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An $mGlu_5$-Positive Allosteric Modulator Rescues the Neuroplasticity Deficits in a Genetic Model of NMDA Receptor Hypofunction in Schizophrenia

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There is substantial evidence that NMDA receptor (NMDAR) hypofunction contributes to the pathophysiology of schizophrenia (SCZ). A recent large-scale genome-wide association study identified serine racemase (SR), the enzyme that produces the NMDAR co-agonist D-serine, as a risk gene for SCZ. Serine racemase knockout (SR−/−) mice, which lack D-serine, exhibit many of the neurochemical and behavioral abnormalities observed in SCZ. Metabotropic glutamate receptor 5 ($mGlu_5$)-positive allosteric modulators (PAMs) are currently being developed to treat cognitive dysfunction. We used *in vitro* electrophysiology to determine whether the $mGlu_5$ PAM VU0409551 directly enhances NMDAR function in hippocampal slices from adult male SR−/− mice. We administered VU0409551 systemically for 5 days to adult male wild-type C57BL/6 animals to determine the optimal dose to test in SR−/− mice. We used western blot analyses and trace-fear conditioning to determine whether 5 days of VU0409551 treatment could reverse the neuroplasticity and learning deficits, respectively, in SR−/− mice. We show that VU0409551 enhances NMDAR function and rescues long-term potentiation in hippocampal slices obtained from SR−/− mice. Systemic treatment with VU0409551 (10 and 30 mg/kg) to wild-type mice causes a dose-dependent increase in the Akt/GS3Kα/β signaling pathway, which is reduced in SR−/− mice and in SCZ. Furthermore, the administration of VU0409551 to SR−/− mice reverses their deficits in several neuroplasticity signaling pathways and improves their contextual fear memory. These results support positive allosteric modulation of $mGlu_5$, particularly with VU0409551, as a viable mechanism to reverse the deficits in NMDAR function, synaptic plasticity, and memory that are known to be impaired in SCZ.
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INTRODUCTION

There is substantial evidence that hypofunction of the *N*-methyl-D-aspartate receptor (NMDAR) is a core pathophysiological mechanism underlying schizophrenia (SCZ) (Balu and Coyle, 2011b). Recent findings from genome-wide association and exome-sequencing studies of persons diagnosed with SCZ have implicated genes encoding proteins involved in glutamatergic neurotransmission and the postsynaptic density (Fromer *et al*, 2014; Kirov *et al*, 2012; Purcell *et al*, 2014), including the enzyme serine racemase (SR) (Schizophrenia Working Group of the Psychiatric Genomics C, 2014). SR is responsible for the synthesis of the NMDAR co-agonist D-serine, which is concentrated in corticolimbic regions of the brain. Mice lacking SR (SR−/−) exhibit many of the neuropathologic features observed in SCZ. SR−/− mice have enlarged lateral ventricles, reduced corticohippocampal volume, and perturbations in neuronal dendritic morphology in the cortex and hippocampus (Balu and Coyle, 2012; Balu *et al*, 2013; Puhl *et al*, 2015). These morphological changes are paralleled by diminished microRNA-132 expression, reduced Akt/mammalian target of rapamycin (mTOR) signaling, and decreased Arc expression (Balu and Coyle, 2014; Balu *et al*, 2013). Behaviorally, SR−/− mice display cognitive impairments with regards to spatial and contextual memory, as well as memory for the order of events (Balu *et al*, 2013; Basu *et al*, 2009; DeVito *et al*, 2011). Furthermore, SR−/− mice recapitulate the anhedonic symptoms associated with SCZ, as they show a blunted reward response to cocaine in an intracranial self-stimulation paradigm (Puhl *et al*, 2015).

The metabotropic glutamate receptor subtype 5 ($mGlu_5$) is expressed widely throughout the brain and is enriched in corticolimbic circuits that are important for memory and

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affect (Herman *et al*, 2012). It is found primarily in the postsynaptic compartment of excitatory and inhibitory neurons, although it is also found in astrocytes (Biber *et al*, 1999; Herman *et al*, 2012). Selective mGlu$_5$ receptor ligands have been the subject of interest for reversing the NMDAR deficits associated with SCZ (Herman *et al*, 2012). It is well known that mGlu$_5$ can enhance NMDAR function through its physical interaction with the NMDAR in the postsynaptic density. This enhancement is facilitated by adaptor proteins, such as Homer and Shank, and is independent of $G_{\alpha q}$ signaling (Gao *et al*, 2013). mGlu$_5$ is currently a leading target for novel therapeutics to treat SCZ and cognitive disorders, particularly with the development of subtype-specific positive allosteric modulators (PAMs) (Nickols and Conn, 2014). PAMs are unique, in that unlike traditional agonists, they do not directly activate the receptor, but rather augment signaling in the presence of an endogenous agonist. This class of compounds has advantages over traditional agonists because they do not cause receptor internalization and have a safer side-effect profile.

We have previously demonstrated that chronic administration of D-serine in adulthood normalizes the electrophysiological, neurochemical, and cognitive deficits in SR−/− mice (Balu *et al*, 2013). A meta-analysis of 26 double-blind placebo-controlled clinical trials reported that glycine modulatory site (GMS) agonists, including D-serine, significantly improved positive, negative, and cognitive symptoms in patients with SCZ, who were receiving concurrent antipsychotic medications (Tsai and Lin, 2010). However, due to the poor pharmacokinetics and brain penetrance, as well as potential nephrotoxicity, there are several concerns about the utility of D-serine as a clinically useful medication for SCZ (Balu and Coyle, 2015). Therefore, we were interested in determining whether the recently developed, potent, and selective mGlu$_5$ PAM VU0409551 (Rook *et al*, 2015) could reverse the neuroplasticity and learning deficits in SR−/− mice.

MATERIALS AND METHODS

Animals

SR−/− mice (Basu *et al*, 2009) were generated as previously described. SR± sires and dams were bred to produce wild-type (WT) and SR−/− offspring. Adult male mice (3–5 months old) were used for all the experiments. Animals were housed in groups of four in polycarbonate cages and maintained on a 12 : 12-h 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.

Drugs

VU0409551 was synthesized as previously described (Rook *et al*, 2015). Mice received once daily, intraperitoneal (i.p.) injections of vehicle (20% hydroxypropyl β-cyclodextran (BCD); CDT Inc., Cat #: THPB-p) or VU0409551 for 5 days at a volume of 10 ml/kg. Twenty percent BCD was dissolved in sterile water 24 h before the formulation of VU0409551. VU0409551 was added to the vehicle and stirred for ~5 min, and then hand-sonicated for 1 min using a microtip attachment. The drug was then subjected to sonication at 40 °C in a mechanical ultrasonic cleaner (Fisher Scientific) for 60 min, or until all the large particles were in solution. A new solution of VU0409551 was made each day immediately before injections. For the *in vivo* pharmacokinetic and dose-finding experiments, WT mice ($n = 5$–6/dose) received vehicle or VU0409551 (10 and 30 mg/kg). For the SR−/− mice reversal studies, WT mice received vehicle and SR−/− mice received either vehicle or VU0409551 (30 mg/kg). All mice were killed 2 h after the last injection on day 5.

Electrophysiology

A stock solution of VU0409551 was dissolved in dimethyl sulfoxide (Fisher Scientific) at a concentration of 30 mM. Working aliquots were stored at −20 °C until use in experiments. This dose was chosen based on previous *in vitro* hippocampal slice electrophysiology (Rook *et al*, 2015). Coronal hippocampal slices (400 μm in thickness) were prepared from adult SR−/− or WT mice (littermates) with a vibratome. Slices were continuously perfused in solution containing (in mM) 125 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.0 $MgSO_4$, 1.25 $NaH2PO_4$, 26 $NaHCO_3$, 10 glucose, and 0.05 picrotoxin, and equilibrated with 95% O_2 and 5% CO_2 (pH 7.3–7.4) at 22 °C. Field excitatory postsynaptic potentials (fEPSPs) were recorded in the stratum radiatum of the CA1 region. Synaptic responses were evoked at 0.033 Hz by stimulation of Schaffer collaterals with a concentric stimulation electrode (Balu *et al*, 2013). Long-term potentiation (LTP) of synaptic transmission was induced by one 1-s train of 100-Hz stimulation after recording of baseline fEPSPs. In these experiments, we measured the initial slope of the fEPSPs' rising phase. The magnitude of LTP was estimated during a 5-min window 45 min after it was induced. NMDAR-mediated field EPSPs (NMDAR-fEPSPs) were recorded in low external Mg^{2+} (0–0.1 mM Mg^{2+} in the ACSF) in the presence of picrotoxin (50 μM) and the AMPAR antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 10 μM; (Papouin *et al*, 2012)). Under these conditions the fEPSPs were mediated by NMDARs, which was confirmed by their sensitivity to a specific NMDAR antagonist (50 μM D-AP5, Figure 2a). To assay the effect of SR ablation on (*S*)-3,5-dihydroxyphenylglycine (DHPG)-induced mGluR-LTD, fEPSPs were recorded at CA3–CA1 synapses in slices from control and mutant mice. After obtaining a stable baseline, DHPG (75 μM) was applied for 10 min. This resulted in significant depression of synaptic responses (DHPG-induced LTD). The average of fEPSP amplitudes during the last 6 min of recordings was used for statistical analysis.

In vivo VU0409551 Pharmacokinetic Analysis

The animals were killed and decapitated, and the brains were removed, thoroughly washed in cold phosphate-buffered saline, and cortex frozen on dry ice; trunk blood was collected into EDTA-treated tubes and centrifuged (1700 r.p.m., 5 min) to provide plasma samples. Samples were shipped on dry ice to Vanderbilt University

for analysis by HPLC–MS/MS (Supplementary Materials and Methods).

Western Blot Analysis

Immunoblotting was performed as previously described (Balu and Coyle, 2011a). Primary antibodies are listed in Supplementary Table S1. Chemiluminescent values of the protein of interest were divided by its corresponding β-actin chemiluminescent values. The ratio of each WT sample was divided by the average of all the WT sample values in each gel and multiplied by 100. The average of the normalized WT values from each gel was 100% ± SEM. The mutant values were normalized to WT values (%WT) collected in parallel from the same gel. The normalized values were then averaged and used for statistical analysis.

Trace Fear Conditioning

One cohort of WT mice received 5 days of either vehicle ($n=8$) or VU0409551 (30 mg/kg, $n=8$) and was subjected to a trace fear-conditioning paradigm during the last 3 days of treatment. Another cohort of WT ($n=19$; vehicle), SR−/− ($n=13$; vehicle), and SR−/− mice treated with VU0409551 (30 mg/kg; $n=12$) was subjected to a trace fear-conditioning paradigm during the last 3 days of treatment. Injections were given 2 h before behavioral testing. On day 1, each conditioning session consisted of a 3-min acclimation period followed by seven trials of the following structure: a 20-s tone (conditioned stimulus; CS) at 75 dB followed by a 20-s trace period followed by a foot-shock (duration 2 s, amplitude 0.7 mA; unconditioned stimulus, US). Trials were roughly 4 min apart. On day 2, mice were placed in the same conditioning chambers as on day 1 and freezing behavior was measured for 8 min. On day 3, mice were placed in chambers with different contextual cues, including a smooth chamber floor, a peppermint odor (peppermint extract diluted 1 : 1 K), and a roof insert to distinguish the testing environment from the conditioning environment. The protocol from day 1 was repeated without the foot-shock. The house light in the chamber was illuminated during all sessions. All testing was performed using The Near Infrared Fear Conditioning System (Med Associates, Inc.; St. Albans, VT). Freezing behavior was quantified using VideoFreeze software.

Statistical Analyses

Two-way ANOVA (genotype × drug) with Newman–Keuls multiple comparisons test was used to compare groups for the electrophysiology results in Figure 1c. Two-way repeated measures ANOVA (genotype × time) with Bonferroni's multiple comparisons test was used to compare groups for the electrophysiology results in Figure 2e. Unpaired Student's *t*-test was used to analyze the electrophysiological data in Figure 2f. Two-way repeated measures ANOVA was used to analyze the data obtained from day 1 of the trace fear-conditioning experiments. Unpaired *t*-tests were used to analyze the contextual and cue freezing data obtained from the WT mice after chronic VU0409551 treatment. Type I (fixed effect) one-way ANOVAs were used to analyze western blot and fear-conditioning (context and cue) results following chronic VU0409551 treatment. Significant one-way ANOVA results were followed up by Newman–Keuls multiple comparison test. Values of $P<0.05$ were considered statistically significant.

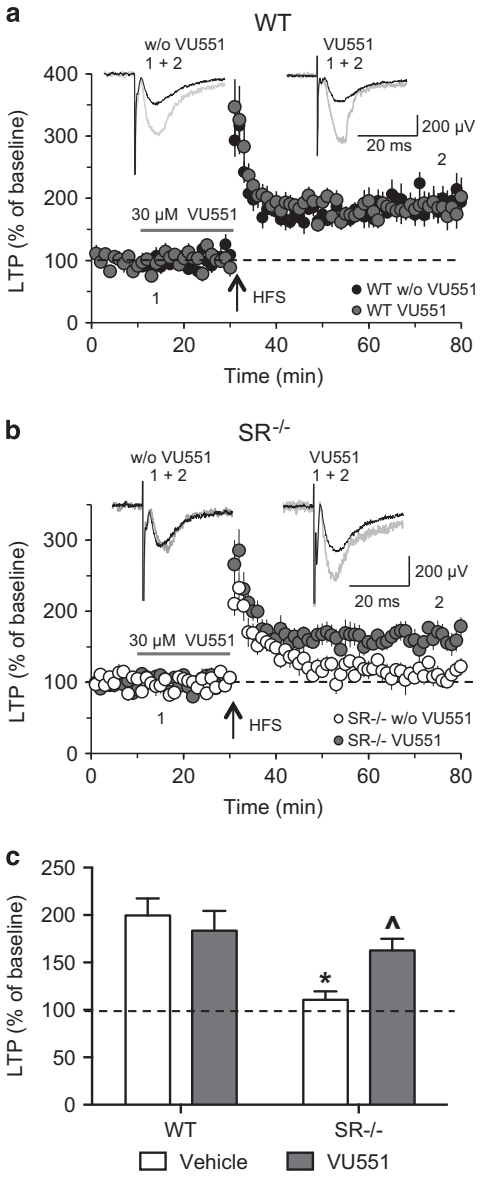

Figure 1 VU0409551 rescues synaptic plasticity deficits in the hippocampus of SR−/− mice. (a) Averaged graphs showing results of LTP experiments performed in slices from WT mice under control conditions (solid black circles, $n=6$ slices from two WT mice) and after VU551 treatment (red circles, $n=7$ slices from three WT mice). Insets show averages of 40 fEPSPs before (black trace) and 10 fEPSPs (thick gray trace) 45 min after LTP induction. (b) Results of LTP experiments in untreated slices (open circles, $n=7$ slices from four SR−/− mice) or slices treated with 30 µm VU0409551 (VU551; red filled circles, $n=9$ slices from five SR−/− mice). Insets show averages of 60 fEPSPs (black trace) before and 10 fEPSPs (gray trace) 45 min after LTP induction under both conditions (with or without the treatment of slices with VU551). (c) Summary of LTP results for the four experimental groups (white bars, vehicle; red bars, VU551). Data are presented as the mean ± SEM. Significant two-way ANOVA results were followed up with Newman–Keuls multiple comparisons tests. Asterisk (*) indicates significant difference from the WT vehicle group ($P<0.05$) and ^ indicates significant difference from the SR−/− vehicle group. A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

RESULTS

VU0409551 Restores NMDA Receptor Function in SR−/− Mice

SR−/− mice display NMDAR hypofunction in several brain regions, including the cortex (Basu *et al*, 2009), hippocampus (Balu *et al*, 2013; Basu *et al*, 2009), and amygdala (Li *et al*, 2013), due to the lack of D-serine availability. Specifically, SR−/− mice have reduced LTP at the juvenile Schaffer collateral CA3–CA1 synapse (Basu *et al*, 2009) and at the adult medial perforant path–dentate gyrus (DG) synapse (Balu *et al*, 2013). To test whether synaptic plasticity deficits in the hippocampus of

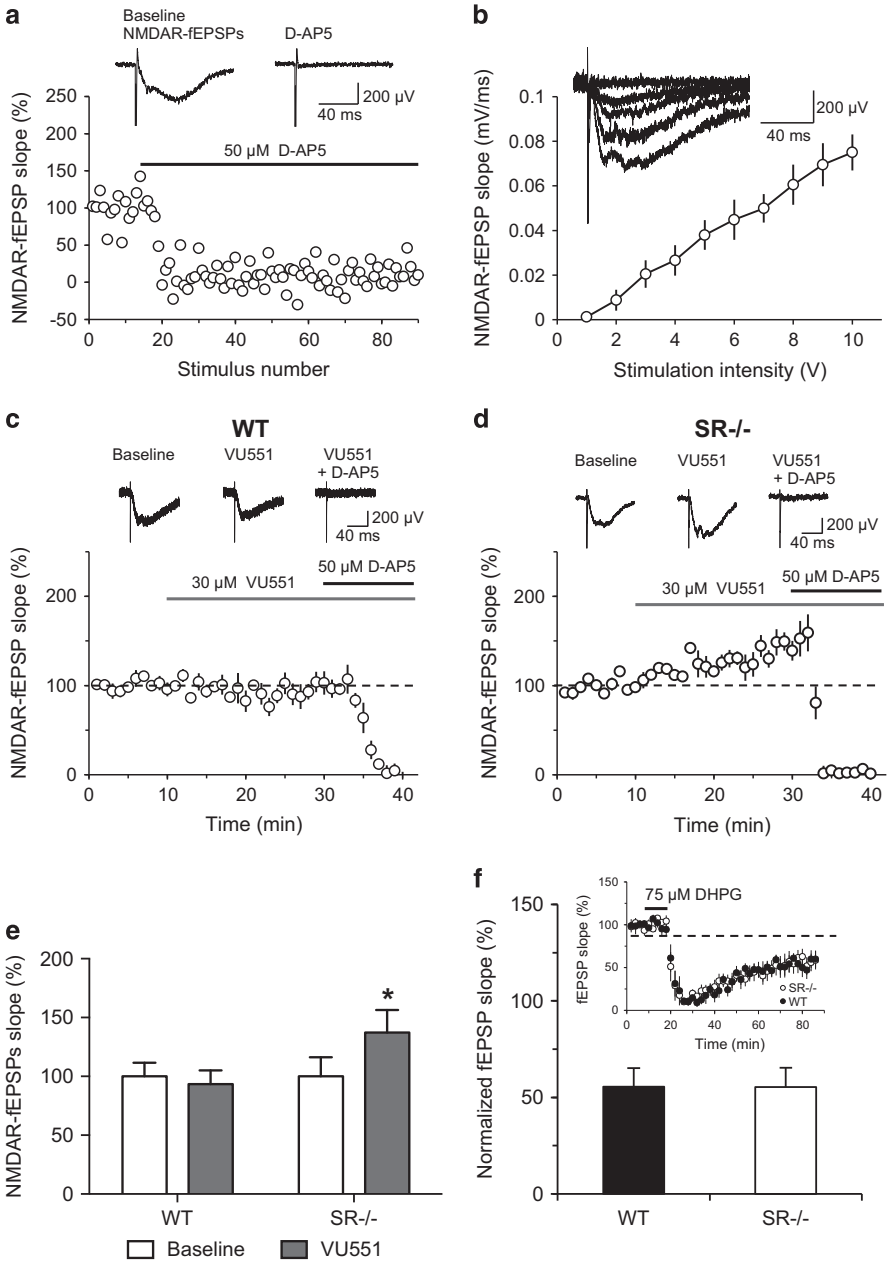

Figure 2 VU0409551 potentiates NMDAR-fEPSPs in SR−/− mice, but not WT mice. (a) Recorded NMDAR-fEPSPs were abolished by the specific NMDAR antagonist D-AP5 (50 μM). Insets show averages 15 NMDAR-fEPSPs during baseline recording (left) and 15 NMDAR-fEPSPs 5 min after the application of NMDAR antagonist (right). (b) The synaptic input–output curve for NMDAR-fEPSPs obtained at CA3–CA1 synapses by presynaptic stimuli of increasing intensity in slices from WT mice ($n=5$ slices). Inset shows averaged NMDAR-fEPSPs (10 traces) at stimulation intensities of 1, 3, 4, 5 and 7 V. (c) Insets show averages of 20 NMDAR-fEPSPs before (left), 10 fEPSPs 15 min after (middle) the application of VU551, and 10 NMDAR-fEPSPs 5 min after (right) the addition of D-AP5 (50 μM). Summary of VU551 (30 μM) tests in WT mice ($n=5$ slices from two WT mice). (d) Insets show averages of 20 NMDAR-fEPSPs before (left), 10 NMDAR-fEPSPs 15 min after (middle) the application of VU0409551 (VU551), and 10 NMDAR-fEPSPs 5 min after (right) the addition of the NMDAR antagonist D-AP5 (50 μM). Summary of VU551 (30 μM) tests in SR−/− mice ($n=7$ slices from three SR−/− mice). (e) Summary of the results for all four groups is shown in c and d; white bars, baseline; red bars, VU551. Significant two-way repeated measures ANOVA results were followed up by Bonferroni's multiple comparisons test to compare groups. (f) Time course of DHPG-induced depression of fEPSPs at CA3–CA1 synapses in slices from WT ($n=4$ slices from three mice; black bar) and SR−/− mice ($n=5$ slices from two mice; white bar). Summary of the experiments is shown in inset (averages of fEPSP amplitudes during the last 6 min of recordings). Data are presented as the mean ± SEM. Asterisk (*) indicates significant difference from the SR −/− baseline group ($P<0.05$). A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

SR−/− mice could be reversed by VU0409551, a highly selective mGlu$_5$ PAM, we performed LTP experiments in WT and SR−/− mice. We show that the magnitude of LTP at the CA3–CA1 synapse was significantly reduced in untreated hippocampal slices from adult SR−/− mice (Figure 1a–c; two-way ANOVA; genotype: $F_{1,25}=12.70$, $P<0.005$; drug: $F_{1,25}=1.38$, $P=0.25$; interaction: $F_{1,25}=4.90$, $p<0.05$; Newman–Keuls multiple comparisons test). The magnitude of LTP in slices from SR−/− mice was 110.8±8.9% of baseline value, compared with 199.6±17.6% of baseline value in slices from WT mice. Treatment with VU0409551 (30 μM) for 20 min during the recording of baseline fEPSPs significantly enhanced fEPSPs and rescued the deficits of synaptic plasticity in SR−/− mice (162.9±12.2%; Newman–Keuls multiple comparisons test). The same treatment in slices from control mice did not affect the magnitude of LTP (183.7±20.9%) compared with untreated slices (Figure 1a and c; Newman–Keuls multiple comparisons test). As mentioned above, in untreated slices from SR−/− mice, the fEPSP magnitude after the delivery of LTP-inducing stimulation was not different from the baseline amplitude value (paired *t*-test, $t_6=1.38$, $P=0.22$ *vs* baseline fEPSPs; Figure 1b and c). The treatment with VU0409551 did not affect the amplitude of baseline fEPSPs (paired *t*-test; WT: $t_7=0.28$, $P=0.79$; SR−/−: $t_9=2.06$, $P=0.07$; fEPSPs during the first 5 min *vs* fEPSPs during the last 5 min of the 20-min-long application of VU0409551; $n=8$ slices from three WT mice and $n=10$ slices from five SR−/− mice), suggesting that VU0409551 did not alter AMPAR-mediated synaptic responses at the CA3–CA1 hippocampal synapses.

To test whether VU0409551 could affect NMDAR-mediated synaptic responses, we assayed the effect of VU0409551 (30 μM) on NMDAR-fEPSPs. First, we demonstrated that the recorded fEPSPs were mediated by NMDARs by showing they were blocked with the NMDAR antagonist AP5 (Figure 2a). We also plotted the synaptic input–output curves for NMDAR-fEPSPs obtained at CA3–CA1 synapses by presynaptic stimuli of increasing intensity in slices from WT mice (Figure 2b). We found that the application of 30 μm VU0409551 for 20 min did not potentiate NMDAR-fEPSPs (baseline fEPSPs *vs* the last 5-min recording of NMDAR-fEPSPs during a 20-min-long application of VU0409551) in slices from WT mice (Figure 2c and e: two-way repeated measures ANOVA genotype: $F_{1,10}=0.96$, $P=0.35$; time: $F_{1,10}=5.22$, $P=0.046$; interaction: $F_{1,10}=10.62$, $p<0.01$), in agreement with recent findings in WT rats (Rook *et al*, 2015). However, application of VU0409551 resulted in significant potentiation of NMDAR-fEPSPs in SR−/− mice (Figure 2d and e: Bonferroni's multiple comparisons test). As shown in Figure 2c and d, the recorded NMDAR-fEPSPs in both WT and SR−/− mice were completely abolished by the NMDAR antagonist (D-AP5, 50 μM), confirming that the recorded fEPSPs were indeed mediated by NMDARs. Finally, we compared the magnitude of DHPG-induced LTD in slices from WT and SR−/− mice to determine whether there was a potential difference in type I mGluR-mediated signaling between genotypes. We found no difference between the groups (Figure 2f; unpaired *t*-test; $t_7=0.01$, $P=0.99$).

In vivo Characterization of VU0409551 in Wild-Type Mice

As VU0409551 was able to restore NMDAR function in SR−/− mice *in vitro*, we next wanted to see whether this compound could rescue other neuroplasticity deficits in SR−/− mice *in vivo*. Rook *et al* (2015) demonstrated a favorable pharmacokinetic profile and brain penetration for VU0409551 in the rat. We first characterized the pharmacokinetic profile of VU0409551 in C57BL/6 mice following subchronic (5 days) intraperitoneal administration. As shown in Supplementary Table S2, there was a dose-dependent increase in the concentration of VU0409551 in the plasma and cerebral cortex, with the 30 mg/kg dose producing concentrations 2.4× and 5× higher than the 10 mg/kg dose, respectively.

After determining that adequate concentrations of VU0409551 were achieved in the brain following chronic dosing, we next tested whether these doses elicited any changes in Akt/GS3Kα/β signaling in the hippocampus and prefrontal cortex (PFC) of WT mice. We chose this signaling pathway because it is impaired in the hippocampus of SR−/− mice (Balu *et al*, 2013). Furthermore, group I mGlu receptor agonists and older-generation mGlu$_5$ PAMs have been shown to increase Akt phosphorylation (Doria *et al*, 2015; Hou and Klann, 2004). VU0409551 dose-dependently increased pAkt (Figure 3a; $F_{2,13}=5.9$, $P<0.05$) and pGS3Kα/β (Figure 3b; α: $F_{2,13}=5.6$, $P<0.05$; β: $F_{2,13}=3.8$, $P<0.05$) in the hippocampus, but only the 30 mg/kg dose achieved significance. VU0409551 had no effect on the phosphorylation status of p-mTOR (Figure 3c; $F_{2,13}=1.34$, $P=0.29$) or TrkB (Figure 3d; $F_{2,13}=0.01$, $P=0.99$). A similar pattern was observed in the PFC (Supplementary Figure S1a–d), except the 30 mg/kg dose of VU0409551 significantly increased only pAkt (Supplementary Figure S1a; $F_{2,25}=3.5$, $P=0.05$). Interestingly, VU0409551 had no effect on extracellular regulated kinase 1/2 phosphorylation (pERK1, $F_{2,13}=0.07$, $P>0.05$; pERK2, $F_{2,13}=0.42$, $P>0.05$). This is similar to what was observed in normal rats *in vivo* (data not shown, PJC), even though VU0409551 was able to stimulate ERK signaling in HEK293 cells stably expressing rat mGlu5 *in vitro* (Rook *et al*, 2015).

VU0409551 Reverses Neurochemical and Learning Deficits in SR−/− Mice

Given that the 30 mg/kg dose of VU0409551 produced the most robust changes, we examined whether it could reverse the neurochemical abnormalities in the hippocampus of SR−/− mice. Indeed, VU0409551 reversed the pTrkB (Figure 4a; $F_{2,23}=5.2$, $P<0.05$) and pAkt (Figure 4b; Ser473, $F_{2,24}=4.5$, $P<0.05$; Thr308, $F_{2,24}=3.7$, $P<0.05$; pAkt1 Ser473, $F_{2,24}=4.3$, $P<0.05$) deficits, as well as the downstream deficits in pGS3Kα/β (Figure 4c; α: $F_{2,25}=5.0$, $P<0.05$; β: $F_{2,24}=5.3$, $P<0.05$) and p-mTOR (Figure 4d; $F_{2,24}=8.1$, $P<0.005$) in SR−/− mice. Furthermore, VU0409551 rescued the deficit in the phosphorylation state of protein kinase A (PKA; Figure 4e; $F_{2,23}=4.8$, $P<0.05$), which is dependent, in part, upon Ca^{2+} influx through NMDARs. The activity-regulated cytoskeletal-associated protein (Arc) is an immediate early gene that is positively regulated by NMDAR activity. Arc protein levels were lower

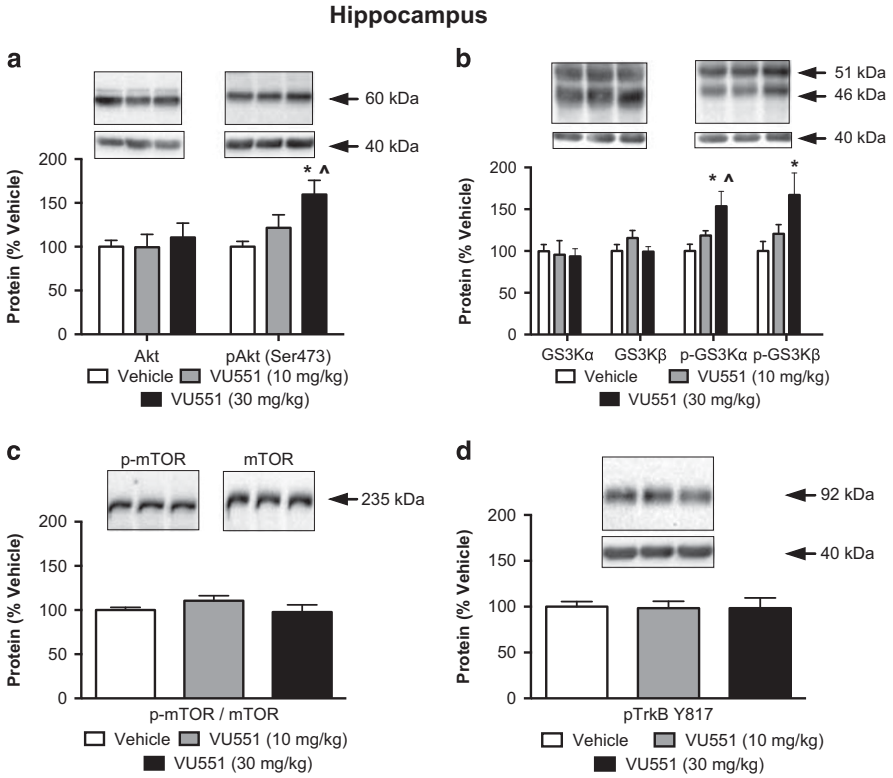

Figure 3 VU0409551 dose-dependently enhances Akt/GS3K signaling in the hippocampus of WT mice. WT mice received 5 days of either vehicle (white bars, $n=5$) or VU0409551 (VU551: 10 mg/kg, gray bars, $n=6$; 30 mg/kg, black bars, $n=5$) and were killed 2 h after the last injection. Protein levels of (a) Akt and p-Akt, Ser 473, (b) glycogen synthase kinase 3 (GS3K) α/β and p-GS3K α/β (α = 51 kDa, β = 46 kDa), (c) p-mTOR/mTOR, and (d) pTrkB Y817 in the hippocampus. Values are expressed as the optical density (OD) normalized to WT values (%WT). Each western blot image includes a representative band of the protein of interest and β-actin (41 kDa) from each of the experimental groups: vehicle, VU551 (10 mg/kg), VU551 (30 mg/kg), respectively. Significant one-way ANOVA results were followed up by Newman–Keuls multiple comparison test. Asterisk (*) indicates significant differences from the vehicle group ($P<0.05$) and ^indicates difference from 10 mg/kg VU551. All values represent the mean ± SEM.

in the hippocampus of SR−/− mice, which was restored to WT levels by VU0409551 (Figure 4f, $F_{2,25}=8.4$, $P<0.005$).

Emotional learning and memory, as well as contextual processing are impaired in patients with SCZ (Hall *et al*, 2007; Maren *et al*, 2013). Classical Pavlovian fear conditioning is one of the most powerful and widely used models for studying emotional memory and associative learning in rodents (LeDoux, 2000). We utilized a trace fear-conditioning assay (temporal gap between the tone and the shock) to determine whether VU0409551 could reverse the contextual memory deficits of SR−/− mice. We first found that in WT mice, VU0409551 affected neither the acquisition (Figure 5a; two-way repeated measures ANOVA; drug: $F_{1,23}=0.74$, $P=0.40$; tone: $F_{6,138}=34.3$, $P<0.0001$; interaction: $F_{6,138}=0.30$, $P=0.93$) nor the expression of fear to the context (Figure 5b; $t_{21}=1.5$, $P=0.15$) and the cue (Figure 5c; $t_{21}=1.39$, $P=0.18$). The amount of freezing between SR−/− mice treated with vehicle or VU551 also did not differ from WT mice during conditioning on day 1 (Figure 5d; two-way repeated measures ANOVA; genotype: $F_{2,42}=0.16$, $P=0.86$; tone: $F_{6,252}=41.8$, $P<0.0001$; interaction: $F_{2,252}=1.43$, $P=0.15$). We found that vehicle-treated SR−/− mice froze less in the conditioning context and VU0409551 was able to rescue this deficit in contextual fear memory in SR−/− mice (Figure 5e; $F_{2,41}=3.68$, $P<0.05$). We did not observe deficits in cued fear conditioning in SR−/− mice and VU0409551 had no effect on freezing behavior (Figure 5f; $F_{2,28}=0.70$, $P=0.51$; average freezing during the first three 20-s tone presentations).

DISCUSSION

The mGlu$_5$ has garnered recent attention as a tractable drug target for several brain disorders with cognitive symptoms, including SCZ (Herman *et al*, 2012). Thus, we examined whether the newly developed mGlu$_5$ PAM VU0409551 would be able to restore the neuroplasticity deficits in SR−/− mice, a genetic NMDAR hypofunction model relevant to SCZ (Schizophrenia Working Group of the Psychiatric Genomics C, 2014). We found that VU0409551 enhanced hippocampal NMDAR function and restored LTP in SR−/− mice. Furthermore, subchronic VU0409551 rescued the neurochemical abnormalities and learning deficits of SR−/− mice.

Genetic (Basu *et al*, 2009) and pharmacologic (Le Bail *et al*, 2015) studies have shown that D-serine is the primary NMDAR co-agonist at adult hippocampal CA3–CA1 synapses and that SR−/− mice display NMDAR hypofunction in this region. We demonstrate here that VU0409551 rescues deficits in NMDAR-mediated synaptic responses in the hippocampus of SR−/− mice. Furthermore, VU0409551 enhanced LTP at the CA3–CA1 synapse in SR−/− mice.

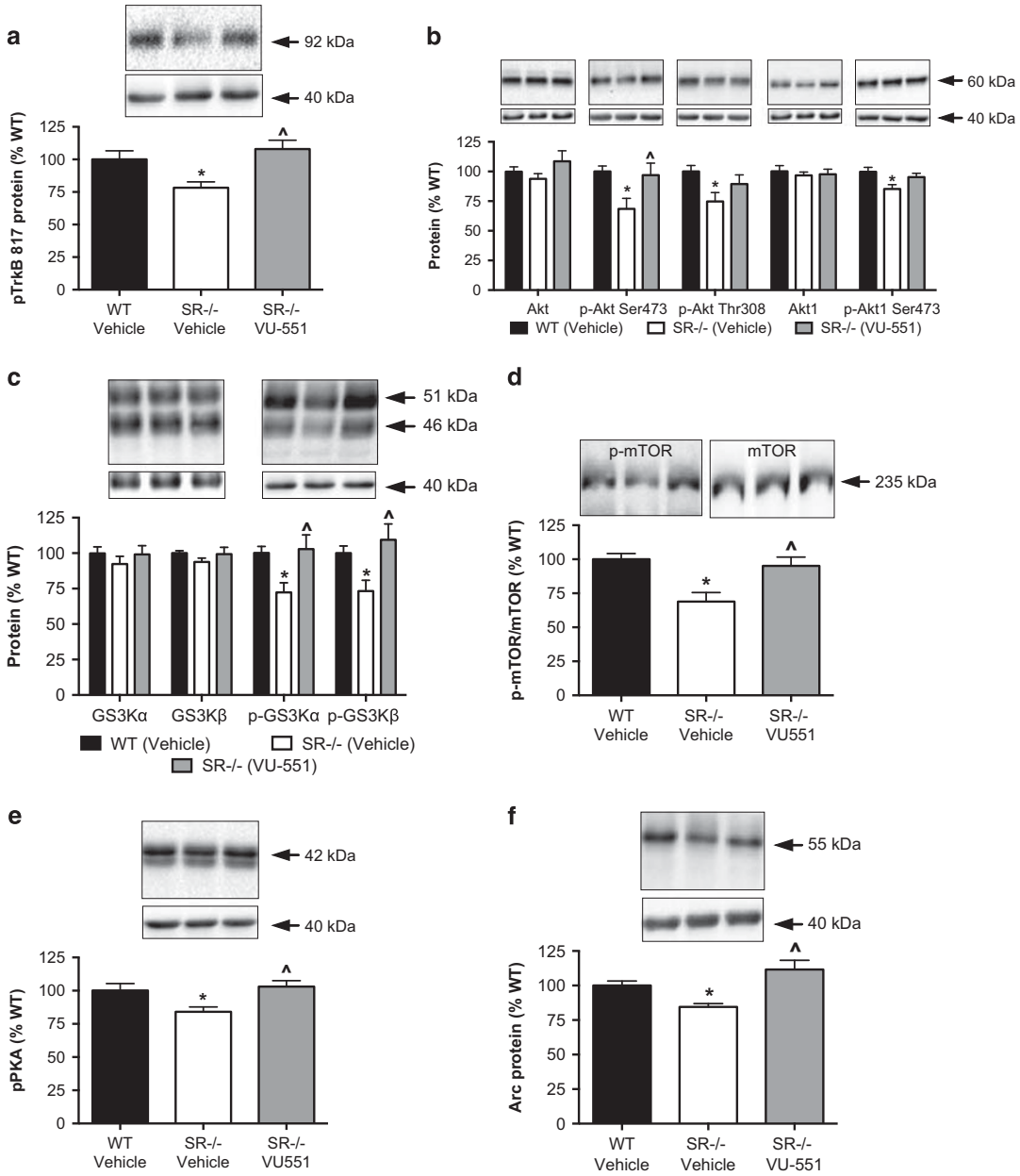

Figure 4 Chronic VU0409551 treatment rescues the hippocampal neurochemical deficits in SR−/− mice. WT ($n=10$) and SR−/− ($n=7$–8) mice received 5 days of either vehicle or VU0409551 (VU551; 30 mg/kg; i.p.) and were killed 2 h after the last injection. Protein levels of (a) phosphorylated tropomysin receptor kinase B (pTrkB) Tyr817, (b) Akt, p-Akt Ser473, and p-Akt Thr308, Akt1, p-Akt1 Ser473, (c) glycogen synthase kinase 3 (GS3K) α/β and p-GS3K α/β (α = 51 kDa, β = 46 kDa), (d) p-mTOR Ser2448/mTOR, (e) p-protein kinase A (PKA), and (f) activity-regulated cytoskeleton-associated protein (Arc) were measured in the hippocampus of WT mice (black bars), SR−/− mice (vehicle; white bars), or SR−/− mice treated with VU551 (gray bars) as the optical density normalized to WT values (%WT). Each western blot image includes a representative band of the protein of interest and β-actin (41 kDa) from each of the experimental groups: WT (vehicle), SR−/− 551 (vehicle), SR−/− (VU551), respectively. Asterisk (*) indicates significant difference from the WT vehicle group ($P<0.05$) and ^ indicates significant difference from the SR−/− vehicle group. Significant one-way ANOVA results were followed up by Newman–Keuls multiple comparison test. All values represent the mean ± SEM.

These results are especially interesting in light of the unique properties of VU0409551 relative to previously reported mGlu$_5$ PAMs. It is well established that mGlu$_5$, through its physical interaction with the NMDAR in the postsynaptic density, can potentiate NMDAR activity. This potentiation is facilitated by adaptor proteins, such as Homer and Shank, and is independent of $G_{\alpha q}$ signaling (Gao *et al*, 2013). However, VU0409551 displays a unique 'stimulus bias' and potentiates coupling of mGlu$_5$ to $G_{\alpha q}$, but does not directly potentiate mGlu$_5$ modulation of NMDAR currents in hippocampus of WT animals (Rook *et al*, 2015). Consistent with this finding in rats, we found that VU0409551 did not enhance NMDAR fEPSPs in hippocampal slices from WT mice. Similarly, VU0409551 was also found not to augment LTP or potentiate NMDAR currents in the hippocampus of normal rats (Rook *et al*, 2015). These findings raise the possibility that constitutively reduced NMDAR activity leads to changes in the composition of receptors, scaffolding, and/

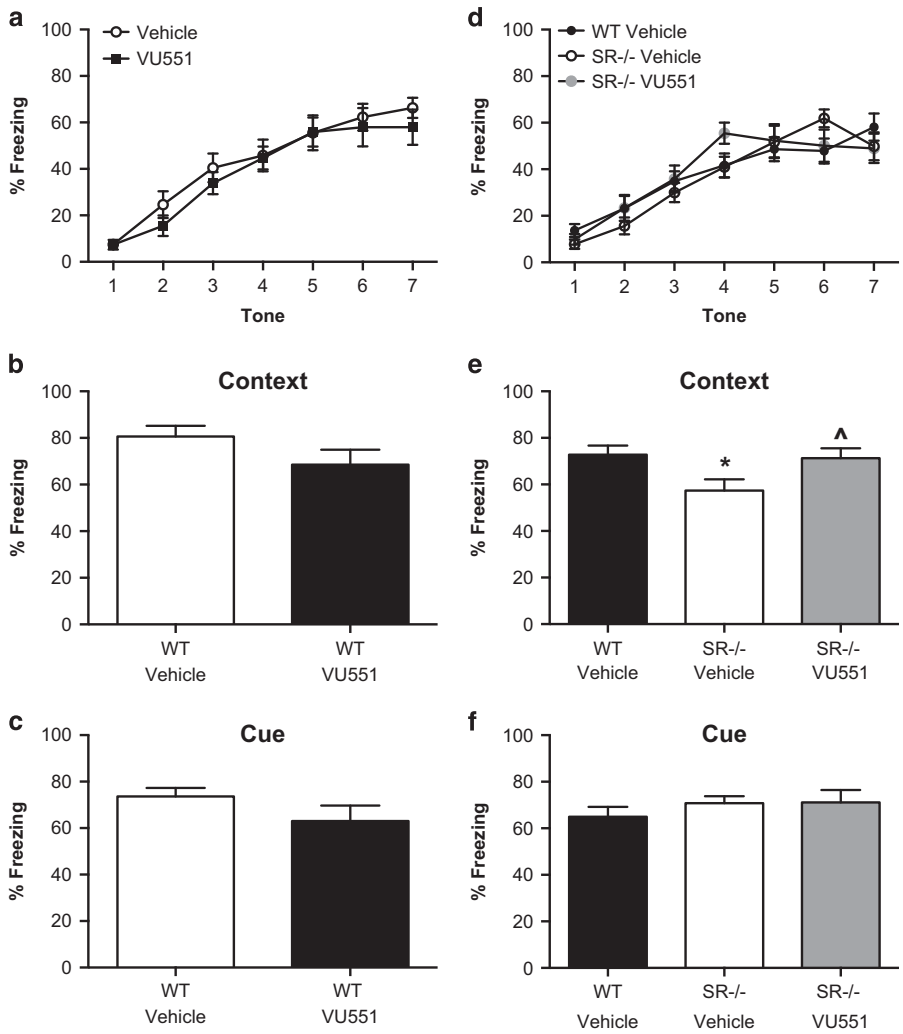

Figure 5 Chronic VU0409551 treatment improves memory in SR−/− mice. WT mice received 5 days of either vehicle (white; $n=8$) or VU0409551 (VU551; 30 mg/kg, black, $n=8$) and were subjected to a trace fear-conditioning paradigm during the last 3 days of treatment. Injections were given 2 h before behavioral testing. (a) The amount of freezing during each of the seven tone presentations was measured for each group. (b) The average time animals froze during the first 4 min of being placed in the chamber on day 2. (c) The average time animals froze during the first three tone presentations on day 3. WT (vehicle, black, $n=19$), SR−/− (vehicle, white, $n=13$), and SR−/− mice treated with VU551 (30 mg/kg, gray, $n=12$) were subjected to a trace fear-conditioning paradigm during the last 3 days of treatment. Injections were given 2 h before behavioral testing. (d) The amount of freezing during each of the seven tone presentations was measured for each group. (e) The average time animals froze during the first 4 min of being placed in the chamber on day 2. (f) The average time animals froze during the first three tone presentations on day 3. Significant one-way ANOVA results were followed up by Newman–Keuls multiple comparison test. Asterisk (*) indicates significant difference from the WT vehicle group ($P<0.05$) and ^ indicates significant difference from the SR−/− vehicle group. All values represent the mean ± SEM.

or signaling molecules in the postsynaptic density (Balu and Coyle, 2011a), such that VU0409551 can augment NMDAR function in this genetic background. Similar findings were recently reported in studies of another mGlu$_5$ PAM 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl) benzamide (CDPPB), which was shown to rescue NMDAR electrophysiological deficits in a Shank2−/− genetic mouse model of autism, without affecting the same measures in WT littermates (Won *et al*, 2012). However, it is also possible that VU0409551 restores deficits in NMDAR-mediated signaling in SR−/− mice by a mechanism that does not require direct modulation of NMDAR currents. Interestingly, recent studies have shown that mGlu$_5$ activation can prime CA3-CA1 synapses for induction of LTP by inducing release of endocannabinoids from CA1 pyramidal cells that act on neighboring inhibitory terminals to depress neurotransmission at inhibitory GABAergic synapses (iLTD) onto the pyramidal cells (Chevaleyre and Castillo, 2003, 2004; Younts *et al*, 2013; Xu *et al*, 2014). This disinhibition induced by mGlu$_5$ can facilitate neurotransmission and induction of NMDAR-dependent LTP at glutamatergic synapses and does not require direct modulation of NMDAR currents, but is dependent on mGlu$_5$ activation of $G_{\alpha q}$ and phospholipase C (Chevaleyre and Castillo, 2003, 2004). Our results demonstrate the critical importance of testing novel compounds for desired therapeutic action in disease-relevant models, where disorder-relevant perturbation(s) in brain physiology have been established.

There are several mechanisms by which subchronic administration of VU0409551 could facilitate the complete reversal of Akt/GS3K/mTOR signaling deficits in SR−/− mice. Akt is activated by binding to phospholipids at the plasma membrane and subsequent phosphorylation at Thr308, by protein dependent kinase 1 (PDK1) in a phosphoinositide 3 kinase (PI3K)-dependent manner, (4) and Ser473 by the mTORC2 complex (formerly known as PDK2). It has been shown that CDPPB increases pAkt *in vitro* (Doria *et al*, 2013) and *in vivo* (Doria *et al*, 2015). The activation of Akt by group I mGlu receptors involves the GTPase PI3K enhancer-long (PIKE-L), which couples PI3K to group I mGlu receptors via the adaptor protein Homer (Rong *et al*, 2003). Stimulation of NMDAR activity or TrkB by VU0409551 treatment could also activate this pathway. Interestingly, VU0409551 did not augment the phosphorylation states of TrkB and mTOR in the hippocampus of WT mice. This suggests that the composition of certain adaptor proteins at the membrane and/or intracellular cascades is altered by the constitutive NMDAR hypofunction of SR−/− mice. Finally, VU0409551 was shown to potentiate $G_{\alpha q}$-mediated calcium release in the presence of glutamate in a HEK293A *in vitro* cell culture system (Rook *et al*, 2015). However, it is unclear whether the effect of VU0409551 on calcium release holds true *in vivo*. Future work will be needed to determine the mechanism(s) that are responsible for the ability of VU0409551 to normalize Akt signaling in the hippocampus of SR−/− mice.

Mouse genetic and pharmacological studies in rats demonstrate the importance of mGlu$_5$ in cognition (Herman *et al*, 2012). Emotional learning and memory and contextual processing are impaired in patients with SCZ (Hall *et al*, 2007; Maren *et al*, 2013). In contrast to delay fear conditioning, trace fear conditioning places a temporal gap between the tone (CS) and the shock (US). The insertion of this trace interval during CS–US presentation alters the brain circuitry recruited for fear learning (Raybuck and Lattal, 2014) and makes the association more difficult than delay conditioning (Kinney *et al*, 2002). This paradigm also engages working memory and attention circuits, which are perturbed in SCZ (Young and Geyer, 2015). The expression of contextual fear memory following trace fear conditioning is dependent on NMDARs in the dorsal hippocampus (Misane *et al*, 2005; Quinn *et al*, 2005). We found that VU0409551 was able to restore the contextual memory impairments of adult SR−/− mice in a trace fear-conditioning paradigm. We believe that the memory-enhancing effects of VU0409551 in SR−/− mice are due to the ability of this compound to reverse the hypofunction of their hippocampal NMDARs. However, mGlu$_5$s are also highly expressed in the medial prefrontal and anterior cingulate cortices, areas that are important for trace fear learning (Raybuck and Lattal, 2014). Future studies will determine in which particular brain regions mGlu$_5$ enhances NMDAR function and where stimulation is important for reversing the learning deficits in SR−/− mice. It should be noted that VU0409551 was found to enhance acquisition of contextual fear conditioning in normal rats (Rook *et al*, 2015). The lack of effect in WT mice could be due to several factors, including species differences, the type of fear conditioning used (contextual *vs* trace), and/or a ceiling effect due to the high level of freezing in vehicle-treated WT mice.

In sum, VU0409551 was as effective as D-serine in normalizing NMDAR function, as well as reversing the neurochemical and learning deficits in SR−/− mice. These findings are of considerable importance because they demonstrate that a primary deficit in NMDAR function, in this case due to a lack of co-agonist, can be overcome by targeting a different receptor. Positive allosteric modulation of mGlu$_5$ not only augments NMDAR function but also stimulates the Akt pathway, which is impaired in SR−/− mice and is also genetically linked to SCZ (Emamian, 2012). VU0409551 has a more favorable safety profile compared with other mGlu$_5$ PAMs, as well as a more favorable pharmacokinetic and pharmacodynamic profile than endogenous GMS agonists, such as D-serine and glycine. Therefore, this study highlights the potential use of this compound to treat disorders, such as SCZ, in which NMDAR hypofunction is a contributing etiological factor. Importantly, VU0409551 ameliorates those processes in the SR−/− mouse that are linked to negative symptoms and cognitive impairments in SCZ, the very ones that are associated with persistent disability and are unresponsive to current antipsychotic medications (Strassnig *et al*, 2015).

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)