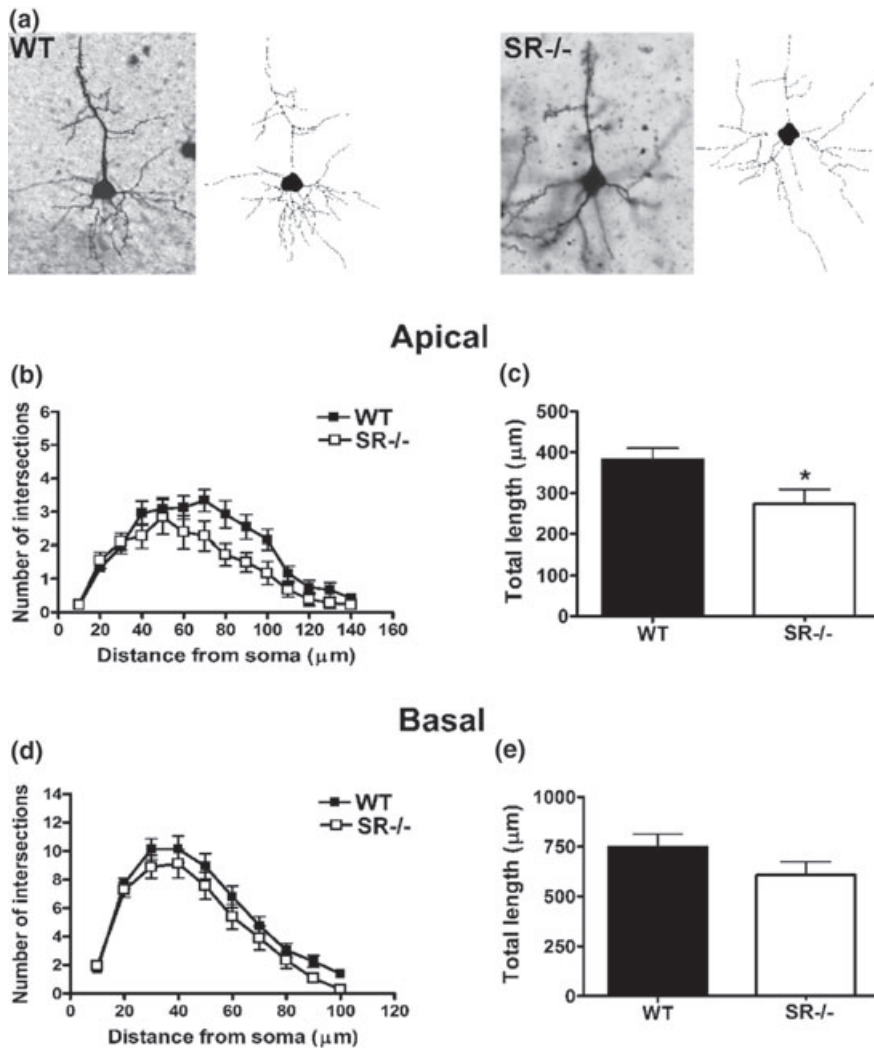


Figure 5: Apical and basilar dendritic morphology of pyramidal neurons in the mPFC. (a) Golgi-stained pyramidal neurons and computer-assisted reconstructions of representative neurons in WT (left) and SR^{-/-} (right) mice. (b,c) Sholl analysis was used to compare the apical dendrites of neurons from WT and SR^{-/-} mice. (d,e) Sholl analysis was also used to compare the basal dendrites of neurons from WT and SR^{-/-} mice. * indicates $P < 0.05$ between groups.

genotype: $F_{1,32} = 7.708$, $P = 0.009$; branch \times genotype: $F_{1,32} = 0.14$, $P = 0.72$; Fig. 6a,b). However, spine density on all branch orders of basal dendrites was unaffected on the neurons of SR^{-/-} mice (branch order: $F_{1,32} = 0.42$, $P = 0.52$; genotype: $F_{2,32} = 0.05$, $P = 0.95$; branch \times genotype: $F_{2,32} = 0.19$, $P = 0.82$; Fig. 6c).

Discussion

NMDARs play an important role in memory and cognition, and hypofunction of these receptors contribute to the pathophysiology of schizophrenia (Goff & Coyle 2001; Konradi & Heckers 2003; Lisman *et al.* 2008). Here, we show that SR^{-/-} mice, in which NMDAR signaling is hypofunctional (Basu *et al.* 2009), display significant alterations in their representation for the order of events that is consistent with compromised function of the PFC. Moreover, pyramidal neurons in the mPFC of SR^{-/-} mice exhibit dendritic abnormalities reminiscent of the pathological changes observed in schizophrenia.

SR^{-/-} mice show impaired representation of the order of events in distinct experiences

Tulving (1983) characterized episodic memory as having a temporal organization, involving the ability to remember the order of events in distinct experiences, and this ability depends on the hippocampus (Fortin *et al.* 2002; Gelbard-Sagiv *et al.* 2008; Lehn *et al.* 2009). Episodic memory has also been characterized as the ability to remember what happened in a particular place and time (Clayton & Dickinson 1998). In the present study, we observed that SR^{-/-} mice have intact memory for object identity, odors (in the odor sequence test) and object location. In contrast, SR^{-/-} mice exhibited an abnormal pattern of preference for the order of events in distinct experiences in two distinct tests, where unlike WT, SR^{-/-} mice showed preference for the objects and odors that were presented more recently during the task. Additionally, in the odor sequence task, despite exhibiting a preference for items occurring later within each sequence, the SR^{-/-} mice displayed a marginal preference for items occurring in Sequence 1 compared to Sequence 2, again, the

Table 1: Detailed segment analysis of apical and basal dendrites of neurons in the mPFC from WT and SR^{-/-} mice

	Branch order						
	1st	2nd	3rd	4th	5th	6th	7th
Total length (μm)							
Apical							
WT	23 ± 4	48 ± 4	85 ± 11	120 ± 18	62 ± 12	32 ± 11	11 ± 6
SR ^{-/-}	17 ± 4	61 ± 11	72 ± 11	74 ± 21	24 ± 8*	15 ± 7	10 ± 8
Basal							
WT	124 ± 12	180 ± 19	227 ± 38	151 ± 23	51 ± 16	N/A	N/A
SR ^{-/-}	105 ± 13	150 ± 25	170 ± 28	73 ± 20*	35 ± 11	N/A	N/A
Number of segments							
Apical							
WT	1.0 ± 0.0	2.0 ± 0.4	2.9 ± 0.3	2.9 ± 0.4	2.1 ± 0.4	1.1 ± 0.3	0.4 ± 0.2
SR ^{-/-}	1.0 ± 0.0	2.0 ± 0.2	2.6 ± 0.4	2.1 ± 0.6	0.9 ± 0.2*	0.4 ± 0.2	0.2 ± 0.2
Basal							
WT	5.1 ± 0.3	6.9 ± 0.5	7.3 ± 0.8	4.0 ± 0.5	1.3 ± 0.4	N/A	N/A
SR ^{-/-}	4.2 ± 0.3	5.5 ± 0.5	5.6 ± 0.8	2.3 ± 0.5*	1.4 ± 0.3	N/A	N/A
Average segment length (μm)							
Apical							
WT	23 ± 4	24 ± 2	27 ± 3	40 ± 5	20 ± 4	11 ± 3	4 ± 2
SR ^{-/-}	17 ± 4	29 ± 5	25 ± 4	25 ± 5*	12 ± 4	7 ± 4	5 ± 4
Basal							
WT	24 ± 2	27 ± 3	27 ± 2	32 ± 3	16 ± 4	N/A	N/A
SR ^{-/-}	23 ± 3	24 ± 3	25 ± 3	20 ± 5	15 ± 5	N/A	N/A

The total length (μm), number of segments and average length of segments (μm) were compared across each of the branch orders. N/A (not applicable) indicates that no data were collected at that branch order.

* Indicates $P < 0.05$ between groups.

opposite pattern to that exhibited by WT mice. This finding of abnormal preference in order tests is unlike the loss of order memory following hippocampal damage (Fortin *et al.* 2002). Instead, these findings suggest that the SR^{-/-} mice have some representation for the order of events, but it reflects an interference with the normal expression of the organization of events in distinct experiences. One possible explanation for the reversal of order preference is that, in the absence of a functional PFC, other brain systems may bias the animals to abnormally prefer the stimulus with the greater relative level of familiarity in object order test, that is, to prefer the object they had less time to forget. In the odor sequence test, one possibility is that, having been repeatedly rewarded for digging in odors in a particular order may dispose the mice to spend considerable time searching for food in the odor for which digging had always previously been rewarded first; that is, they may have had a high expectancy of reward in the earlier odor, as was the case repeatedly during the exposure phase. Conversely, they might switch quickly away from digging in a non-rewarded probe odor that had only previously been rewarded after reward on the alternate probe in the exposure phase; that is, they may have had a low expectancy of reward in an odor that had always been preceded by the alternate odor and reward.

The pattern of abnormality in order memory observed here is similar to that observed in animals with damage to the PFC (Hannesson *et al.* 2004; Mitchell & Laiacona 1998). We have previously reported that while hippocampal damage eliminates memory for order, damage to the PFC results in the

pattern of preference for more recently experienced odors when the lag between items is short, similar to our current findings in the SR^{-/-} mice (DeVito & Eichenbaum, under review). Thus, these cognitive impairments are most likely due to a disruption within the hippocampal–prefrontal circuit. Additionally, this disruption in information processing is similar to the clinical presentation of cognitive disorganization in schizophrenia.

Intact relational memory and social recognition

The hippocampus and surrounding cortical structures are importantly involved in relational memory, the ability to bind together overlapping items in order to recreate the full episode (Dusek & Eichenbaum 1997; Heckers *et al.* 2004). The SR^{-/-} mice learned the overlapping odor pairs as readily as their WT littermates and performed at comparable levels on both the transitive and non-transitive probe tests, showing intact relational memory. These results contrast with previous findings indicating that animals with hippocampal damage (DeVito *et al.* 2010; Van der Jeugd *et al.* 2009) and schizophrenic patients (Titone *et al.* 2004) have profound deficits in transitive inference. Although the ability to associate overlapping events is an important aspect of episodic memory in that it allows for the flexible expression of information in novel situations, it seems to be an aspect of memory with which these animals display little difficulty.

SR^{-/-} mice showed normal social preferences in both the sociability and social novelty tests, which is in contrast to previous reports indicating that NMDAR hypofunction

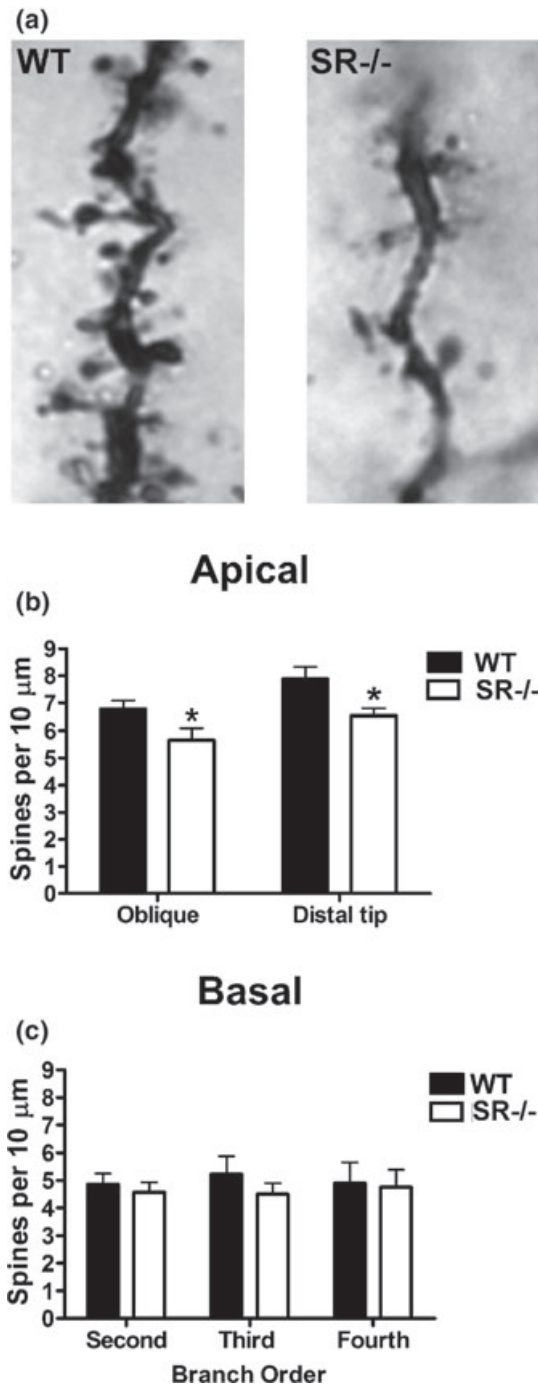


Figure 6: Spine density analysis of pyramidal neurons in the mPFC. (a) Apical dendritic spines of a Golgi-stained pyramidal neuron in a WT (left) and a SR^{-/-} mouse (right). (b) Spine density analysis of both oblique branches and the distal end of apical dendrites in WT and SR^{-/-} mice. (c) Spine density analysis of branch order of basilar dendrites of pyramidal neurons in WT and SR^{-/-} mice. Spine density was normalized to the number of spines per 10 μm of dendrite. * indicates $P < 0.05$ between groups.

disrupts social behaviors (Labrie *et al.* 2009; Mohn *et al.* 1999). Although all of these models, including ours, aim to induce NMDAR hypofunction, the various strategies ultimately affect NMDAR signaling differently and could therefore produce distinct behavioral phenotypes.

Dendritic abnormalities in the mPFC of SR^{-/-} mice

SR^{-/-} mice had less complex dendrites with reduced spine density in the mPFC. These findings bear similarity with the observation that, in schizophrenic subjects, neurons in deep layer 3 of the dorsolateral PFC (DLPFC) have specific reductions in both basilar dendritic material and spine density, whereas no perturbations were found in the primary visual cortex (Glantz & Lewis 2000). Whether this same pattern holds true in our genetic model has yet to be determined; however, importantly, similar dendritic abnormalities were observed in the somatosensory cortex of the SR^{-/-} mice (Balu *et al.* 2009). It is possible that somatosensory cortex could have contributed to performance on the object tests, in that whisking may have provided cues to identify the objects. However, if this was the case, one would expect similar deficits in SR^{-/-} mice on all the object-cued tasks, and not a selective effect on object order memory, as was observed. Combining this finding with the similar results on the odor sequence task, wherein somatosensory cortex is unlikely to have contributed significantly, we conclude it is most likely that the cognitive deficits are due to the abnormalities in the PFC.

Interestingly, the SR^{-/-} mice had pyramidal neurons in the mPFC with abnormal apical, but not basal, dendrites. Others have reported divergence in the effects of genetic manipulation on the patterning of apical and basilar dendrites in the infralimbic region of the mPFC in serotonin transporter KO mice (Wellman *et al.* 2007). The soma and basilar dendritic tree of layer II/III neurons are innervated by thalamic projections (Shibata 1993), while the apical dendrites are preferentially contacted by afferents from limbic structures, such as the hippocampus (Swanson & Cowan 1977). Therefore, as SR is highly expressed in limbic regions, including hippocampus, but virtually absent in the thalamus (Basu *et al.* 2009), it is plausible that the apical dendrites of mPFC pyramidal neurons would be the most severely affected because they receive projections from areas where D-serine plays a more prominent role in NMDAR-dependent transmission.

The reduced spine density of pyramidal neurons in the cortex of SR^{-/-} mice would be expected to lead to abnormalities in glutamatergic transmission (Fiala *et al.* 2002). The complexity of dendritic branching is thought to represent the extent of neuronal connectivity. Dendritic arborization is enhanced by NMDAR-dependent transmission and downstream Ca²⁺ sensitive signaling pathways (Wayman *et al.* 2006, 2008). We previously showed NMDAR hypofunction in SR-KO mice by showing that the mutants had reduced occupancy of the GMS, reduced global NMDAR-mediated transmission by a biotin-switch assay, and reduced hippocampal LTP (Basu *et al.* 2009). Although we have not measured NMDAR expression in our mice, NMDAR protein levels were found to be unaltered in whole tissue homogenates in other mouse models of SR deficiency

(Inoue *et al.* 2008; Labrie *et al.* 2009). As excitatory activity influences the density of dendritic spines (Zito *et al.* 2009), reduced NMDAR-dependent signaling in the SR^{-/-} mice could be responsible for the reductions in spine density. Future studies should shed light on molecular and physiological abnormalities specifically in the mPFC of SR^{-/-} mice.

Conclusion

SR^{-/-} mice showed an altered representation of the order of events in distinct experiences, a feature of episodic memory associated with PFC function. In addition, pyramidal neurons in the mPFC of SR^{-/-} mice displayed dendritic perturbations and reduced spine density, suggesting that there is significantly altered glutamatergic signaling in this region. The results presented here show that D-serine modulation of NMDAR function is important for specific aspects of cognition, as well as the dendritic characteristics of cortical pyramidal neurons, and suggest that NMDAR hypofunction might, in part, be contributing to the cognitive and synaptic changes observed in schizophrenia (Coyle 1996, 2006; Javitt 2006).

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