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Original Article

ORIGINAL ARTICLE

Asc-1 Transporter Regulation of Synaptic Activity via the Tonic Release of D-Serine in the Forebrain

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Abstract

D-Serine is a co-agonist of NMDA receptors (NMDARs) whose activity is potentially regulated by Asc-1 (SLC7A10), a transporter that displays high affinity for D-serine and glycine. Asc-1 operates as a facilitative transporter and as an antiporter, though the preferred direction of D-serine transport is uncertain. We developed a selective Asc-1 blocker, Lu AE00527, that blocks D-serine release mediated by all the transport modes of Asc-1 in primary cultures and neocortical slices. Furthermore, D-serine release is reduced in slices from Asc-1 knockout (KO) mice, indicating that D-serine efflux is the preferred direction of Asc-1. The selectivity of Lu AE00527 is assured by the lack of effect on slices from Asc-1-KO mice, and the lack of interaction with the co-agonist site of NMDARs. Moreover, in vivo injection of Lu AE00527 in P-glycoprotein-deficient mice recapitulates a hyperekplexia-like phenotype similar to that in Asc-1-KO mice. In slices, Lu AE00527 decreases the long-term potentiation at the Schaffer collateral-CA1 synapses, but does not affect the long-term depression. Lu AE00527 blocks NMDAR synaptic potentials when typical Asc-1 extracellular substrates are present, but it does not affect AMPAR transmission. Our data demonstrate that Asc-1 mediates tonic co-agonist release, which is required for optimal NMDAR activation and synaptic plasticity.

Key words: glycine, long-term potentiation, neurodegeneration, NMDA receptor, synaptic plasticity

Introduction

The binding of co-agonists (D-serine or glycine) to NMDA-type glutamate receptors (NMDARs) is required for several NMDAR-related processes, ranging from normal neurotransmission to neurotoxicity (Johnson and Ascher 1987; Mothet et al. 2000; Wolosker et al. 2008; Traynelis et al. 2010). The mechanisms regulating the release of co-agonists and their relative roles in NMDAR activity are yet to be fully elucidated (Mothet et al. 2015). In the adult CA1 hippocampus, endogenous D-serine was proposed to

be the major co-agonist at synaptic NMDARs (Yang et al. 2003; Papouin et al. 2012), though glycine also stimulates synaptic NMDARs in the same region (Rosenberg et al. 2013; Le Bail et al. 2015). In the lateral amygdala, a combination of the tonic release of D-serine and the activity-dependent release of glycine is required for the optimal activation of synaptic NMDARs (Li et al. 2013).

Neurons contain D-serine and its biosynthetic enzyme serine racemase (SR) (Kartvelishvily et al. 2006; Miya et al. 2008; Benneyworth et al. 2012; Ehmsen et al. 2013; Balu et al. 2014). Neuronal D-serine dynamics was proposed to be regulated by neutral amino acid transporters such as the Asc-1 antiporter (Helboe et al. 2003; Matsuo et al. 2004). Along with its ancillary subunit 4F2hc, Asc-1 mediates the bidirectional transport of D-serine coupled with the counter-transport of small neutral amino acids, referred to as the exchange mode (Fukasawa et al. 2000; Pineda et al. 2004). However, it can also release neutral amino acids by passive transport via facilitated diffusion (Fukasawa et al. 2000; Pineda et al. 2004; Rosenberg et al. 2010, 2013).

Asc-1 was initially proposed to be involved in D-serine uptake rather than release (Fukasawa et al. 2000; Rutter et al. 2007). However, endogenous D-serine release via Asc-1 has been demonstrated by the addition of D-amino acid substrates that selectively activate the exchange transport mode of Asc-1 (Rosenberg et al. 2013). Using D-isoleucine (D-Ile) as a tool to stimulate D-Ile/D-serine exchange in acute slices, we recently showed that Asc-1-mediated D-serine release increases NMDAR synaptic activity at the hippocampal CA1 (Rosenberg et al. 2013). However, D-Ile is not present in the brain and its effects do not warrant a definitive conclusion on the preferred direction of Asc-1 transport. Consequently, the role of Asc-1 in regulating NMDARs via D-serine release is still uncertain. In addition, Asc-1 is unique among amino acid exchangers in its ability to mediate the efflux of D-serine from cells via facilitated diffusion (Rosenberg et al. 2013). However, a possible role of this pathway in mediating D-serine release in physiological preparations and its functional implications are yet to be determined.

In addition to its role in D-serine transport, Asc-1 also works on additional substrates, including glycine and L-serine (Fukasawa et al. 2000; Helboe et al. 2003; Safory et al. 2015). Asc-1-knockout (KO) mice display a global reduction in brain glycine (Safory et al. 2015). This is associated with glycinergic inhibitory transmission impairment and a fatal hyperekplexia-like phenotype due to deficient glycine synthesis from L-serine (Safory et al. 2015). The Asc-1-KO neuromotor phenotype is reversed by glycine, but not by D-serine administration, indicating that Asc-1 is mainly involved in glycine metabolism. D-Serine administration also does not reverse the glycine deficits in the brains of Asc-1-KO mice (Safory et al. 2015). These data raise further questions on the previously proposed roles of Asc-1 in NMDAR activity via changes in D-serine homeostasis (Rosenberg et al. 2010, 2013). On the other hand, it is also possible that the strong glycine-related phenotype of Asc-1-KO mice obscures more subtle changes in NMDAR-dependent processes mediated by D-serine via the Asc-1 transporter.

In the present report, we developed Lu AE00527, a new selective Asc-1 inhibitor that blocked all transport modes of Asc-1 without stimulating the exchange activity, and did not change the total glycine content in acute slice preparations. Using this novel inhibitor, as well as Asc-1-KO mice as additional controls, we carried out a detailed investigation on the role of Asc-1 in D-serine dynamics and NMDAR activity in forebrain slices. Our study concludes that acute Asc-1 inhibition decreases the tonic release of D-serine, indicating that this is the preferred direction of Asc-1-mediated D-serine transport, and Asc-1 activity is required for optimal NMDAR activation and synaptic plasticity.

Materials and Methods

Materials

L-Serine and D-Ile were purchased from Bachem. D-[^{3}H]Serine (lot 1311127) was purchased from American Radiolabeled Chemicals. [^{3}H]Glycine (lots 3632835 and 1817306) and [^{3}H]MDL 105 519 [(*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1[3*H*]-indole-2-carboxylic acid] (lot 643251) were obtained from PerkinElmer Life and Analytical Sciences. L-alanine, L-cysteine, veratridine, choline chloride and glycine were obtained from Sigma-Aldrich. D-Serine was purchased from Bachem or Sigma-Aldrich. (*R*)-2-({3-[1-(4-Chloro-phenyl)-6,7-difluoro-indan-1-yl]-propyl}-methyl-amino)-3-thiazol-4-yl-propionic acid (Lu AE00527) stock solution (10 mM) was prepared in DMSO and stored at –20 °C. All the other reagents were of analytical grade.

Human Asc-1 Screening Assay and Identification of Lu AE00527

To enable high throughput screening in a scintillation proximity assay (SPA) format, we developed a method based on [^{35}S]-cysteine uptake, due to the significantly higher specific activity of this isotope ([^{35}S]-cysteine, specific activity >1000 Ci/mmol; Amersham, cat. SJ15232). For the SPA, HEK293 cells expressing human Asc-1 were detached from culture flasks by the addition of 25 mM EGTA in PBS and washed in assay buffer (120 mM choline chloride, 1.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM glucose, 25 mM triethylammonium bicarbonate, and 10 mM HEPES pH 7.4) by centrifugation. The uptake assay was performed in 96-well plates (Isoplates, PerkinElmer). To each well, the following were added: 5×10^5 cells, 0.5 mg wheat germ agglutinin-coated SPA beads (RPMQ0001, PerkinElmer), test compound, and 10^5 dpm [^{35}S]-cysteine in a total volume of 200 μL. The plates were incubated for 2 h at RT and counted in a MicroBeta counter (PerkinElmer). Using this assay, we identified (*R*)-2-({3-[(*R*)-1-(4-Chloro-phenyl)-6,7-difluoro-indan-1-yl]-propyl}-methyl-amino)-3-thiazol-4-yl-propionic acid (Lu AE00527) as an Asc-1 inhibitor. Its detailed synthesis route will be disclosed in a subsequent publication. The identification of Lu AE00527 was carried out using ^{1}H NMR and LC–MS. ^{1}H NMR spectra were recorded on a DRX-500 (Bruker) equipped with a 5-mm QNP probe with a Z-gradient operating at 500.13 MHz for ^{1}H. Tetramethylsilane was used as an internal reference for ^{1}H. LC–MS was run on a Sciex API150EX equipped with an APPI source operating in the positive ion mode. The HPLC consisted of LC10-ADvp LC pumps (Shimadzu), an SPD-M20A PDA detector (Shimadzu, operating at 254 nm), an SCL-10A system controller (Shimadzu), Gilson 21 5autosampler (Gilson), a 7990R column oven (Jones Chromatography), and a Sedex 55 Evaporative Light Scattering detector (Sedere). The LC was carried out using an Atlantis dC-18, 4.6 × 30 mm column, 3 μm (Waters) at 40 °C on 3.3 mL/min of a binary gradient (2% B to 100% B in 2.4 min followed by 2% B in 0.4 min). The buffers consisted of 0.05% tetrahydrofurane (TFA) in water (A) and 0.05% TFA and 95% acetonitrile in water (B), with a total running time of 2.8 min.

Amino Acid Uptake in HEK293 Transfected Cells

HEK293 cells cultured in DMEM, 10% FBS, and penicillin/streptomycin were seeded in poly-l-lysine-coated 96-well plates and then transfected with several constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

When co-transfected with Asc-1 (SLC7a10) and its ancillary subunit 4F2hc (SLC3A2), a 1 : 1 or 3 : 2 cDNA ratio was employed. Thirty-six to 48 h after transfection, the cells were washed 2–3 times with HEPES-buffered saline (HBSS; in mM: 137 NaCl, 5.4 KCl, 0.44 KH_2PO_4, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 1 $CaCl_2$, 5.6 D-glucose, 4.2 $NaHCO_3$, 0.34 Na_2HPO_4, 1 pyruvate, and 10 HEPES, pH 7.4) at 25 °C. Then, the uptake of D-[^{3}H]serine or [^{3}H]glycine was started by adding 80 µL of uptake buffer containing the radiolabeled amino acid substrate at 100 nM concentration at 25 °C. After the incubation, the uptake was stopped by removing the uptake solution and quickly washing the cells 4 times with 100 µL of ice-cold uptake buffer per well. The cells were lysed by the addition of 150 µL of Optiphase Supermix scintillation liquid (PerkinElmer, MA, USA) or first lysed with 100 µL of water followed by Ultima Gold scintillation liquid (PerkinElmer), and subsequently counted on a scintillation counter. Both lysis methods released the entire radioactivity from the cells. The protein concentration in each well was measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc., IL, USA). The amino acid uptake was monitored at the linear phase (10–15 min uptake) and normalized to the protein concentration in each well. For amino acid uptake in the absence of Na^+, we used a Na^+-free HBSS medium in which NaCl was replaced by choline chloride. The specific uptake was determined by subtracting the basal [^{3}H]amino acid uptake from the cells transfected with a suitable control plasmid (GFP or 4F2hc gene alone).

cDNAs

HEK293 cells were transfected with rat Asc-1 (SLC7A10)–pExpress-1 (Open Biosystems), rat 4F2 heavy chain (4F2hc, SLC3A2)–pCMV–SPORT6 (Open Biosystems), human Asc-1-pCMV-XL5 (Origene), human 4F2hc-pCMV6-XL5 (Origene), rat GlyT1b–pExpress-1 (Open Biosystems), rat GlyT2–pRc/RSV (provided by Prof. N. Nelson, Tel Aviv University, Tel Aviv, Israel), and rat ASCT2–pRK5–KS (provided by Prof. S. Broer, Australian National University, Canberra, Australian Capital Territory, Australia). GFP subcloned into pRK5 was a gift from Prof. S. Engelender (Technion, Israel). The cells were used 48–72 h after transfection.

D-[^{3}H] Serine Transport in Rat Brain Membranes

The cerebral cortices were dissected from the brains of the male Wistar rats (150–200 g) and the tissue was homogenized in 0.40 M sucrose and centrifuged at 1000 × *g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 40 000 × *g* for 20 min and resuspended in an assay buffer consisting of 120 mM choline chloride, 1.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM D-glucose, 25 mM triethylammonium bicarbonate, and 10 mM HEPES. Test compounds and tissue (1 mg tissue/well) were added to the 96-well plates and incubated with 100 nM D-[^{3}H]-serine for 5 min at 25°C. The samples were filtered on Unifilter GF/B glass fiber and washed 3 times with 0.25 mL of ice-cold assay buffer.

Mice Models

P-glycoprotein (P-gp, Mdr1a, Abcb1a)-deficient male mice (Crl: CF1-Abcb1a^{mds} mutants) and CF-1 outbred background mice [wild type (WT)-expressing P-gp] were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA; 20–25 g body weight). All the animals arrived at the animal facility at least 5 days before initiation of the experiments. During acclimatization, the animals were housed in pairs under controlled conditions of temperature (21 ± 2 °C) and relative humidity (55 ± 5%) and a 12-h light/dark cycle (lights on at 6:00 AM).

Asc-1-KO mice were obtained from Deltagen (San Mateo, CA, USA). The mice were maintained as heterozygotes in a mixed strain background (C57BL/6J-129/OlaHsd) with the approval of the Committee for the Supervision of Animal Experiments (Technion—Israel Institute of Technology). Genotyping was carried out using the same primers and protocol previously described (Rutter et al. 2007). Homozygotes and WT littermates were produced, genotyped, and used at postnatal days 9–10, before any neurological symptoms appeared.

Primary Cultures

Primary neuronal cultures from the cortex were prepared from E18 Sprague-Dawley rat embryos as previously described (Kartvelishvily et al. 2006). Briefly, pregnant Sprague-Dawley rats were anesthetized with isoflurane and decapitated in accordance with the Committee for the Supervision of Animal Experiments (Technion—Israel Institute of Technology). The embryos (E18) were harvested and the cortices were quickly dissected. The neurons were cultured in Neurobasal medium supplemented with 2% B27. Such cultures typically contain <2% contaminant astrocytes (Kartvelishvily et al. 2006). Primary astrocyte cultures were obtained from the cortices of the neonatal Sprague-Dawley rats as described previously (Kartvelishvily et al. 2006). The neuronal and astrocytic cultures were used 14–20 days after plating.

Amino Acid Release From Cell Cultures

For monitoring the kinetics of amino acid release from cultures, primary neurons (DIV 10–14) were first preloaded with 100 nM D-[^{3}H]serine or [^{3}H]glycine for 20 min. In the last 5 min of the preloading phase, the Lu AE00527 or DMSO vehicle was added in the same preloading medium. Subsequently, the wells were washed 3 times with cold HBSS and then transitioned to a new HBSS buffer supplemented with different drugs for 1–2 min at RT. The released [^{3}H]amino acids (AA_{out}) were monitored in the release medium by scintillation counting. The remaining intracellular [^{3}H]amino acid (AA_{in}) was determined after cells lysis with 100 µL of water per well. The fractional release was calculated using the formula: $(AA_{out} \times 100)/(AA_{out} + AA_{in})$.

Plasma and Brain Exposure Studies

P-gp-deficient and WT mice were given a single subcutaneous 10 mg/kg dose of Lu AE00527 ($n = 3$ in each group). Lu AE00527 was dissolved in 10% hydroxylpropyl-β-cyclodextrin and dosed in a volume of 10 mL/kg. One hour after dosing, the animals were anesthetized using isoflurane, and cardiac blood samples were taken in EDTA-coated tubes. Immediately afterwards, the mice were decapitated and the brains removed and gently rinsed on filter paper. Blood samples were centrifuged for 10 min at 4 °C, after which the plasma was harvested and kept at –80 °C together with the brains until bioanalysis. Prior to analysis, brain homogenate was prepared by homogenizing the whole brain with 70% acetonitrile (1 : 4 v/v), followed by centrifugation and collection of the supernatant. The plasma and brain concentrations of Lu AE00527 were determined using liquid chromatography followed by tandem mass spectrometry detection (LC–MS/MS) using a Sciex-API 4000 mass spectrometer (AB Sciex, Foster City, CA, USA). The lower limit of quantification was 1 ng/mL in plasma and 5 ng/g in the brain samples.

In Vitro Permeability Studies

Madin-Darby Canine Kidney (MDCK) cells transfected with MDR1, the gene encoding for P-gp, were used to assess the membrane transport characteristics of Lu AE00527 according to a previously described methodology (Risgaard et al. 2013). Briefly, 2 μM of Lu AE00527 was applied to the apical (A) or basolateral (B) side of the cell monolayer. The apparent permeability in both directions (A to B and B to A) was determined in triplicate following 150 min incubation at 37 °C and quantification by LC–MS/MS analysis. The efflux ratio was calculated from the permeability ratio between B to A and A to B.

Amino Acid Content and Release From Neocortical Slices

Two- to 3-month-old male Sprague-Dawley rats or 9- to 10-day-old Asc-1-KO and WT mice littermates were anesthetized with isoflurane and killed by decapitation. The neocortices were dissected and chopped into strips measuring 400 μm by 400 μm using a McIlwain tissue chopper (Gonzalez-Alvear and Werling 1994). After 3 washes with oxygenated modified Krebs-HEPES buffer (MKB; in mM: 127 NaCl, 1.3 NaH_2PO_4, 15 HEPES, 10 D-glucose, 1 $MgCl_2$, 5 KCl, and 2.5 $CaCl_2$, pH 7.4), the slices were transferred to the Suprafusion 1000 (SF-6) apparatus (Brandel). Subsequently, the slices were equilibrated in the presence of either 10 μM Lu AE00527 or the DMSO vehicle by 20–30 min perfusion with oxygenated MKB in 0.3 mL chambers at a flow rate of 0.6 mL/min at 37 °C. After equilibration, the samples were collected at 1.6 min intervals before and after stimulation with drugs. The endogenous D-serine or glutamate release was monitored in the perfusate by HPLC as described (Hashimoto et al. 1992). The total amino acid loading was estimated after the slices were incubated for 20 min with 0.1 M HCl to release all the intracellular free amino acids. The release was normalized by the total amino acid content in the slices from each channel, and expressed as % total. In experiments where L-serine, L-alanine and L-cysteine were included in the artificial cerebrospinal fluid (aCSF), the perfusates were treated with 0.010 mg/mL of recombinant l-serine dehydratase prior to the HPLC analysis, as previously described (Radzishevsky and Wolosker 2012). This enzyme degrades l-serine into pyruvate and ammonia, but leaves D-serine intact, and therefore allows precise determination of released D-serine. For the release of D-[^{3}H]serine or [^{3}H]glycine, the slices were first preloaded with 5 μM D-[^{3}H]serine or [^{3}H]glycine by a 20-min incubation in oxygenated MKB at 37 °C prior to the equilibration step. The slices were then washed 3 times with oxygenated MKB and transferred to the perfusion apparatus. D-[^{3}H]serine or [^{3}H]glycine was monitored by liquid scintillation counting and the values expressed as fractional release.

Ligand Binding

The binding of [^{3}H]MDL 105 519 (final concentration of 15 nM) to total rat brain membranes was carried out at 4 °C in 10 mM Tris–acetate buffer, pH 7.4, and monitored by filtration assay as described previously (Baron et al. 1996). Briefly, after incubation with membranes for 30 min, the reaction was terminated by rapid filtration through glass fiber filters (Whatman GF/B), followed by 5 washes with 4 mL of ice-cold Tris–acetate buffer using a Brandel Harvester (Brandel, Gaithersburg, MD, USA). Nonspecific binding was monitored by the addition of [^{3}H]MDL 105 519 in the presence of 1 mM glycine and subtracted from each experimental point.

Ex Vivo Electrophysiology

Experiments were carried out in accordance with the European Council Directive (63/2010) regarding the care and use of animals for experimental procedures. Animals were housed under a 12-h light–dark cycle in a temperature of 20 ± 2 °C and humidity of 45 ± 15% controlled environment with food and water ad libitum.

Transverse hippocampal slices (400 μm) were obtained from young adult (3–5 months old) Sprague-Dawley rats. The animals were anesthetized with halothane before decapitation. Slices were prepared in ice-cold aCSF and placed in a holding chamber for at least 1 h. The composition of aCSF was as follows (in mM): NaCl 124, KCl 3.5, $MgSO_4$ 1.5, $CaCl_2$ 2.3, $NaHCO_3$ 26.2, NaH_2PO_4 1.2, and glucose 11, at pH 7.4. A single slice was transferred to the recording chamber one at a time and continuously submerged with aCSF pre-gassed with 95% O_2/5% CO_2 and perfused at 2 mL/min.

Extracellular recordings were obtained at RT from the apical dendritic layer of the CA1 area using micropipettes filled with 2 M NaCl. Presynaptic fiber volleys (PFVs) and field excitatory postsynaptic potentials (fEPSPs) were evoked by electrical stimulation of the Schaffer collaterals and commissural fibers located in the "stratum radiatum." Specific NMDAR-mediated fEPSPs were isolated in slices perfused with low-Mg^{2+} (0.1 mM) aCSF supplemented with NBQX (10 μM). The averaged slope of 3 PFVs and fEPSPs was measured using Win LTP software (Anderson and Collingridge 2001). To evaluate the level of receptor activation, the fEPSP/PFV ratio was plotted against stimulus intensity (300, 400, and 500 μA). The effects of exogenous Lu AE00527 (10 μM), D-Ile (1 mM), neutral amino acids, including L-alanine (14 μM), L-serine (36 μM), and L-cysteine (2 μM) (A,S,C), and of D-serine (100 μM) were assessed by determining the fEPSP/PFV ratio 15 min after the addition of the amino acid to the aCSF.

Paired-pulse facilitation (PPF) of the synaptic transmission was induced by electrical stimulation of afferent fibers with paired pulses (interstimulus interval of 30 ms). PPF was calculated as the ratio of the slope of the second response over that of the first one.

To investigate the long-term potentiation (LTP) of synaptic transmission, a test stimulus was applied every 10 s in a control medium and adjusted to obtain a fEPSP with a baseline slope of 0.1 V/s. The averaged slope of 3 fEPSPs was measured for 15 min before the delivery of a high-frequency stimulation (HFS), consisting of 1 train at 100 Hz pulses for 1 s at the test intensity. In some experiments, HFS consisted in 2 trains at 100 Hz pulses separated by 20 s interval. In the pharmacological experiments, Lu AE00527 (10 μM) was added to the aCSF 10 min before the establishment of the baseline in the absence or presence of D-serine (100 μM) or L-alanine (14 μM), L-serine (36 μM), and L-cysteine (2 μM), and maintained throughout recording. Long-term depression (LTD) was assessed by low-frequency conditioning stimulation at 2 Hz delivered for 10 min. In further pharmacological experiments, Lu AE00527 (10 μM) with or without D-amino-5-phosphonovalerate acid (D-APV, 80 μM) was added to the aCSF 10 min before the establishment of the baseline, and maintained throughout recording.

Behavioral Tests

Adult male P-gp mice were injected either with 70 mg/kg s.c. Lu AE00527 or vehicle (n = 15 in each group), and analyzed for tremors and acoustic startle after 4 h. The drug was solubilized in 15% hydroxylpropyl-β-cyclodextrin and dosed in a volume of 10 mL/kg. There was no significant difference in body weight between the treatments (35 ± 4 g, SD, vehicle, and 37 ± 4 g, SD,

drug-injected; $P > 0.3$). The tests were held in a sound-proof and ventilated box (Kinder Scientific, CA, USA). The session started with a 3-min acclimatization period with a 57-dB background noise level, delivered continuously throughout the test session. Spontaneous tremors were evaluated by 15 "no stimuli" trials. To evaluate the startle response, 10 trials consisting of single 20 ms 120 dB "pulse alone" startle stimuli (intertrial interval 1 min) were employed.

Statistical Analysis

All results are expressed as means ± SEM. In biochemical experiments, we employed the repeated-measures ANOVA and Bonferroni's multiple comparison test or paired, two-tailed Student's *t*-test using GraphPad Prism software version 6.03 (GraphPad, Inc.). The significance of changes in LTP magnitude between groups was determined by comparing the last 15 min of recordings. To take into account the correlations inherent in repeated measures, *P*-values were calculated using multivariate analyses of variance followed by Tukey's post hoc tests. A paired Student's *t*-test was used to evaluate the significance of pharmacological treatments on NMDA or AMPA synaptic transmission. Tremors and acoustic startle response were evaluated by unpaired two-tailed *t*-test. In all cases, differences were considered significant when $P \leq 0.05$.

Results

Identification of the Asc-1 Inhibitor Lu AE00527

Screening for amino acid transport inhibitors and medicinal chemistry optimization identified Lu AE00527 (Fig. 1*A*; Materials and Methods) as an inhibitor of Asc-1-mediated amino acid transport. In a screening assay using an SPA-based [^{35}S]-cysteine uptake, the estimated Lu AE00527 IC_{50} on human Asc-1 was 5 μM (pIC_{50} 5.3 ± 0.27, $n = 4$). The rat Asc-1 transporter appeared to be more sensitive to Lu AE00527 than its human counterpart. We found that 10 μM of Lu AE00527 partially inhibits D-[^{3}H]serine uptake in HEK293 cells transiently expressing human Asc-1 and its ancillary subunit 4F2hc, whereas the same concentration practically abolishes D-[^{3}H]serine uptake in HEK293 cells expressing rat Asc-1/4F2hc (Fig. 1*B*,*C*). The IC_{50} for Lu AE00527 on D-[^{3}H]serine uptake in HEK293 cells transfected with rat Asc-1/4F2hc was 350 nM (pIC_{50} 6.5 ± 0.2, $n = 5$; Fig. 1*D*). Similar to that observed in transfected cells, Lu AE00527 inhibited D-[^{3}H]serine uptake in rat cortical membranes with an apparent Ki around 550 nM (pKi 6.3 ± 0.05, $n = 5$). Since Asc-1 does not require Na^+ for activity (Fukasawa et al. 2000), we employed an uptake media devoid of Na^+ to inhibit all other transporters in the rat cortical membranes. Furthermore, we found that Lu AE00527 does not inhibit rat ASCT2, a Na^+-dependent neutral amino acid transporter that also uses D-serine as a substrate (Fig. 1*E*).

Since Asc-1 also uses glycine as a substrate (Fukasawa et al. 2000; Safory et al. 2015), we investigated if the inhibitor affects glycine uptake. We found that Lu AE00527 inhibits the uptake of [^{3}H]glycine in rat Asc-1/4F2hc-transfected HEK293 cells (Fig. 1*F*), but does not inhibit the rat glycine transporters GlyT1 and GlyT2 when tested at 10 μM (Fig. 1*G*,*H*).

Inhibition by Lu AE00527 was more pronounced at lower D-serine concentrations (Fig. 2*A*). A Dixon plot analysis suggested a competitive type inhibition (Fig. 2*B*). We found that Lu AE00527 inhibits the uptake of D-serine in rat cortical neuronal cultures (Fig. 2*C*), but does not affect the uptake of D-serine in purified astrocyte cultures (Fig. 2*C*), indicating that the drug may selectively affect neuronal D-serine dynamics. This is in agreement with the expression of Asc-1 in neurons in vivo (Helboe et al. 2003; Matsuo et al. 2004).

In addition to its role as an antiporter, Asc-1 mediates net amino acid release by facilitated diffusion (Fukasawa et al. 2000; Pineda et al. 2004; Rosenberg et al. 2013). We found that in the absence of extracellular Asc-1 substrates, Lu AE00527 decreases the basal release of D-serine from D-[^{3}H]serine-preloaded neurons (Fig. 2*D*). A similar blockade by Lu AE00527 is observed in neurons preloaded with [^{3}H]glycine, which is also a substrate of Asc-1 (Fig. 2*E*). The addition of D-Ile, which artificially activates the exchange transport mode of Asc-1 (Fukasawa et al. 2000; Pineda et al. 2004; Rosenberg et al. 2013), accelerates the release of preloaded D-[^{3}H]serine or [^{3}H]glycine by D-Ile/ D-Ser or Gly counter-transport (Fig. 2*D*,*E*). We found that Lu AE00527 abrogates the enhancement of D-serine and glycine release promoted by D-Ile (Fig. 2*D*,*E*). Thus, unlike D-Ile, Lu AE00527 is not a substrate for the amino acid exchange transport mode, and blocks all transport modes of Asc-1.

Effect of Lu AE00527 in Acute Brain Slices

Lu AE00527 ability to inhibit all transport modes of Asc-1 makes it particularly useful to probe the directionality of the Asc-1 transporter in physiological preparations. Asc-1 mediates either the uptake (Fig. 1) or release of D-serine/glycine (Fig. 2) from cells, but cell cultures may not replicate the physiological distribution of extracellular and intracellular amino acid pools. Therefore, in order to investigate the preferred direction of D-serine fluxes mediated by Asc-1, we applied Lu AE00527 to acute neocortical slices from adult rats and monitored endogenous D-serine release. In the case that Asc-1 predominantly mediates D-serine re-uptake into neurons, one expects that the drug will increase the extracellular D-serine concentration found in the perfusate of treated slices. Instead, we found that Lu AE00527 decreases the basal rates of endogenous D-serine release (Fig. 3*A*,*C*). A similar decrease was observed in slices preloaded with D-[^{3}H]serine (Fig. 3*B*,*D*), which labels endogenous pools and is not significantly metabolized during the experiment (Rosenberg et al. 2010). Thus, Asc-1 appears to mediate D-serine release from neocortical slices rather than re-uptake.

We also investigated the effect of Lu AE00527 in D-serine release from slices promoted by the exchange mode. The addition of D-Ile artificially increases the rate of D-serine release from acute neocortical slices by activating a D-Ile/ D-Ser exchange via Asc-1 (Figs 3*A*,*B*; Fukasawa et al. 2000; Pineda et al. 2004; Rosenberg et al. 2013). We found that Lu AE00527 abrogates the enhancement of D-serine release promoted by D-Ile, and this effect is detectable both with endogenous (Fig. 3*A*) and preloaded D-[^{3}H]serine pools (Fig. 3*B*).

Although D-Ile is an artificial Asc-1 substrate, the extracellular milieu in the brain contains almost saturating levels of typical Asc-1 substrates (Lindroth et al. 1985) that are likely to activate Asc-1 in a similar fashion. For instance, the extracellular concentrations of serine, alanine, and cysteine in the brain were reported to be 14, 36, and 2 μM, respectively (Lindroth et al. 1985). Altogether, these values are above the K_m of Asc-1 (Fukasawa et al. 2000). Similar to that seen with D-Ile, supplementation of the perfusion media with physiological concentrations of alanine, serine, and cysteine (A,S,C) increases the rate of release of both endogenous D-serine (Fig. 3*C*) and preloaded D-[^{3}H]serine pools (Fig. 3*D*), presumably by activating the exchange transport mode. In addition to reducing the basal release of D-serine, we found that Lu AE00527 also attenuates the enhancement of D-serine release promoted by typical Asc-1 substrates, indicating

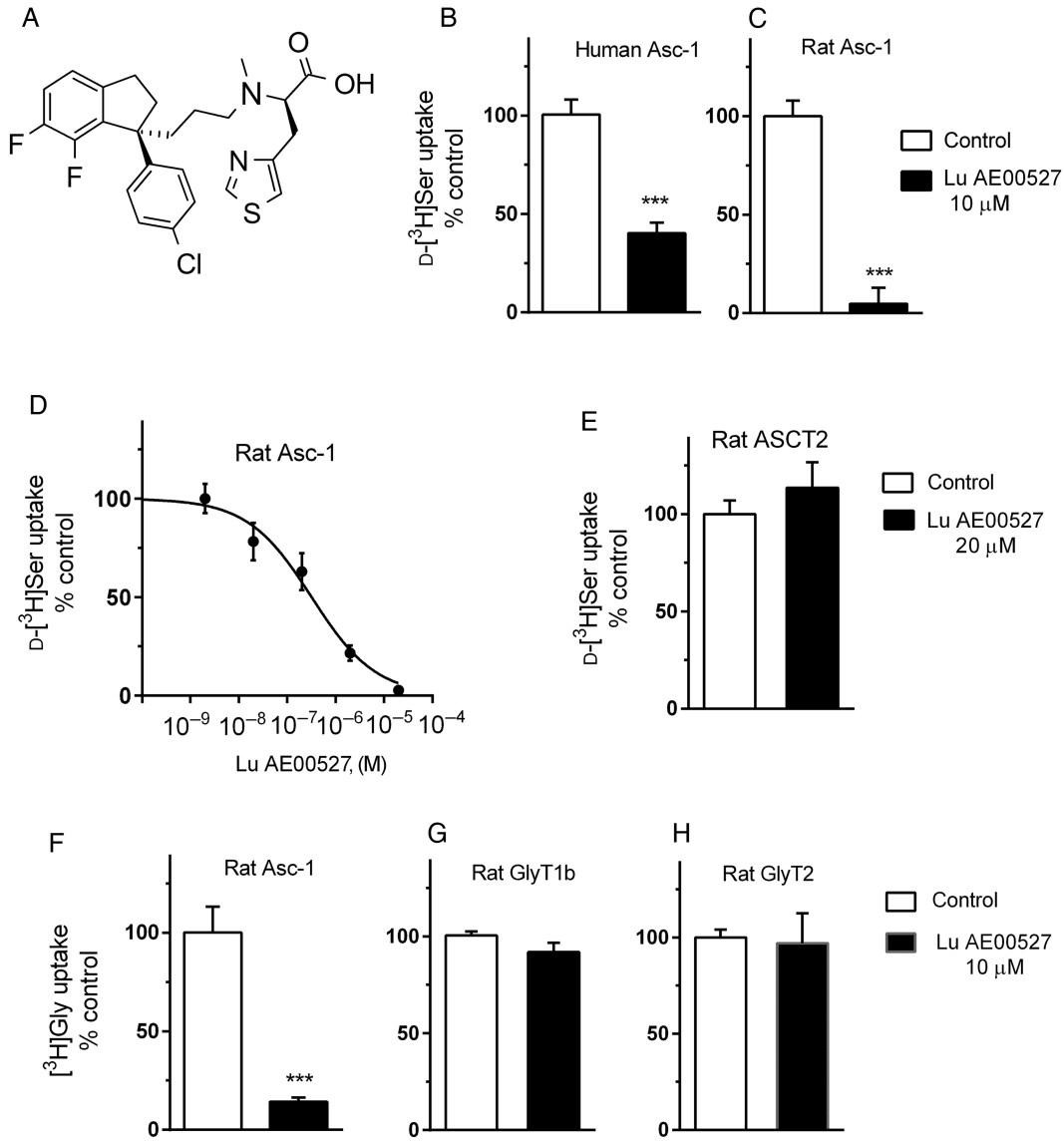

Figure 1. Lu AE00527, a novel Asc-1 inhibitor. (*A*) Structure of Lu AE00527. (*B* and *C*) Lu AE00527 inhibits D-serine uptake in HEK293 cells transiently expressing human and rat Asc1 along with its ancillary subunit 4F2hc. (*D*) Dose-dependent inhibition of D-[^{3}H]serine in HEK293 cells transiently expressing rat Asc1/4F2hc. (*E*) Lu AE00527 (20 μM) does not inhibit rat ASCT2 transporter transiently transfected into HEK293 cells and assayed in Na^+-containing medium. (*F*) Lu AE00527 (10 μM) inhibits glycine uptake in rat Asc-1/4F2hc HEK293 transfected cells assayed in a medium lacking Na^+ and supplemented with 100 nM [^{3}H]glycine. (*G* and *H*) Lu AE00527 (10 μM) does not affect rat GlyT1b or rat GlyT2 transporters transfected into HEK293 cells assayed with 100 nM [^{3}H]glycine in Na^+-containing media. The values were calculated by subtracting blanks consisting of cells transfected with 4F2hc or GFP. The values are the mean ± SEM of 3–6 experiments with different cultures. ***, different from control at $P < 0.001$ (paired Student's *t*-test).

that Asc-1 contributes to amino acid-mediated D-serine release (Fig. 3C,*D*).

To investigate the effect of Lu AE00527 on glycine release, we preloaded neocortical slices with [^{3}H]glycine, as it should not be significantly metabolized under our experimental conditions (Rosenberg et al. 2013). We found that Lu AE00527 inhibits both the basal and the exchange-mediated [^{3}H]glycine release (Fig. 3E). However, endogenous glycine release from slices induced by D-Ile was undetectable above the basal glycine concentration in the slice perfusate (0.48 ± 0.03 μM, SEM, $n = 6$). Therefore, if release of endogenous glycine occurs via Asc-1, it should be locally restricted or not high enough to appear in the slice perfusate under our experimental conditions.

Previous studies have demonstrated that D-serine is also released from slices by neuronal depolarization (Rosenberg et al. 2010). We found that Lu AE00527 does not affect D-serine release by the Na^+-channel activator veratridine (Fig. 3F). The data are compatible with the conclusion that Asc-1 does not mediate a depolarization-dependent increase in D-serine release.

D-Serine Release From Asc-1-KO Mice and the Selectivity of Lu AE00527

Experiments using Asc-1-KO mice as controls provide further evidence that Asc-1 mediates tonic D-serine release. We found that slices from Asc-1-KO mice display lower D-serine release when compared with WT littermates, and this is more evident in the basal D-serine release rate prior to stimulation by neutral amino acids (Fig. 4A). Under our experimental conditions, the total amount of D-[^{3}H]serine loaded was not different from slices

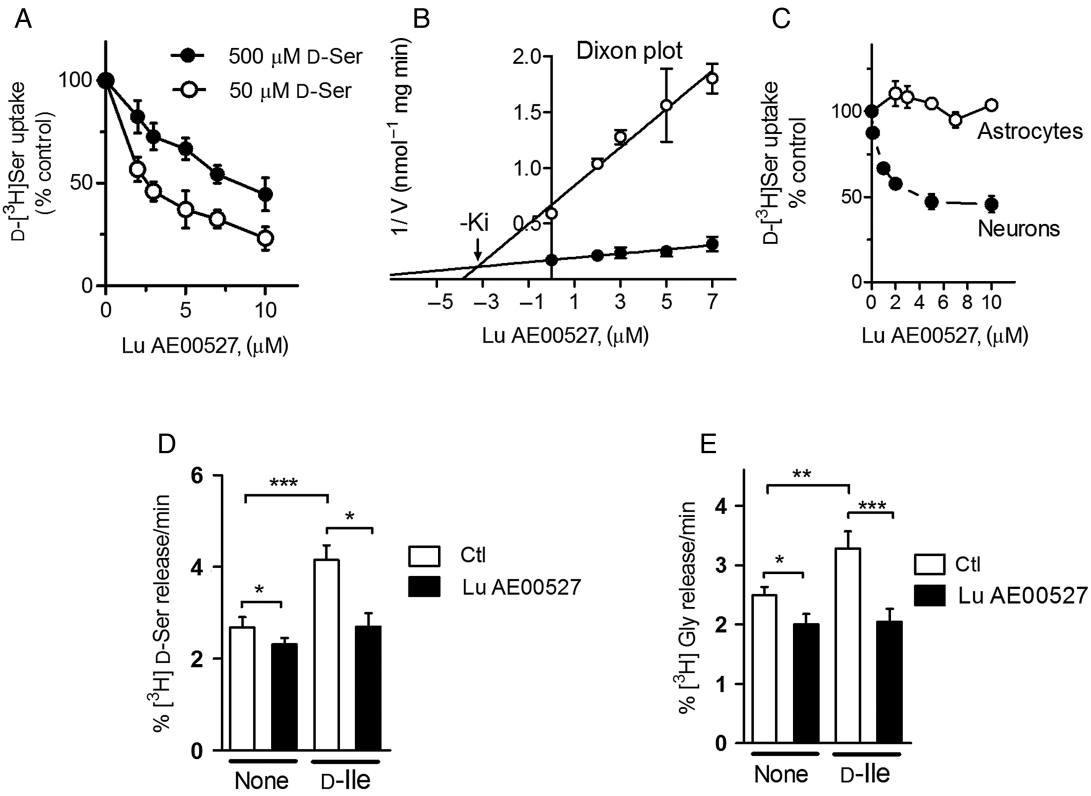

Figure 2. Inhibition kinetics of Lu AE00527 and its effects on Asc-1-mediated amino acid release. (*A*) Inhibition of D-serine uptake by Lu AE00527 in the presence of either 50 (open circles) or 500 μM (closed circles) D-[^{3}H]serine. D-Serine uptake assayed in HEK293 cells transfected with rat Asc-1/4F2hc and increasing concentrations of Lu AE00527 in media lacking Na^+. (*B*) Dixon plot of the data from (*B*). (*C*) Lu AE00527 inhibits the uptake of D-serine by primary neuronal cultures but not astrocytes. Primary cortical neuronal cultures (closed circles) were assayed in a medium lacking Na^+ and supplemented with 100 nM D-[^{3}H]serine, whereas primary astrocytes (open circles) were incubated with a Na^+-containing medium. (*D*) Lu AE00527 (10 μM) inhibits the release of D-serine from neurons preloaded with D-[^{3}H]serine, in the presence or in the absence of D-Ile. (*E*) Lu AE00527 (10 μM) inhibits the release of glycine from neurons preloaded with [^{3}H]glycine. *, **, and *** are different from the control at $P < 0.05$, 0.01, and 0.001, respectively (repeated-measures ANOVA with Bonferroni's post hoc test).

of WT and Asc-1-KO mice (Fig. 4*B*). In contrast with the significant lower basal D-serine release in the Asc-1-KO mice slices, the ability of neutral amino acids to stimulate D-serine release via exchange was little affected (Fig. 4A). This result might reflect compensatory increases in d-serine/amino acid heteroexchange by unrelated neutral amino acid transporters in Asc-1-KO mice, such as ASCT-like transporters that are obligatory amino acid exchangers (Rutter et al. 2007).

To evaluate further the selectivity of Lu AE00527, we monitored its effects in slices from Asc-1-KO mice. We found that incubation with Lu AE00527 does not affect D-serine release from slices obtained from Asc-1-KO mice (Fig. 4C), indicating that the drug is indeed selective for Asc-1. Furthermore, Lu AE00527 did not interact with the NMDAR co-agonist site under our experimental conditions. The incubation of rat brain membranes with Lu AE00527 (up to 10 μM) has no effect on the binding of [^{3}H]MDL 105 519, a selective ligand of the co-agonist site (Fig. 4*D*). In contrast, sub-micromolar D-serine concentrations strongly displace MDL 105 519 binding (Fig. 4*D*).

We additionally found that Asc-1 does not alter glutamate dynamics. The basal release of glutamate from rat neocortical slices is not affected by either Lu AE00527 or D-Ile (Fig. 4E). Furthermore, Lu AE00527 has no effect on veratridine-induced glutamate release from slices (Fig. 4F).

Asc-1-KO mice display a chronic global reduction in brain glycine (Safory et al. 2015). In contrast with Asc-1-KO mice, we found that acute treatment of slices with Lu AE00527 does not change the tissue levels of glycine and other amino acids (Table 1), making this drug suitable for electrophysiological experiments by avoiding confounding changes in tissue amino acid levels.

Effect of Lu AE00527 on NMDAR Synaptic Activity

We next investigated whether the tonic release of co-agonists via the Asc-1 transporter is involved in NMDAR-dependent synaptic plasticity. In the hippocampal CA1, we found that preincubation with Lu AE00527 (10 μM) significantly decreases the magnitude of 1 × 100 Hz-induced LTP ($F_{1,32} = 4.7$, $P < 0.05$; Fig. 5A,B). The addition of saturating concentrations of D-serine prevents the reduction by Lu AE00527 (Fig. 5A; $F_{1,27} = 3{,}76$, $P < 0.05$), indicating that LTP alteration may be related to a decrease in co-agonist release. The decrease in LTP promoted by Lu AE00527 is more prominent when a stronger LTP protocol (2 × 100 Hz) is employed ($F_{1,18} = 5.8$, $P < 0.03$; Fig. 5C,D). Furthermore, supplementation of the aCSF with physiological concentrations of the neutral amino acids (A,S,C) increases the magnitude of the 1 × 100 Hz-induced LTP when compared with control aCSF ($F_{1,25} = 4.5$, $P < 0.05$; Fig. 5E,F), presumably by increasing co-agonist availability through activation of the exchange transport mode. Lu AE00527 prevents the LTP enhancement promoted by typical Asc-1 substrates ($F_{1,23} = 29.9$, $P < 0.0001$; Fig. 5E,F), indicating that Asc-1 contributes to the enhancement of synaptic plasticity mediated by neutral amino acids. However, blocking Asc-1 with Lu AE00527 (10 μM) did not affect the magnitude of NMDAR-dependent LTD expression

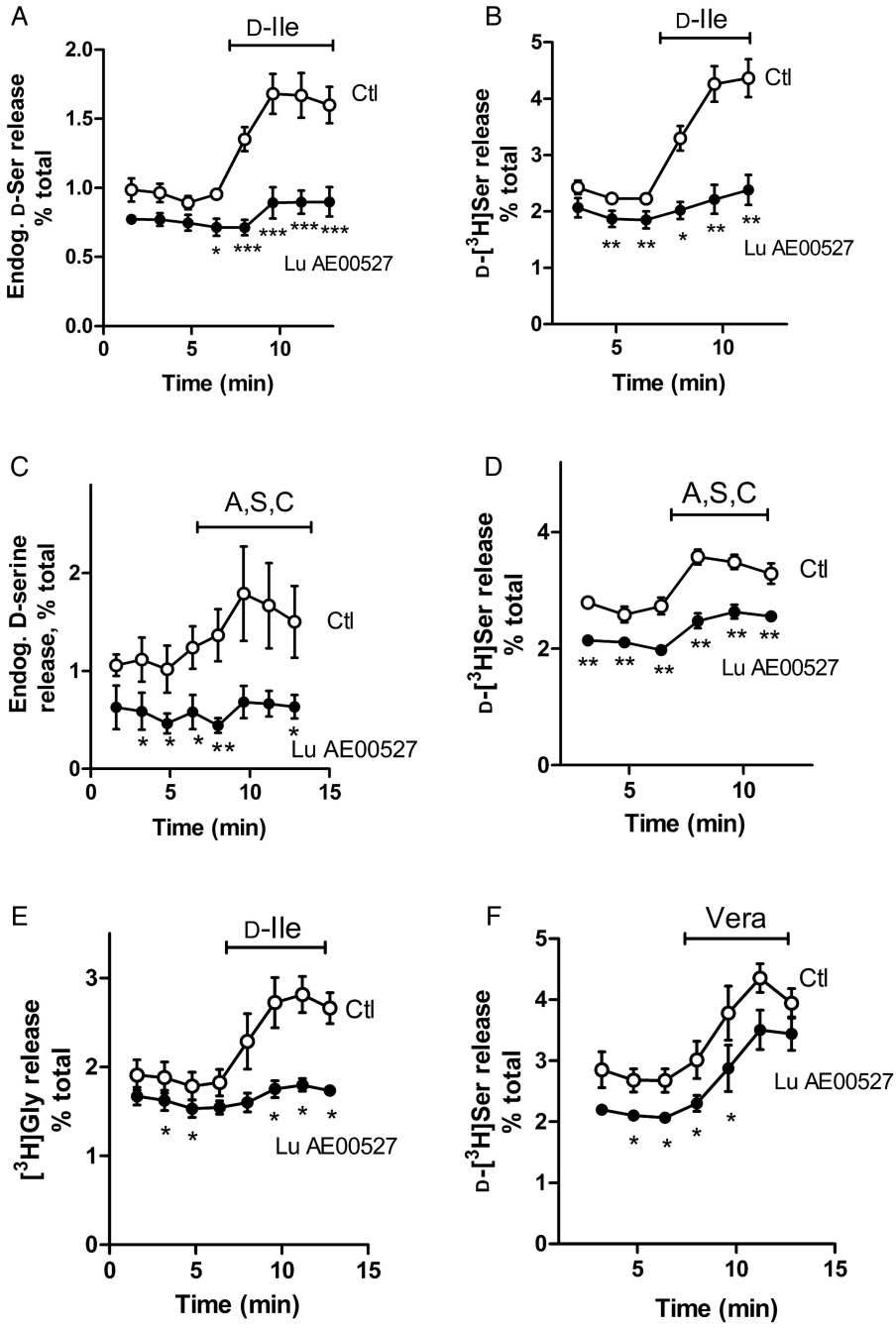

Figure 3. The blockade of Asc-1 decreases the release of co-agonists in acute neocortical slices. (*A*) Lu AE00527 blocks the release of endogenous D-serine. The perfusion of adult rat neocortical slices with oxygenated HEPES-modified Krebs buffer supplemented with 1 mM D-Ile at the indicated times (horizontal bar) induces the release of endogenous D-serine (open circles). The addition of 10 μM Lu AE00527 blocks both the basal and D-Ile-induced D-serine release (closed circles). (*B*) Lu AE00527 blocks the release of preloaded D-[^{3}H]serine (closed circles) when compared with untreated slices (open circles). (*C*) Endogenous D-serine release induced by perfusion with 14 μM L-alanine, 36 μM L-serine, and 2 μM L-cysteine (A,S,C) (open circles) is abolished by 10 μM Lu AE00527 (closed circles). (*D*) D-[^{3}H]Serine release induced by perfusion with A,S,C (open circles) is decreased by 10 μM Lu AE00527 (closed circles). (*E*) The release of preloaded [^{3}H]glycine (open circles) is decreased by perfusion with a medium containing 10 μM Lu AE00527 (closed circles). (*F*) The effect of Lu AE00527 on D-[^{3}H]serine release induced by perfusion with 50 μM veratridine. The values are the mean ± SEM of 4–6 independent experiments with different slice preparations. *, **, and *** are different from the control values at $P < 0.05$, 0.010, and 0.001, respectively (paired Student's *t*-test).

(Fig. 6A,B), while d-APV prevented LTD (Fig. 6B; $F_{1,17} = 10{,}9$, $P < 0.05$). At the concentrations used in the LTP and LTD experiments, Lu AE00527 does not inhibit the isolated AMPAR potentials, suggesting that it does not alter basal synaptic transmission (Fig. 6C). Furthermore, Lu AE00527 does not affect the PPF, suggesting a lack of effect on presynaptic glutamate release mechanisms (Fig. 6D).

We also investigated if Asc-1 directly affects NMDAR activation at low levels of synaptic activity by analyzing isolated NMDAR-mediated fEPSPs. Activation of the Asc-1 exchange mode by D-Ile increases these responses by 20% [Fig. 7A; see also Rosenberg et al. (2013)]. Likewise, a similar 25–30% increase in NMDAR fEPSPs is observed by supplementing the aCSF with

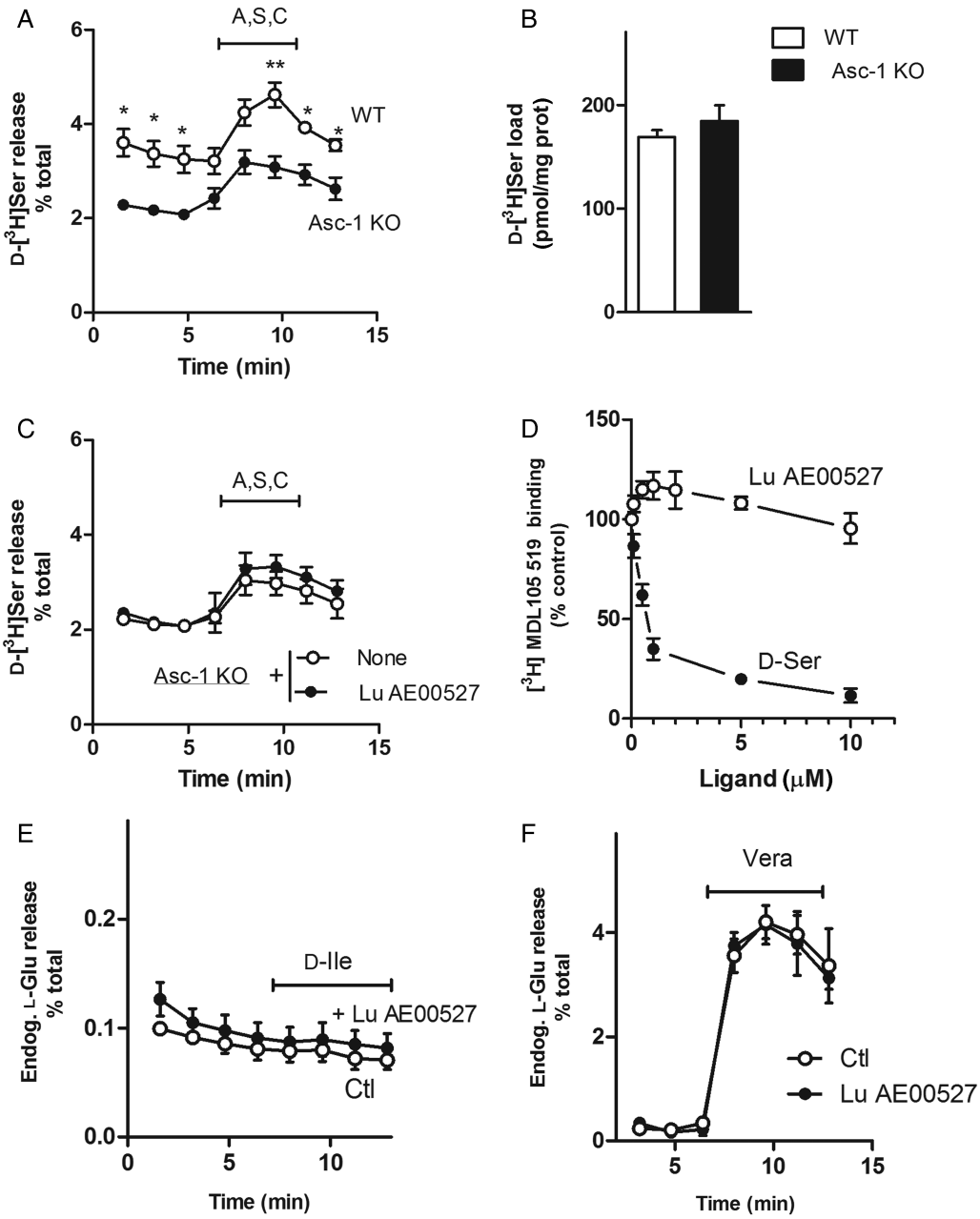

Figure 4. Kinetics of D-serine release in Asc-1 KO mice and the selectivity of Lu AE00527. (*A*) Neocortical slices from Asc-1 KO mice (closed circles) display a reduced D-[^{3}H] serine release when compared with WT littermates (open circles) before and after stimulation with 14 μM L-alanine, 36 μM L-serine, and 2 μM L-cysteine (A,S,C). (*B*) Total levels of preloaded D-[^{3}H]serine were unchanged in slices from Asc-1-KO mice. (*C*) Lu AE00527 does not affect D-[^{3}H]serine release from slices of Asc-1 KO mice stimulated with A,S,C. (*D*) Lu AE00527 does not affect [^{3}H]MDL 105 519 binding to total brain membranes, while the addition of D-serine prevented specific [^{3}H]MDL 105 519 binding. (*E*) Lack of effect of D-Ile or Lu AE00527 on endogenous glutamate release from rat neocortical slices. (*F*) Lu AE00527 does not affect depolarization-mediated endogenous glutamate release induced by perfusion with 50 μM veratridine. The values are the mean ± SEM of 3 (*A*, *C*, and *F*), 4 (*D* and *E*), and 5 (*B*) independent experiments using different slice preparations. *, ** are different from the Asc-1-KO values at $P < 0.05$ and 0.010, respectively (Student's *t*-test).

physiological levels of neutral amino acids, suggesting that they are required for optimal NMDAR activation (Fig. 7*B*). Preincubation with Lu AE00527 prevents the effects of both D-Ile and neutral amino acids (Fig. 7*C*,*D*), confirming the role of Asc-1 in mediating co-agonist release promoted by typical Asc-1 substrates via the exchange mode. In the absence of extracellular ligands, however, Asc-1 has no effect or only promotes a small (10%) increase in NMDAR potentials depending on the stimulus strength (Fig. 7*E*). Altogether, the data indicate that the release of NMDAR co-agonists via Asc-1 is an important pathway regulating synaptic NMDAR activation, and this is mediated mainly by typical Asc-1 substrates present in the extracellular milieu.

Blood–Brain Barrier Transport Characteristics of Lu AE00527

In vitro bi-directional transport data across MDCK cells transfected with P-gp showed that Lu AE00527 exhibited a low apparent permeability in the apical-to-basolateral direction ($0.7 \pm 0.03 \times 10^{-6}$ cm/s) when compared with the reverse direction

Table 1 Effect of Lu AE00527 on the total levels of endogenous amino acids in slices

Amino acid	Control	Lu AE00527	*t*-test
Glycine	40.05 ± 4.19	41.65 ± 3.41	n.s.
d-Serine	5.57 ± 0.39	5.93 ± 0.39	n.s.
l-Serine	48.22 ± 3.82	55.30 ± 2.68	n.s.
l-Glutamate	187 ± 31	192 ± 330	n.s.

Note: Cortical slices were perfused for 30 min with and without 10 μM Lu AE00527 and the tissue amino acids were analyzed by HPLC. The data are expressed as nmol of amino acid/mg slice protein and represent the average ± SEM of 6 independently perfused slices obtained from 2 mice.
n.s., not a significant difference.

($14 \pm 0.7 \times 10^{-6}$ cm/s). The resulting efflux ratio of 19 ± 0.6 suggests that Lu AE00527 may be a strong substrate for P-gp, which is present at the blood–brain barrier and extrudes several organic molecules from the brain (Koepsell 1998). To evaluate this possibility in vivo, we employed P-gp-deficient mice, which show a 17-fold higher brain concentration of Lu AE00527 when compared with their WT counterparts, but without any significant changes in the systemic plasma concentrations between the genotypes (see Supplementary Fig. 1). In WT mice, the brain to plasma exposure ratio was 0.05 ± 0.01, which indicates extrusion from the CNS in P-gp-competent animals.

In P-gp-deficient mice, we found that administration of 30 mg/kg s.c of Lu AE00527 gives a total brain concentration of 8 μM when measured 4 h after the injection. Therefore, we tested if in vivo administration of Lu AE00527 recapitulates the hyperekplexia-like phenotype observed in Asc-1-KO mice that is caused by impairment of glycinergic inhibitory transmission at the brainstem and spinal cord (Safory et al. 2015). For this purpose, we monitored spontaneous tremor episodes and acoustic startle response 4 h after Lu AE00527 injection. We found a significant increase in tremors ($t_{(28)} = 3.3$, $P < 0.003$) and acoustic startle response ($t_{(28)} = 4.47$, $P < 0.0001$) in Lu AE00527-injected P-gp-deficient mice (see Supplementary Fig. 1). Although less dramatic than the alterations seen with homozygous Asc-1-KO mice (Safory et al. 2015), these observations suggest that Lu AE00527 mimics phenotypic abnormalities that are typical of glycinergic dysfunction at the brainstem and spinal cord, such those exhibited by Asc-1-KO mice.

Discussion

In the present study, we identified a selective Asc-1 inhibitor, Lu AE00527, and demonstrated that Asc-1 is involved in the tonic release of NMDAR co-agonists contributing to NMDAR-mediated LTP in the hippocampal CA1. Controls with Asc-1-KO mice confirmed the role of this transporter in mediating constitutive D-serine release. We showed that Lu AE00527 is selective for Asc-1, since it did not inhibit other putative D-serine or glycine transporters and did not bind to the co-agonist site of NMDARs. Furthermore, Lu AE00527 did not alter glutamate release or affect AMPAR at the concentrations used in this study. Most importantly, Lu AE00527 did not affect D-serine release in Asc-1-KO mice, indicating that this inhibitor is useful for determining the role of Asc-1 in D-serine dynamics. In contrast to other Asc-1 modulators that artificially activate the exchange mode (Rosenberg et al. 2010, 2013) or may be unsuitable for use in physiological preparations (Brown et al. 2014), Lu AE00527 is a selective and nonsubstrate inhibitor that is useful for defining the directionality of the Asc-1 transporter in cell cultures and brain slices.

Our results demonstrate that the decrease in LTP expression by Lu AE00527 is preventable by exogenous D-serine, suggesting a deficient NMDAR co-agonist release through Asc-1. The inhibitory effect of Lu AE00527 appeared to be more pronounced when a stronger LTP protocol is used or when typical Asc-1 substrates are added to the aCSF, indicating that Asc-1 might be more active under these conditions. However, we found that LTD was not affected by Lu AE00527. This may be due to the lower activation of NMDARs required for inducing LTD, as opposed to the stronger activation of NMDAR-dependent Ca^{2+} influx, which controls the LTP magnitude (Malenka 1994). Furthermore, glycine appears to be more important than D-serine for LTD (Papouin et al. 2012). Malinow and coworkers also proposed that LTD may not depend on co-agonist binding [Nabavi et al. 2013; but see Volianskis et al. (2015)]. In either case, our data indicate that LTD apparently does not rely on Asc-1-derived D-serine or glycine.

Our biochemical experiments demonstrate that Lu AE00527 inhibits the basal release of both endogenous and radiolabeled D-serine, as well as the release induced by the exchange mode promoted by D-Ile or neutral amino acids in the aCSF. We thus propose that physiological Asc-1-mediated amino acid exchange is possible by the presence of substantial levels of typical Asc-1 substrates in the extracellular milieu in vivo (Lindroth et al. 1985). These amino acid substrates are generally thought to be inert and are systematically omitted from aCSFs used in electrophysiology. Our results demonstrate that the inclusion of physiological levels of neutral amino acids significantly increased LTP magnitude and enhanced isolated NMDAR fEPSPs, and this effect is prevented by the Asc-1 inhibitor. Thus, the Asc-1 exchange mode appears to contribute to NMDAR activation. The results also indicate that routine slice recording conditions lacking neutral amino acids do not recapitulate all the pathways involved in co-agonist release, and therefore preclude optimal NMDAR activation.

In the absence of neutral amino acid substrates in the aCSF, we found that Lu AE00527 decreases the expression of NMDAR-dependent LTP at hippocampal CA1—an effect that is preventable by adding exogenous D-serine. It is possible that the strong stimulation for inducing LTP augments the local concentration of extracellular neutral amino acids to levels that activates Asc-1, since depolarization also releases moderate levels of neutral amino acids, like L-serine (Rosenberg et al. 2010). However, the drug has little or no effect on the isolated NMDAR fEPSPs, unless typical Asc-1 substrates are present. The data therefore suggest that in the absence of extracellular substrates, Asc-1 activity is limited by the local availability of neutral amino acids within the slices and this varies depending on the experimental conditions. When extracellular Asc-1 substrates are below the threshold for Asc-1, it is also possible that the transporter will work in the direction of D-serine uptake. The later might explain why Asc-1 inhibition in the absence of extracellular substrates promotes a small (10%) increase, rather than a decrease, in the isolated NMDAR fEPSPs measured at strong stimuli intensity.

Other amino acid transporters, such as ASCT1 or ASCT2 (Ribeiro et al. 2002; Rutter et al. 2007), might also promote D-serine release via amino acid heteroexchange. Our data also do not exclude that additional D-serine release pathways participate in synaptic plasticity, such as glial vesicular release (Martineau et al. 2013), nonvesicular release stimulated by depolarization (Rosenberg et al. 2010), volume-regulated channels (Rosenberg et al. 2010), or TRP1A-mediated activation (Shigetomi et al. 2013).

Asc-1 is not selective for D-serine and uses glycine and L-serine among other neutral amino acids. This may explain why the phenotype of Asc-1-KO is more severe than that of

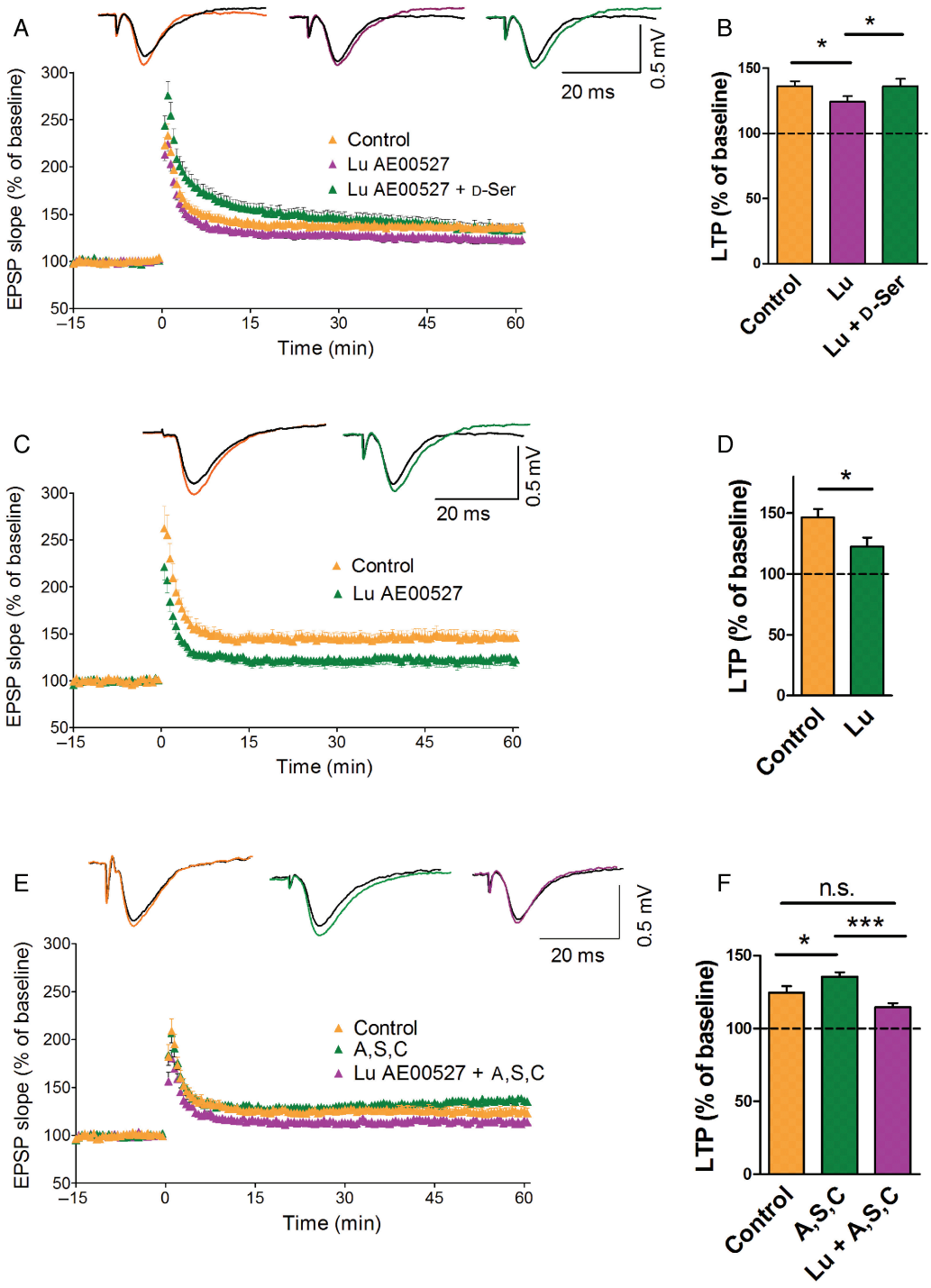

Figure 5. The blockade of Asc-1 decreases LTP expression in hippocampal slices. (*A*) LTP induced by 1 × 100 Hz in the hippocampal CA1 field of young rats is significantly reduced by 10 μM Lu AE00527 when compared with the control slices ($n = 17$ in each group). This decrease in LTP magnitude by Lu AE00527 is prevented when D-serine (100 μM) is present in the aCSF ($n = 12$). The inset depicts representative traces of fEPSPs recorded before (black traces) and 60 min after 1 × 100 Hz in a control slice (orange) and in the presence of Lu AE00527 without (magenta) or with D-serine (green). (*B*) Summary of the LTP experiments shown in *A* (average ± SEM). (*C*) LTP induced by 2 × 100 Hz in the hippocampal CA1 field of young rats is significantly reduced by 10 μM Lu AE00527 ($n = 10$) when compared with the control slices ($n = 9$). Traces show representative fEPSPs recorded before (black traces) and 60 min after 2 × 100 Hz in a control slice (orange) and in the presence of Lu AE00527 (green). (*D*) Summary of the LTP experiments shown in *C* (average ± SEM). (*E*) LTP induced by 1 × 100 Hz in the hippocampal CA1 field of young rats is significantly enhanced by 14 μM L-alanine, 36 μM L-serine, and 2 μM L-cysteine (A,S,C) ($n = 13$) when compared with the control slices ($n = 14$). The addition of 10 μM Lu AE00527 ($n = 12$) abrogates the stimulatory effect of neutral amino acids. Traces show representative fEPSPs recorded before (black traces) and 60 min after 1 × 100 Hz in a control slice (orange) and in the presence of A,S,C (green) or Lu AE00527 in the presence of A,S,C (magenta). (*F*) Summary of the LTP experiments shown in *E* (average ± SEM). *, *** are different from the control values at $P < 0.05$ and 0.001, respectively (unpaired Student's *t*-test).

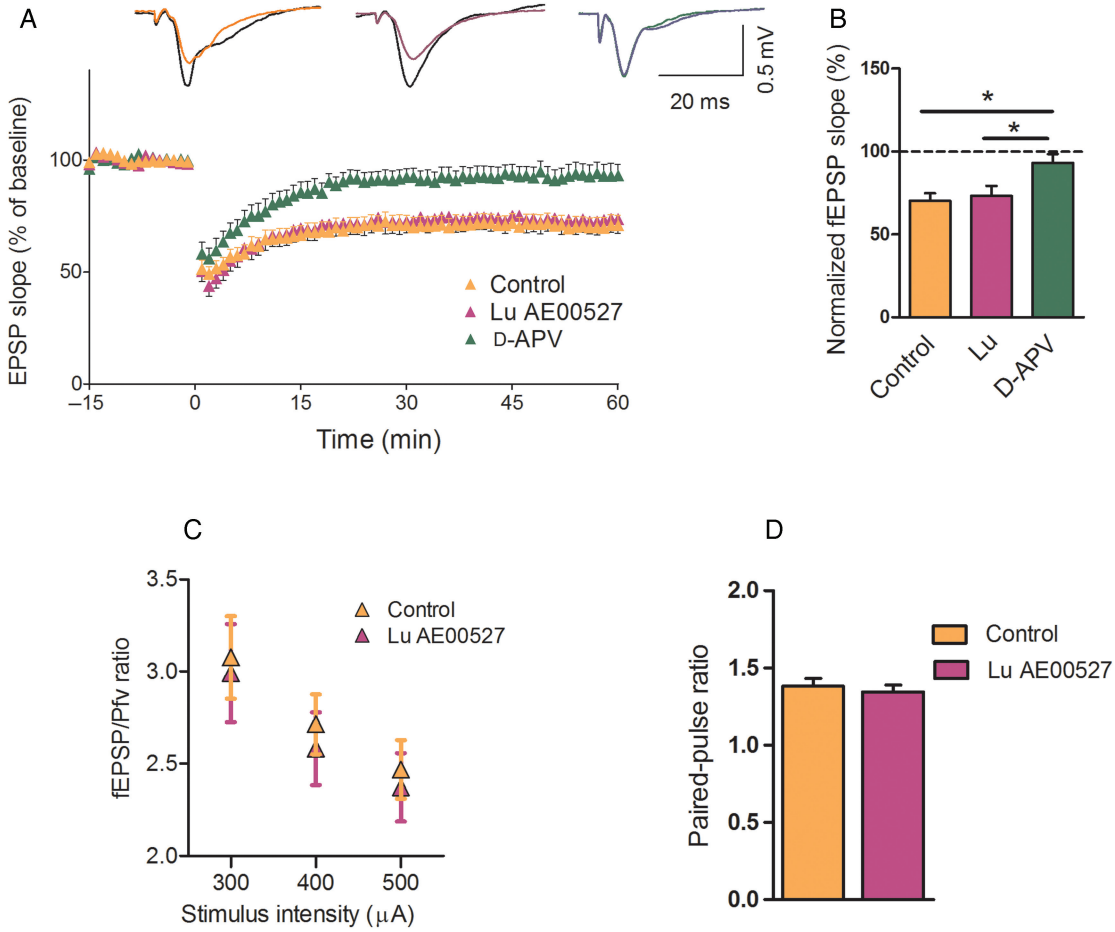

Figure 6. The blockade of Asc-1 does not affect LTD, basal synaptic transmission, or PPF in hippocampal slices. (*A*) LTD induced by low-frequency stimulation is unaffected by 10 μM Lu AE00527 when compared with the control slices ($n = 12$ in each group). The addition of the NMDAR antagonist D-APV (80 μM) blocks LTD ($n = 7$). Traces show representative fEPSPs recorded before (black traces) and after 2 Hz for 10 min in a control slice (orange), in the presence of Lu AE00527 (magenta), or in the presence of both Lu AE00527 and D-APV (green). (*B*) Summary of the LTD experiments shown in *A* (average ± SEM). (*C*) The basal synaptic transmission is not affected by Lu AE00527. The AMPA fEPSP/PFV ratio was calculated at increasing stimuli intensity before and 15 min after the application of 10 μM Lu AE00527. The values are mean ± SEM of recordings from 14 slices. (*D*) Lu AE00527 does not affect the PPF determined at 30 ms stimulation interval in slices from adult rats. The values are the mean ± SEM of recordings from 14 slices. *, different from the control values at $P < 0.05$ (Student's *t*-test).

SR-KO mice. Asc-1-KO mice display a fatal hyperekplexia-like phenotype and die around the third postnatal week because of impaired inhibitory glycinergic transmission (Safory et al. 2015). This is due to a chronic decrease in the brain levels of glycine, apparently because of a deficient conversion of L-serine into glycine in vivo (Safory et al. 2015). Our observation that injection of Lu AE00527 causes spontaneous tremors and enhanced acoustic startle responses in P-gp-deficient mice indicates that the drug recapitulates a hyperekplexia-like phenotype due to impairment of the inhibitory glycinergic transmission, though less prominent than previously observed in Asc-1-KO mice (Safory et al. 2015). This makes the inhibitor of little value to study learning and memory in vivo. Even partial inhibition of glycine receptors, such as that induced by subconvulsive doses of strychnine, is known to enhance the performance of mice in numerous hippocampal-dependent learning tasks, an effect that may be attributed to the general stimulatory effects of glycinergic blockade (McGaugh and Roozendaal 2009).

Because glycine is also required for NMDARs, Asc-1 is likely to affect NMDAR activity, both during the synthesis and release of co-agonists. The use of Lu AE00527 allowed us to examine the role of Asc-1 specifically plays in co-agonist release, since the drug does not affect the levels of glycine in the slices during the incubation times employed in this study. However, although exchange-mediated [^{3}H]glycine release via Asc-1 was detectable, the endogenous glycine release via the exchange mode of Asc-1 was not above the basal glycine concentration in the perfusate (0.48 μM), and was below the level for the complete saturation of GluN2A-containing NMDARs (Matsui et al. 1995). These observations do not allow conclusions to be drawn on the role of Asc-1 in releasing glycine. The action of the powerful glycine transporter GlyT1 may prevent glycine buildup in the extracellular medium (Berger et al. 1998).

Our results with Lu AE00527 also ascertain the directionality of the Asc-1 transporter. In contrast to other Asc-1 modulators (Rosenberg et al. 2013), Lu AE00527 does not activate the exchange mode, and its effects are attributable to a complete blockade of the transporter. Our results show that the acute inhibition of Asc-1 causes a decrease in D-serine release that alters the NMDAR activation of forebrain neurons, suggesting that Asc-1 inhibitors may be useful to prevent overactivation of NMDARs in neurodegenerative conditions.

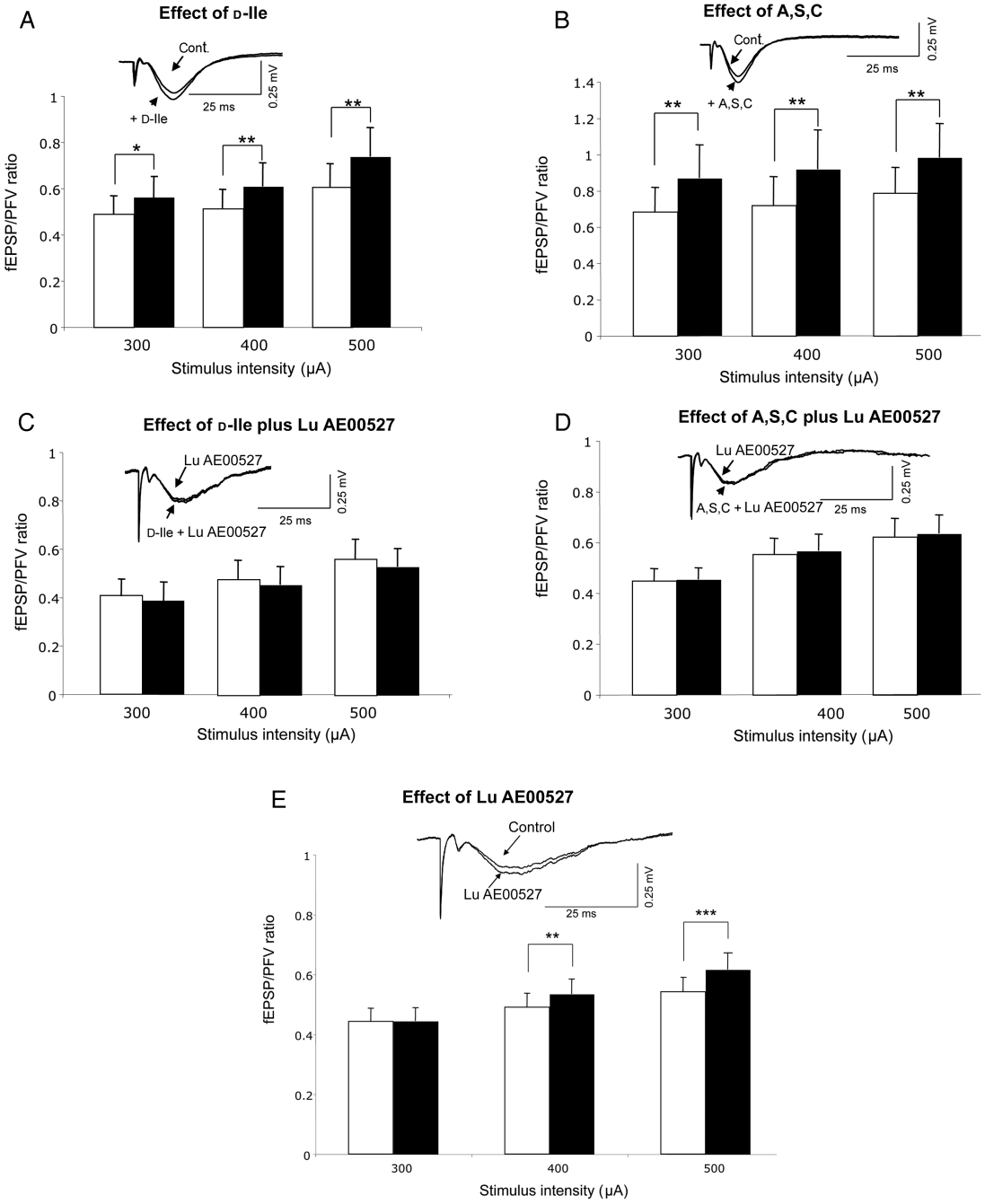

Figure 7. Lu AE00527 prevents the increase in NMDAR synaptic potentials in hippocampal slices induced by D-Ile or by typical Asc-1 substrates. (*A*) D-Ile increases NMDAR potentials isolated in a low-magnesium medium supplemented with NBQX. The fEPSP/PFV ratios were monitored at increasing stimuli intensity before (open bars) and 15 min (filled bars, $n = 12$) after the addition of 1 mM D-Ile. The inset depicts representative traces of NMDAR fEPSP recorded before (cont.) and after the addition of D-Ile (D-Ile). (*B*) The typical Asc-1 substrates L-Ala, L-Ser, and L-Cys (A,S,C) at 14, 36, and 2 μM, respectively, increase isolated NMDAR potentials. The fEPSP/PFV ratios were monitored at increasing stimuli intensity before (open bars) and 15 min (filled bars, $n = 12$) after the addition of A,S,C. The inset depicts representative traces of NMDAR fEPSP recorded before (cont.) and after the addition of A,S,C. (*C*) Lu AE00527 prevents the increase in NMDAR potentials by D-Ile. The fEPSP/PFV ratios were monitored at increasing stimuli intensity in the presence of Lu AE00527 before (open bars) and 15 min (filled bars, $n = 15$) after the addition of 1 mM D-Ile. The inset depicts representative traces of NMDAR fEPSPs recorded before (Lu AE00527) and after the addition of D-Ile (D-Ile + Lu AE00527). (*D*) Lu AE00527 prevents the increase in NMDAR potentials by A,S,C. The fEPSP/PFV ratios were monitored at increasing stimuli intensity in the presence of Lu AE00527 before (open bars) and 15 min (filled bars, $n = 15$) after the addition of A,S,C. The inset depicts representative traces of NMDAR fEPSPs recorded before (Lu AE00527) and after the addition of A,S,C (Lu AE00527 + A,S,C). (*E*) Lu AE00527 has only a minor effect on NMDAR potentials in the absence of extracellular Asc-1 substrates. The fEPSP/PFV ratios were monitored at increasing stimuli intensity in the absence (open bars) or in the presence of 10 μM Lu AE00527 (filled bars, $n = 31$). The inset depicts representative traces of NMDAR fEPSP recorded before (cont.) and after the addition of Lu AE00527. The results are the average ± SEM of the indicated number of slices. *, **, and *** are different from the control values at $P < 0.05$, 0.01, and 0.001, respectively (paired Student's *t*-test).

Supplementary Material

Supplementary material can be found at http://www.cercor.oxfordjournals.org/.

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Notes

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