Cellular/Molecular

Neuronal D-Serine and Glycine Release Via the Asc-1 Transporter Regulates NMDA Receptor-Dependent Synaptic Activity

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D-Serine and glycine are coagonists of NMDA receptors (NMDARs), but their relative contributions for several NMDAR-dependent processes are unclear. We now report that the alanine–serine–cysteine transporter-1 (Asc-1) mediates release of both D-serine and glycine from neurons, and, in turn, this modulates NMDAR synaptic activity. Asc-1 antiporter activity is enhanced by D-isoleucine (D-Ile), which releases D-serine and glycine from Asc-1-transfected cells, primary neuronal cultures, and hippocampal slices. D-Ile has no effect on astrocytes, which do not express Asc-1. We show that D-Ile enhances the long-term potentiation (LTP) in rat and mouse hippocampal CA1 by stimulating Asc-1-mediated endogenous D-serine release. D-Ile effects on synaptic plasticity are abolished by enzymatically depleting D-serine or by using serine racemase knock-out (SR-KO) mice, confirming its specificity and supporting the notion that LTP depends mostly on D-serine release. Conversely, our data also disclose a role of glycine in activating synaptic NMDARs. Although acute enzymatic depletion of D-serine also drastically decreases the isolated NMDAR synaptic potentials, these responses are still enhanced by D-Ile. Furthermore, NMDAR synaptic potentials are preserved in SR-KO mice and are also enhanced by D-Ile, indicating that glycine overlaps with D-serine binding at synaptic NMDARs. Altogether, our results disclose a novel role of Asc-1 in regulating NMDAR-dependent synaptic activity by mediating concurrent non-vesicular release of D-serine and glycine. Our data also highlight an important role of neuron-derived D-serine and glycine, indicating that astrocytic D-serine is not solely responsible for activating synaptic NMDARs.

Introduction

NMDA receptors (NMDARs) are key to several physiological and pathological processes, including learning and memory and neurotoxicity (Traynelis et al., 2010). In addition to glutamate, NMDARs require the binding of a coagonist (glycine or D-serine) for channel opening (Johnson and Ascher, 1987; McBain et al., 1989). Several studies indicate that endogenous D-serine is the main coagonist for NMDARs (Mothet et al., 2000; Shleper et al., 2005; Junjaud et al., 2006; Inoue et al., 2008; Basu et al., 2009; Henneberger et al., 2010). D-Serine is present in astrocytes (Schell et al., 1995) and is synthesized by the enzyme serine racemase (SR) (Wolosker et al., 1999).

The roles of glia and neurons in D-serine dynamics are yet to be determined. Several reports suggest that D-serine is a gliotransmitter (Mothet et al., 2005; Panatier et al., 2006; Papouin et al., 2012). Conversely, recent data indicate that SR is predominantly expressed in glutamatergic neurons, challenging the notion that D-serine is exclusively released from astrocytes (Kartvelishvily et al., 2006; Miya et al., 2008; Balu and Coyle, 2012; Benneyworth et al., 2012).

D-Serine and glycine appear to target different types of NMDARs (Papouin et al., 2012) and are thought to be regulated by distinct uptake and release pathways. Glycine levels are regulated by glycine transporters GlyT1 and GlyT2 (Tsai et al., 2004). GlyT1 is widely expressed in the forebrain, whereas GlyT2 is more restricted to glycinergic terminals (Betz et al., 2006). D-Serine levels are regulated by two types of antiporters: the Na^+-dependent ASCT1 and ASCT2 (Ribeiro et al., 2002) and the Na^+-independent alanine–serine–cysteine transporter-1 (Asc-1) (Fukasawa et al., 2000). These antiporters catalyze amino acid hetero-exchange, in which the uptake or release of D-serine is coupled to the transport of a neutral amino acid in the opposite direction. ASCT1 and ASCT2 antiporters are widely expressed but exhibit low affinity for D-serine (Ribeiro et al., 2002). Conversely, Asc-1 is restricted to neurons and display high affinity for

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both D-serine and glycine (Fukasawa et al., 2000; Helboe et al., 2003). Data obtained with Asc-1 knock-out (KO) mice indicate that Asc-1 is the main D-serine transporter in the brain (Rutter et al., 2007). These mice exhibit a 70–80% reduction in the synaptosomal uptake of D-serine (Rutter et al., 2007), along with seizures that result in early postnatal death (Xie et al., 2005).

In the present report, we investigated the role of Asc-1 in controlling the levels of D-serine in the context of NMDAR-dependent synaptic activity. We identify D-isoleucine (D-Ile) as a selective activator of Asc-1 antiporter activity, which enhances long-term potentiation (LTP) at the hippocampal CA1–CA3 via release of endogenous D-serine. Moreover, we found that Asc-1 regulates NMDAR potentials, which involves glycine release as well. Our data indicate that D-serine and glycine share a common release pathway via Asc-1 to regulate NMDARs. This pathway highlights the role of neurons in releasing D-serine and glycine and may underlie their partial overlapping roles in modulating NMDARs. In this framework, Asc-1 can be a useful pharmacological target to modulate NMDAR activity and counteract neurotoxicity.

Materials and Methods

Materials. L-Serine and D-Ile were purchased from Bachem. D-[^{3}H]Serine (lots 110818 and 111107) and D-[^{14}C]isoleucine (lot 110628) were obtained from American Radiolabeled Chemicals. [^{3}H]Glycine (lot 3632835), [^{3}H]glutamic acid (lot 674819), [^{3}H]MDL 105,519 [(*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1[3*H*]-indole-2-carboxylic acid] (lot 643251), [^{3}H]dopamine (lot 677942), and [^{3}H]GABA (lot 675800) were purchased from PerkinElmer Life and Analytical Sciences. D-and L-Alanine, catalase, choline chloride, dopamine, ((*R*)-*N*-[3-(4′-fluorophenyl)-3-(4′phenylphenoxy)) sarcosine hydrochloride (NFPS), GABA, glycine, glutamate, 3-methyl-2-oxopentanoic acid (2-keto 3-methylvaleric acid), and pyruvate were obtained from Sigma-Aldrich. D-2-Amino-5-phosphonovalerate (D-APV) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione (NBQX) were purchased from Tocris Bioscience. D-Serine was provided by Bachem or Sigma-Aldrich. All pharmacological agents were bath applied from appropriate stock solutions stored at −20°C.

Primary cultures. Animals were killed by decapitation after isoflurane anesthesia. All animal procedures were in accordance with the Committee for the Supervision of Animal Experiments (Technion–Israel Institute of Technology). Primary neuronal cultures from the hippocampus were prepared from E18 Sprague Dawley rat embryos as described previously (Kartvelishvily et al., 2006). 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 hippocampus of P0–P2 Sprague Dawley rats as described previously (Kartvelishvily et al., 2006). The neuronal and astrocytic cultures were used 14–20 d after plating.

Cell transfection. HEK293 cells were cultured in DMEM supplemented with 10% FBS and antibiotics. For transfection, cells were seeded at 70–90% confluence and then transfected using Lipofectamine 2000 (Invitrogen) with rat Asc-1 (SLC7A10)–pExpress-1 (Open Biosystems), rat 4F2 heavy chain (4F2hc)–pCMV–SPORT6 (Open Biosystems), rat GlyT1b–pExpress-1 (Open Biosystems), rat GlyT2–pRc/RSV (provided by Prof. N. Nelson, Tel Aviv University, Tel Aviv, Israel), human ASCT1–pCMV5 (provided by Prof. S. G. Amara, University of Pittsburgh, Pittsburgh, PA), and rat ASCT2–pRK5–KS (provided by Prof. S. Bröer, Australian National University, Canberra, Australian Capital Territory, Australia). Cells were used 48 h after transfection.

Amino acid uptake and release by cell cultures. Primary cultures or transfected HEK293 cells were cultured in 96-well plates and loaded with 100 nM to 5 μM D-[^{3}H]serine in HEPES-buffered saline (HBS) (in mM: 137 NaCl, 5.4 KCl, 0.34 K_2HPO_4, 0.44 KH_2PO_4, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 1.07 $CaCl_2$, 5.6 D-glucose, and 10 HEPES, pH 7.4) at 25°C. D-[^{3}H]Serine uptake was terminated by washing the cells four times with ice-cold HBS. The radioactivity was monitored after lysing the cells with 100 μl of water per well for 10 min. For uptake in the absence of Na^+, we used an Na^+-free HBS medium in which cholineCl was substituted for Na^+, and the pH was adjusted with Tris base. Blanks were performed by incubating the primary or transfected cells with ice-cold uptake medium, and they typically accounted for <10% of the total radioactivity. The values were corrected for protein content in each well. When monitoring D-[^{3}H]serine uptake in HEK293 transfected cells, specific uptake was determined by subtracting the basal D-[^{3}H]serine uptake from cells transfected with a suitable control plasmid (GFP or 4F2hc gene alone). Kinetic constants were calculated by nonlinear regression curve fit using GraphPad Prism 5 program. Competitive inhibition was analyzed using the following equation: $K_{mapp} = K_m \times (1 + [I]/K_i)$, where K_{mapp} is the K_m in the presence of the inhibitor.

For D-[^{3}H]serine release experiments, the cells were preloaded with 5 μM D-[^{3}H]serine for 20–40 min. Subsequently, the wells were washed three times with cold HBS and exposed for 1–5 min to release medium (HBS with or without Na^+) supplemented with different drugs at room temperature. Released D-[^{3}H]serine (D-serine$_{out}$) was monitored in the medium by scintillation counting. Remaining intracellular D-[^{3}H]serine (D-serine$_{in}$) was determined after lysing the cells with 100 μl of water per well. Fractional D-serine release was calculated by using the formula: (D-serine$_{out}$ × 100)/(D-serine$_{out}$ + D-serine$_{in}$). To avoid any D-serine reuptake in some experiments, we included 10 μg/ml recombinant *Escherichia coli* D-serine dehydratase (DsdA) along with 4 mM pyruvate in the release media. This enzyme converts any released D-[^{3}H]serine into [^{3}H]pyruvate that is further diluted by the excess unlabeled pyruvate included in the release media (Rosenberg et al., 2010). Typically, no more than 7–10% D-serine was released during stimulation.

Endogenous amino acid release from slices. Two- to 3-month-old male Sprague Dawley rats were anesthetized with isoflurane and killed by decapitation. Hippocampi were dissected, chopped into strips measuring 400 μm by 400 μm using a McIlwain tissue chopper (Gonzalez-Alvear and Werling, 1994), and washed with oxygenated modified Krebs–HEPES buffer (MKB) (in mM: 127 NaCl, 1.3 NaH_2PO_4, 15 HEPES, 10 glucose, 5 KCl, and 2.5 $CaCl_2$, pH 7.4 adjusted with NaOH). Subsequently, the slices were equilibrated by a 30 min perfusion with oxygenated MKB in 0.3 ml chambers at a flow rate of 0.6 ml/min in a Suprafusion 1000 (SF-6) apparatus (Brandel) at 37°C. After equilibration in perfusion medium, samples were collected at 1.6 min intervals, and the endogenous D-serine peak was monitored by HPLC as described previously (Rosenberg et al., 2010). To estimate the total D-serine content, the slices were incubated for 20 min with 0.2 M HCl to release all intracellular endogenous D-serine. The results were expressed as percentage D-serine release/time unit. Endogenous glutamate release was monitored by HPLC using the same methodology described above for D-serine.

D-[^{3}H]Serine release from slices. Hippocampal slices were loaded with 5 μM D-[^{3}H]serine by 20 min incubation in oxygenated MKB at 37°C. The slices were then washed three times with oxygenated MKB and transferred to the superfusion apparatus. D-[^{3}H]Serine release was monitored by liquid scintillation counting. Total amino acid loading was estimated as described above for endogenous D-serine release. In some experiments, the slices were preloaded with D-[^{3}H]serine using sodium-free MKB (in mM: 127 cholineCl, 1.3 KH_2PO_4, 15 HEPES, 10 glucose, 3.7 KCl, and 2.5 $CaCl_2$, pH 7.4). To monitor subsequent D-[^{3}H]serine release, regular MKB was returned during the equilibration step at the perfusion apparatus. D-[^{3}H]Serine is not significantly metabolized in forebrain slices (Rosenberg et al., 2010).

Glycine release from slices. Hippocampal slices were loaded with 2 μM [^{3}H]glycine essentially as described above for D-serine. To ensure that the released radioactivity correspond to authentic glycine, the amino acids were separated by HPLC (Rosenberg et al., 2010), and the peak corresponding to glycine was manually collected and monitored by scintillation counting.

Ligand binding. [^{3}H]MDL 105,519 binding to isolated rat brain membranes was performed at 4°C in 10 mM Tris-acetate buffer, pH 7.4, and monitored by filtration assay as described previously (Baron et al., 1996).

Ex vivo *electrophysiology.* Experiments were performed in accordance with the European Council Directive (86/809/EEC) regarding the care and use of animals for experimental procedures and approved by the local ethics committee. Transverse hippocampal slices (400 μm) were

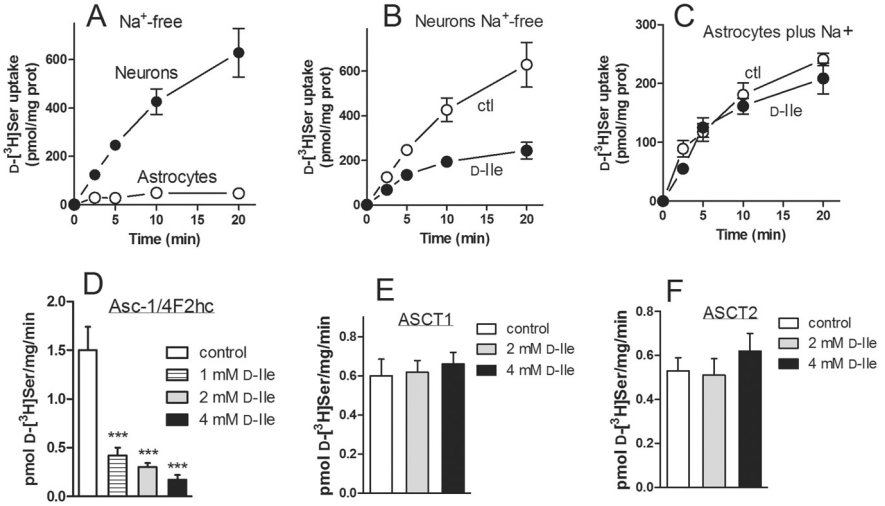

Figure 1. D-Ile inhibits D-serine transport by Asc-1. ***A***, Comparison of D-serine uptake by primary cultures of rat hippocampal neurons (●) and astrocytes (○) in the absence of Na^+. The uptake was performed with 5 μM D-[^{3}H]serine in a Na^+-free medium, in which NaCl was substituted by choline Cl. ***B***, D-Ile inhibits D-serine uptake by neurons. D-[^{3}H]Serine uptake was performed in Na^+-free medium, in either the absence (○) or presence (●) of 1 mM D-Ile. ctl, Control. ***C***, Na^+-dependent D-serine uptake by glia is insensitive to D-Ile. D-[^{3}H]Serine uptake in primary astrocytes was monitored in Na^+-containing medium, in either the absence (○) or presence (●) of 1 mM D-Ile. ***D***, D-Ile inhibits Asc-1 in transfected HEK293 cells. D-Serine uptake in Asc-1 and 4F2hc transfected cells was assayed in medium lacking Na^+ and supplemented with 100 nM D-[^{3}H]serine. The values were calculated by subtracting blanks consisting of cells transfected with 4F2hc only, which accounted for <10% of the total D-[^{3}H]serine accumulation. ***E***, D-Ile does not inhibit ASCT1 in HEK293 transfected cells. Uptake of D-[^{3}H]serine (100 nM) was assayed in Na^+-containing media. The values were calculated by subtracting blanks consisting of cells transfected with GFP, which accounted for ~30% of the total D-[^{3}H]serine accumulation. ***F***, D-Ile does not inhibit ASCT2 in HEK293 transfected cells. Uptake of D-[^{3}H]serine (100 nM) was assayed in Na^+-containing media. The values were calculated by subtracting blanks consisting of cells transfected with GFP, which accounted for ~35% of the total D-[^{3}H]serine accumulation. The values are the mean ± SEM of four to six experiments with different cultures. ***$p < 0.001$, different from control.

obtained from Sprague Dawley rats or C57BL/6 wild-type (WT) and SR-KO mice (3- to 4-month-old animals). The animals were anesthetized with halothane before decapitation. Slices were prepared in ice-cold artificial CSF (aCSF) and placed in a holding chamber for at least 1 h. The composition of aCSF was as follows (in mM): 124 NaCl, 3.5 KCl, 1.5 $MgSO_4$, 2.3 $CaCl_2$, 26.2 $NaHCO_3$, 1.2 NaH_2PO_4, and 11 glucose, pH 7.4. A single slice was transferred to the recording chamber at a time and continuously submerged with aCSF pregassed with 95% O_2/5% CO_2.

Extracellular recordings were obtained at 25–28°C from the apical dendritic layer of the CA1 area using micropipettes filled with 2 M NaCl. Presynaptic fiber volleys (PFVs) and field EPSPs (fEPSPs) were evoked by electrical stimulation of Schaffer collaterals and commissural fibers located in the stratum radiatum. 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 three 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 D-Ile (1 or 2 mM) and D-serine (100 μM) were assessed by determining the fEPSP/PFV ratio 15 min after the addition of the amino acid to the aCSF.

To investigate LTP of synaptic transmission, a test stimulus was applied every 10 s in control medium and adjusted to get an fEPSP with a baseline slope of 0.1 V/s. In experiments performed on rat slices, the averaged slope of three fEPSPs was measured for 15 min before theta-burst stimulation (TBS), consisting of five trains of four 100 Hz pulses each, separated by 200 ms and delivered at the test intensity. This sequence was repeated three times with an interburst interval of 10 s. In experiments done on hippocampal slices from mice, the conditioning stimulation consisted on one high-frequency train at 100 Hz for 1 s. In recordings from both rats and mice, testing with a single pulse was then resumed for 60 min to determine the level of LTP. In pharmacological experiments, D-APV (80 μM), D-Ile (1 mM), or D-serine (100 μM) were added to the aCSF 10 min before the establishment of the baseline and maintained throughout recording.

In some experiments, NMDAR-mediated synaptic potentials as well as LTP were monitored in slices preincubated for at least 90 min with 20 μg/ml purified recombinant DsdA to deplete D-serine contents. The purified recombinant enzyme was prepared as described previously (Shleper et al., 2005).

Statistical analyses. This was performed by repeated-measures ANOVAs, followed by Tukey's *post hoc* test or by paired and unpaired *t* tests.

Results

Asc-1 as a D-serine release pathway from neurons

The plasma membrane Asc-1 (SLC7A10) is exclusively found in neurons *in vivo* and is the main transporter that mediates neuronal D-serine uptake (Fukasawa et al., 2000; Helboe et al., 2003; Matsuo et al., 2004; Rutter et al., 2007; Rosenberg et al., 2010). In contrast to astrocytic D-serine transporters, neuronal Asc-1 displays high affinity for D-serine and does not require Na^+ for activity (Fukasawa et al., 2000; Helboe et al., 2003; Matsuo et al., 2004; Rutter et al., 2007; Rosenberg et al., 2010). Accordingly, we found that our hippocampal neuronal cultures take up D-serine in the absence of Na^+, whereas D-serine transport in primary astrocyte cultures is strictly Na^+ dependent (Fig. 1*A*).

In light of the recent data indicating the existence of a neuronal pool of D-serine (Kartvelishvily et al., 2006; Rosenberg et al., 2010; Benneyworth et al., 2012) and the unique Na^+-independent uptake of D-serine in neurons, we performed a low-throughput screening for compounds that affect D-serine transport in neurons by Asc-1, focusing on D-amino acids. We found that D-Ile specifically inhibits neuronal D-serine uptake without affecting astrocytic transport (Fig. 1*B*,*C*).

To confirm that D-Ile acts on Asc-1, we first analyzed its specificity in HEK293 cells transfected with different transporters. We found that D-Ile inhibits the uptake of D-serine in cells expressing Asc-1 and its ancillary subunit 4F2hc that is essential for Asc-1 activity (Fig. 1*D*). Conversely, D-Ile had no effect in cultures transfected with the Na^+-dependent transporters ASCT1 (Fig. 1*E*) or ASCT2 (Fig. 1*F*), which are present in glia but also presumably in neurons (Bröer et al., 1999; Weiss et al., 2001; Sakai et al., 2003; Gliddon et al., 2009).

We wondered whether D-Ile is a transportable competitive substrate of Asc-1. We found that the inhibition of the neuronal D-serine transport by D-Ile was more pronounced at lower D-serine concentrations, indicating that D-Ile may compete with D-serine (Fig. 2*D*). Dixon plot analysis suggests a competitive inhibitory pattern (Fig. 2*E*). Further kinetic analysis revealed a K_m of 43 ± 4 μM for D-serine, which is almost the same as that reported previously for Asc-1 (Fukasawa et al., 2000). D-Ile increased the K_m to 110 ± 15 μM without changing the V_{max} and exhibited an apparent K_i of 0.98 ± 0.1 mM (Fig. 2*F*). Double-reciprocal plot analysis confirmed a competitive inhibition (Fig. 2*G*).

As an additional indication that D-Ile is a competitive substrate, we found that Asc-1/4F2hc transfected cells take up

D-[^{14}C]Ile, and this was prevented by adding D-serine (Fig. 2*A*). Preincubation of D-[^{14}C]Ile with D-amino acid oxidase (DAO), an enzyme that converts D-Ile into 3-methyl valeric acid, abolished the specific uptake, indicating no contamination of the D-[^{14}C]Ile by L-isomers (Fig. 2*A*). Primary neuronal cultures also transported D-[^{14}C]Ile (Fig. 2*B*), and this was prevented by D-serine or by excess unlabeled D-Ile. Likewise, D-alanine, a highly selective Asc-1 substrate (Rutter et al., 2007), inhibited neuronal uptake of D-[^{14}C]Ile (Fig. 2*B*), confirming the role of D-Ile as a transportable Asc-1 ligand.

Conceivably, D-Ile uptake is coupled to D-serine release via Asc-1 antiporter activity. Using primary neuronal cultures previously loaded with D-serine, we found that D-Ile induces robust release of D-serine, reflecting a D-Ile/D-serine exchange (Fig. 2*C*). In contrast, D-Ile slightly inhibited D-[^{3}H]serine release from astrocytes (Fig. 2*C*), confirming that these cells do not release D-serine via Asc-1.

D-Ile-induced D-serine release by Asc-1 was confirmed in Asc-1/4F2hc transfected HEK293 cells, whereas cells transfected with the 4F2hc subunit alone were insensitive to D-Ile (Fig. 3*A*,*B*). Furthermore, because Asc-1 mediates L-alanine efflux by facilitated diffusion from *Xenopus* oocytes (Fukasawa et al., 2000), we investigated whether D-serine leaks through this transporter by a similar mechanism. Accordingly, Asc-1/4F2hc transfection doubled the rate of D-serine efflux even in the absence of extracellular Asc-1 substrates, reflecting net release of D-serine uncoupled from the antiporter activity (Fig. 3*A*). In contrast to Asc-1, transfection of the obligatory exchanger ASCT1 did not release any D-serine when compared with GFP in the absence of extracellular amino acid substrates (Fig. 3*C*). ASCT1 was also insensitive to D-Ile, whereas L-alanine promoted significant D-serine release by ASCT1 when compared with GFP, indicating that ASCT1 is functional when expressed in HEK293 cells (Fig. 3*C*,*D*). Thus, D-serine leakage resulting from Asc-1 transfection was probably not attributable to activation of hetero-exchange by extracellular amino acids in the unstirred layer around the cells. The data are consistent with the notion that Asc-1 mediates D-serine release from cells by both antiporter and facilitated diffusion mechanisms.

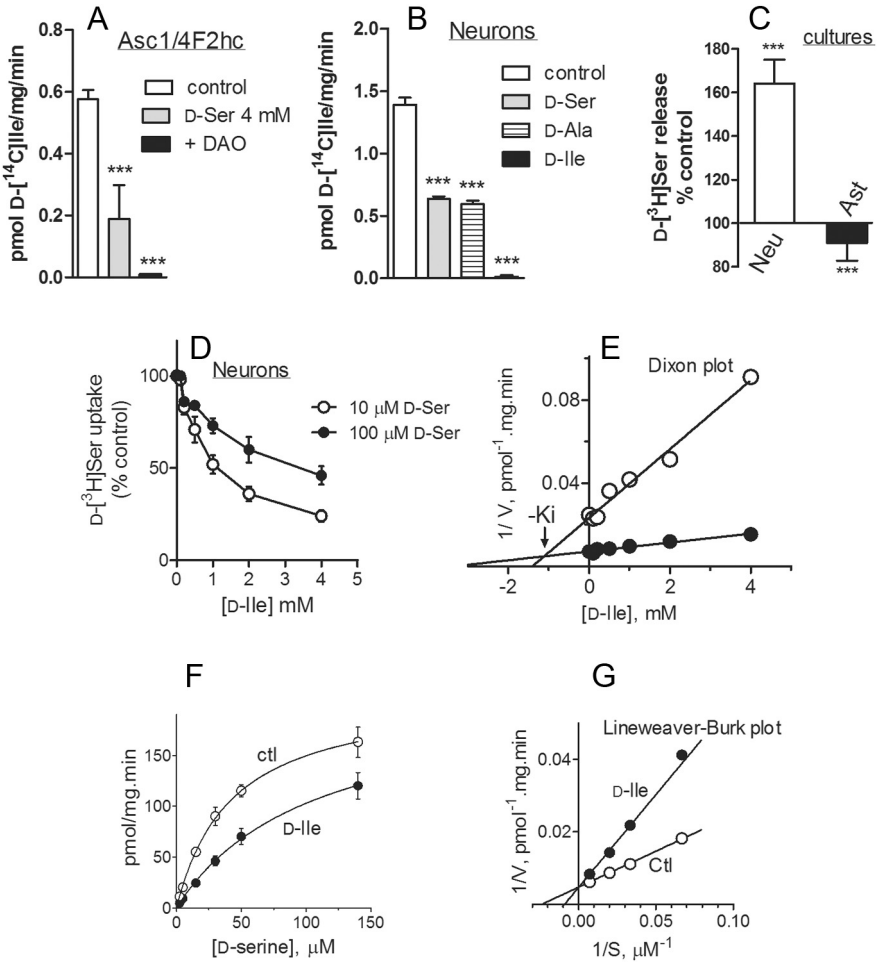

Figure 2. D-Ile is a competitive Asc-1 substrate that promotes D-serine release from neurons. ***A***, D-Ile uptake in HEK293 cells transfected with Asc-1/4F2hc. D-Ile transport was assayed in a Na^+-free medium containing 2 μM D-[^{14}C]Ile and was inhibited by adding 4 mM D-serine (gray bar). Incubation for 30 min with 20 μg/ml DAO and 2 μg/ml catalase completely degraded D-[^{14}C]Ile and abolished its transport (black bar). The medium contained 10 mM unlabeled 3-methyl valeric acid to block the reuptake of the corresponding keto acid generated by DAO activity. ***B***, D-Ile uptake in primary neuronal cultures. D-[^{14}C]Ile uptake in neurons was prevented by 2 mM D-serine, 2 mM D-alanine, or 4 mM D-Ile. ***C***, D-Ile elicits D-serine release from neurons but not astrocytes. Primary cultures were preloaded for 30 min with 5 μM D-[^{3}H]serine and, after washing, further incubated for 2–5 min in the absence or presence of 1 mM D-Ile. The results are expressed as the percentage of fractional D-[^{3}H]serine release observed in the absence of D-Ile. Neu, Primary neuronal culture; Ast, primary astrocytic culture. ***D***, increasing the concentration of D-serine protects from D-Ile inhibition. D-[^{3}H]Serine transport into primary neuronal cultures was performed in the presence of 10 μM (○) or 100 μM (●) D-serine as described in the legend to Figure 1. ***E***, Dixon plot of the data from ***D*** suggests a competitive-type inhibition by D-Ile. ***F***, Initial rate of D-[^{3}H]serine transport as function of D-[^{3}H]serine concentration in the presence (●) and absence (○) of 1.5 mM D-Ile. ctl, Control. ***G***, Lineweaver–Burk plot of the data from ***F*** reveals a competitive inhibition of D-[^{3}H]serine transport by D-Ile. The values are the mean ± SEM of three to six experiments with different culture preparations. ***$p < 0.001$, different from control.

D-Serine and glycine dynamics are linked

To investigate whether D-Ile can be used as a tool to study the role of Asc-1 in more physiological preparations, we monitored the rate of endogenous D-serine release from acute hippocampal slices. Perfusion of slices with D-Ile more than doubled the rate of endogenous D-serine release monitored by HPLC (Fig. 4*A*). Conversely, D-Ile did not affect the rate of endogenous glutamate release, indicating that it does not interfere with glutamate transporters (Fig. 4*B*). These data are consistent with endogenous D-serine release from slices through Asc-1.

Despite its high affinity for glycine (Fukasawa et al., 2000; Helboe et al., 2003), a possible role of Asc-1 in glycine dynamics has not been investigated previously. We also found that D-Ile promotes glycine release from slices through activation of Asc-1 antiporter activity (Fig. 4*C*). To confirm that the radioactivity in the perfusate consisted of authentic [^{3}H]glycine, we validated its identity by HPLC analysis (Fig. 4*D*), ruling out significant metabolism of [^{3}H]glycine throughout the experiment. The data are consistent with the notion that Asc-1 antiporter activity promotes a concurrent release of neuronal D-serine and glycine from acute brain slices.

To confirm that D-Ile directly affects glycine fluxes through Asc-1, we characterized its actions in primary cultures and trans-

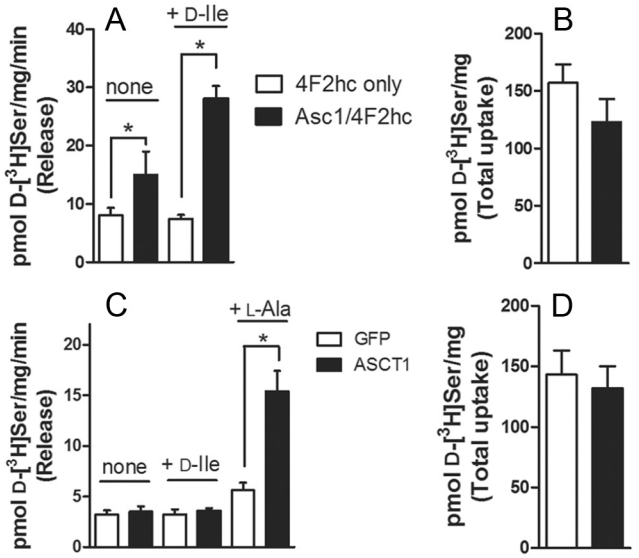

Figure 3. Characteristics of D-serine release from cells expressing Asc-1 and ASCT1 transporters. ***A***, Transfection of HEK293 cells with Asc-1/4F2hc (filled bars) accelerates the rate of D-serine release when compared with 4F2hc alone (open bars). D-Ile induces D-serine release from cells transfected with Asc-1/4F2hc but not from those transfected with 4F2hc alone. Total levels of D-[^{3}H]serine loading are shown in ***B*** and were attained by preloading the cells with 2 μM D-[^{3}H]serine for 30 min (4F2hc only, open bars) or 6 min (Asc-1/4F2hc, filled bars). ***C***, Transfection of HEK293 cells with ASCT1 (filled bars) does not affect basal D-serine release when compared with GFP control (open bars). D-Ile does not stimulate release from ASCT1 or GFP transfected cells, whereas addition of the substrate L-alanine (1 mM) increases the rate of D-[^{3}H]serine release from ASCT1 transfected cells when compared with GFP. Total levels D-[^{3}H]serine loading are shown in ***D*** and were attained by incubating the cells with 2 μM D-[^{3}H]serine for 30 min (GFP) or 10 min (ASCT1). The values are the mean ± SEM of three to six experiments with different culture preparations. *$p < 0.050$, different from control.

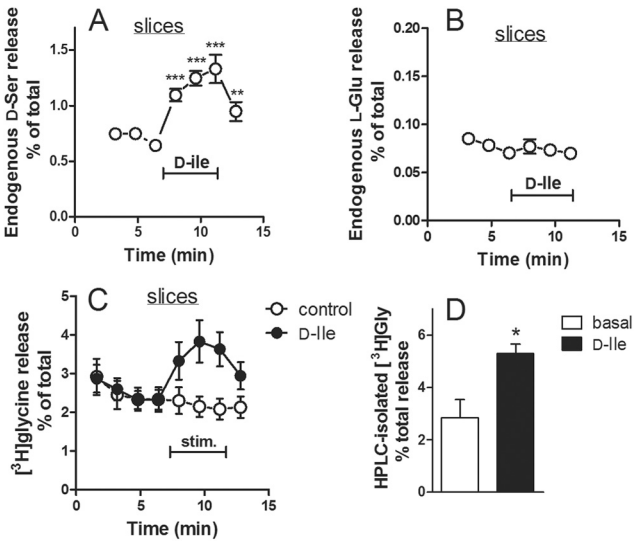

Figure 4. Asc-1-mediates release of D-serine and glycine from hippocampal slices without affecting glutamate dynamics. ***A***, D-Ile induces endogenous D-serine release from slices. Perfusion of acute hippocampal slices with oxygenated MKB supplemented with D-Ile (1 mM) at the indicated times (horizontal bar) induced release of endogenous D-serine. ***B***, D-Ile has no effect on endogenous L-glutamate release. ***C***, D-Ile elicits release of glycine from slices. Hippocampal slices were prelabeled for 20 min with 2 μM [^{3}H]glycine. The slices were then perfused with either none (○) or 1 mM D-Ile (●) at the indicated times. ***D***, Quantification of authentic [^{3}H]glycine release by HPLC analysis from basal (open bar) or D-Ile-stimulated fractions (filled bar). The results are mean ± SEM of 4–10 experiments with different preparations. * $p < 0.05$, ***$p < 0.001$, different from control.

fected cells. We found that D-Ile inhibits glycine uptake by neuronal cultures (Fig. 5*A*) but does not affect glycine transport in astrocytes, which lack Asc-1 activity (Fig. 5*B*). As expected, the uptake of glycine in astrocytes was decreased by the GlyT1 transporter inhibitor NFPS (Fig. 5*B*). The role of Asc-1 in mediating glycine transport was also confirmed in transfected cells. D-Ile inhibited glycine uptake by Asc-1/4F2hc transfected HEK293 cells (Fig. 5*C*) but had no effect in cells transfected with ASCT1 or ASCT2 (Fig. 5*D*,*E*). Furthermore, D-Ile does not affect the glycine transporters GlyT1b and GlyT2 (Fig. 5*F*,*G*).

Selectivity of D-Ile

We further evaluated D-Ile selectivity by investigating its effect on additional neurotransmitter systems. D-Ile does not affect neuronal or astrocytic glutamate uptake (Fig. 6*A*,*B*), nor does it change neuronal GABA or dopamine transport (Fig. 6*C*,*D*). In addition, D-Ile does not displace the binding of the selective NMDAR coagonist site ligand MDL 105,519 to washed brain membranes, indicating that it does not interact with the NMDAR coagonist binding site (Fig. 6*E*). The latter is in agreement with previous data showing that D-Ile does not affect the activity of recombinant NMDARs expressed in *Xenopus* oocytes (McBain et al., 1989).

The Na^+-independent transport of D-serine is mostly abolished in Asc-1 KO mice, indicating that Asc-1 accounts for >90% of the transport of D-serine in the absence of Na^+ (Rutter et al., 2007). To confirm that D-Ile is indeed acting on Asc-1 in slices, we inhibited all other transporters that are Na^+ dependent by monitoring D-serine release in an Na^+-free medium. Although the basal rate of D-serine release increases by omission of Na^+, we found that the effect of D-Ile was not attenuated (Fig. 7*A*). Quantitative analysis of the maximal net D-serine release after subtracting the basal rate shows that the D-Ile-stimulated D-serine release in the absence of Na^+ is at least as high as that observed in normal Na^+ (Fig. 7*B*). D-Alanine transport is also disrupted in Asc-1-KO mice, indicating that it is mediated by Asc-1 (Rutter et al., 2007). Thus, as an additional control, we applied D-alanine during preloading with D[^{3}H]-serine and found that it significantly decreased D-Ile-induced D-serine release (Fig. 7*C*). The data support the notion that Asc-1 is the main target for D-Ile.

D-Ile mimics the activation of Asc-1 by the endogenous substrates

The extracellular concentrations of the main Asc-1 substrates L-alanine, L-serine, and L-cysteine in the brain are 14, 36, and 2 μM, respectively (Lindroth et al., 1985). Altogether, these concentrations are higher than the apparent K_m of Asc-1 for these amino acids (11, 23, and 24 μM for L-alanine, L-serine, and L-cysteine, respectively) (Fukasawa et al., 2000). Thus, it is likely that Asc-1 antiporter activity is operational *in vivo*. However, we found that the concentrations of the main Asc-1 substrates in the hippocampal slice perfusates measured by HPLC were only 0.3 ± 0.05 and 0.5 ± 0.01 μM for L-alanine and L-serine, respectively. These concentrations are ~40-fold lower than their physiologic extracellular values, suggesting that the Asc-1 antiporter activity may be limited by the washout of extracellular substrates in the *ex vivo* perfused slices. In agreement, we found that perfusing the slices with physiological-like extracellular concentrations of L-alanine, L-serine, and L-cysteine enhances the rate of D-serine release to values similar to those obtained with D-Ile, suggesting that they restore the antiporter activity of Asc-1 (Fig. 7*D*). Thus, D-Ile mimics the physiologic activation of Asc-1 by typical extracellular endogenous substrates.

Effects of D-Ile on synaptic plasticity

Does D-serine or glycine release by Asc-1 play a role in synaptic plasticity? To answer this question, we monitored the effects of D-Ile on NMDAR-dependent LTP in the Schaffer collateral–CA1 synapse in adult rats. We found that D-Ile significantly increases the magnitude of NMDAR-dependent LTP induced by TBS (D-Ile, 132 ± 4.3% of baseline when averaged for the last 15 min of recordings vs control, 118.9 ± 4.1%, $p = 0.02$) (Fig. 8*A*). D-APV blocked the enhancement of LTP by D-Ile (99.9 ± 2.5% of baseline), confirming that it depends on NMDARs (Fig. 8*A*). D-Ile did not change the AMPAR-mediated fEPSPs evoked at different stimulus intensities, indicating that it does not affect basal glutamatergic transmission (Fig. 8*B*). Furthermore, D-Ile effect is not attributable to an increase in presynaptic release of glutamate, because it does not affect paired-pulse facilitation, an electrophysiological paradigm used to determine whether the drug affects the mechanisms of glutamatergic release (Fig. 8*C*).

Because D-serine seems to be the dominant coagonist for LTP in acute slices (Mothet et al., 2006; Henneberger et al., 2010), we wondered whether the enhancement of LTP by D-Ile is attributable to acceleration of endogenous D-serine release via Asc-1. For this purpose, we incubated slices with the recombinant enzyme DsdA. This enzyme selectively destroys endogenous D-serine and prevents the NMDAR-dependent neurotoxicity in organotypic slices and NMDAR potentials in the retina (Shleper et al., 2005; Gustafson et al., 2007). We found that treatment of the hippocampal slices with DsdA abolished TBS-induced LTP (DsdA, 106.3 ± 3.2% of baseline vs control, 120.9 ± 5.7%, $p = 0.0001$), confirming the critical role of D-serine in synaptic plasticity (Fig. 8*D*). Most importantly, D-Ile had no effect on LTP in slices treated with DsdA (D-Ile, 102.4 ± 2.4%) (Fig. 8*E*). These results indicate that D-Ile-mediated LTP enhancement is attributable to a specific increase in synaptic D-serine via Asc-1.

To confirm whether D-Ile effects on synaptic plasticity can be ascribed to a specific change in D-serine dynamics, we monitored LTP in mice with deletion of the SR gene (SR-KO). We used a single tetanus of 100 Hz for 1 s to induce reproducible NMDAR-dependent LTP in these animals. As expected, we found that D-Ile enhances LTP in WT mice (D-Ile, 124.4 ± 3.3% of baseline vs control, 113.6 ± 4.3%, $p = 0.02$), and this was blocked by D-APV (93.3 ± 2.7% of baseline) (Fig. 8*F*). In contrast, D-Ile did not enhance LTP in SR-KO mice (D-Ile, 107.8 ± 4.1% of baseline vs control, 110.3 ± 4.7%), confirming its specificity toward D-serine (Fig. 8*G*). Conversely, addition of exogenous D-serine enhanced LTP in WT and SR-KO mice by 10.7 and 18.6%, respectively (Fig. 8*H*,*I*), indicating that SR-KO mice respond to exogenous D-serine at least as well as WT.

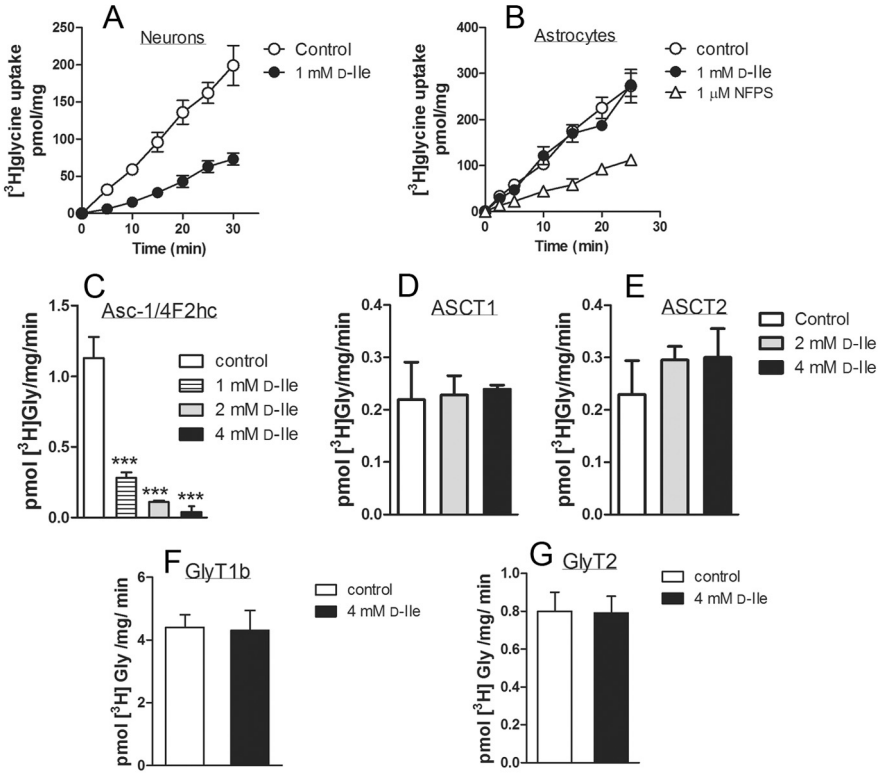

Figure 5. D-Ile inhibits glycine transport by Asc-1. ***A***, D-Ile inhibits glycine uptake in primary neuronal cultures. The assay was performed with 2 μM [^{3}H]glycine in a Na^+-free medium, in either the absence (○) or presence (●) of 1 mM D-Ile. ***B***, D-Ile has no effect on glycine uptake by rat primary astrocyte cultures. The uptake was performed with 2 μM [^{3}H]glycine in an Na^+-containing medium with none (○), 1 mM D-Ile (●), or 1 μM of the GlyT1 inhibitor NFPS (△). ***C***, D-Ile inhibits glycine uptake by Asc-1 in transfected HEK293 cells. Glycine uptake was monitored in Na^+-free medium containing 100 nM [^{3}H]glycine. The values were calculated by subtracting blanks consisting of cells transfected with 4F2hc only, which accounted for <10% of the total D-[^{3}H]serine accumulation. ***D–G***, D-Ile has no effect on glycine uptake by ASCT1 (***D***), ASCT2 (***E***), GlyT1b (***F***), or GlyT2 (***G***) transfected HEK 293 cells. These transport activities were monitored in Na^+-containing media, in the presence of 100 nM [^{3}H]glycine. The values were calculated by subtracting blanks consisting of cells transfected with GFP alone, which accounted for ~10–30% of the total [^{3}H]glycine accumulation. The values are the mean ± SEM of four to six experiments with different cultures. ***$p < 0.001$, different from control.

We also found that the LTP in the absence of D-Ile was the same in 2- to 3-month-old WT and SR-KO mice (Fig. 8, compare *H*, *I*). This contrasts with previous data that found LTP deficits in SR-KO using juvenile mice (21–28 d old) induced by a pairing protocol (Basu et al., 2009) or the striking decrease in LTP promoted by acute depletion of endogenous D-serine during treatment with DsdA (Fig. 8*D*) or DAO enzymes (Mothet et al., 2006; Henneberger et al., 2010). Altogether, the data support the notion that endogenous D-serine release mediates D-Ile effects on LTP, but that adult SR-KO mice may have adaptations on the LTP mechanisms to cope with the lower D-serine availability.

Role of Asc-1 in NMDAR-dependent synaptic activity

Does Asc-1 modulate synaptic NMDAR responses? We found that D-Ile also significantly increased the isolated NMDAR-dependent fEPSPs in rat hippocampal CA1 slices recorded in the presence of the AMPA antagonist NBQX and low magnesium concentration. D-Ile increased NMDAR fEPSPs by 25% whatever the intensity of stimulation in 11 of 16 tested slices (Fig. 9*A*). By comparison, addition of exogenous D-serine at saturating concentrations increased NMDAR-mediated fEPSPs by 45% in 12 of 15 slices (Fig. 9*B*). It is noteworthy that NMDAR fEPSPs were drastically decreased by depleting endogenous D-serine by DsdA treatment (between 60 and 70% reduction depending on

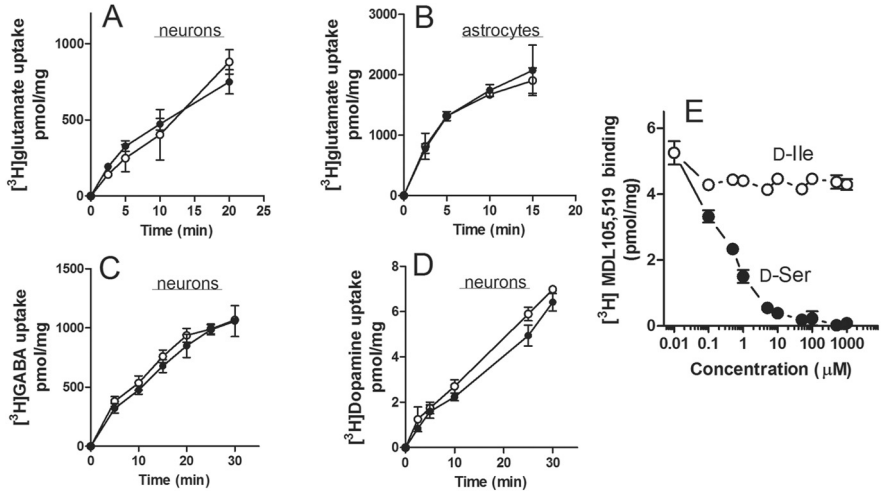

Figure 6. D-Ile has no effect on other neurotransmitter transporters and does not bind to the NMDAR coagonist site. ***A***, Glutamate uptake in rat primary neuronal cultures assayed with 1 μM [^{3}H]glutamate in Na^+-containing medium, in either the absence (○) or presence (●) of 1 mM D-Ile. ***B***, Glutamate uptake in rat primary astrocytic cultures in either the absence (○) or presence (●) of 1 mM D-Ile. ***C***, GABA uptake in rat primary neuronal cultures in the presence of 1 μM [^{3}H]GABA and in the absence (○) or presence (●) of 1 mM D-Ile. ***D***, Dopamine uptake in rat primary neuronal cultures performed in the presence of 1 μM [^{3}H]dopamine and in either the absence (○) or presence (●) of 1 mM D-Ile. ***E***, D-Ile does not affect the binding of the selective NMDAR coagonist site ligand [^{3}H]MDL 105,519 (100 nM) to washed rat brain membranes. Binding was abolished by D-serine (IC_{50} ~300 nM). The values are the mean ± SEM of three to four experiments.

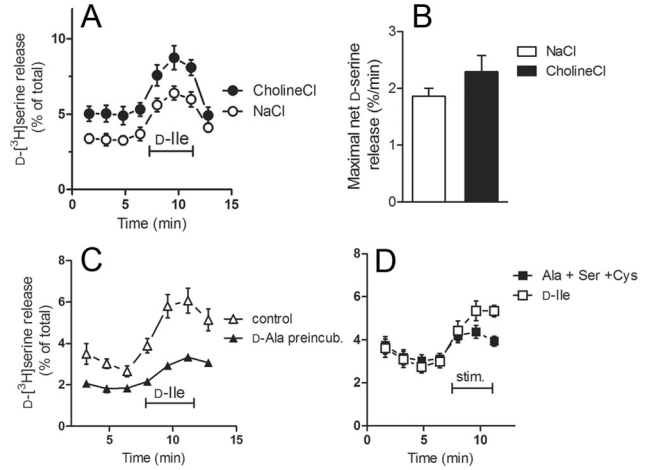

Figure 7. D-Ile elicits D-serine release from an Asc-1-sensitive pool and mimics the effects of typical extracellular Asc-1 substrates. ***A***, D-Ile-induced D-serine release from acute hippocampal slices in media containing NaCl (○) or cholineCl (●). Slices were preloaded with 5 μM D-[^{3}H]serine pre-equilibrated as described in Materials and Methods. Removal of Na^+ (●) was attained by a 15 min perfusion with cholineCl-containing medium before stimulation with 1 mM D-Ile. ***B***, Maximal net release of D-serine by D-Ile calculated from the data of ***A*** was similar in slices perfused with normal Na^+ (open bar) and Na^+-free (filled bar) media. ***C***, Preincubation with 1 mM D-alanine during loading with D-[^{3}H]serine decreases D-Ile-induced D-serine release. ***D***, Comparison between D-serine release promoted by D-Ile with that induced by perfusing the slices with L-alanine (14 μM), L-serine (36 μM), and L-cysteine (2 μM). The values are mean ± SEM of four to six experiments. ***$p < 0.001$, different from control.

stimulus intensity), demonstrating that D-serine is required for synaptic NMDAR activation (Fig. 9*C*). Nevertheless, depletion of endogenous D-serine by DsdA treatment did not abolish the stimulatory effect of D-Ile on NMDAR fEPSPs, regardless of the stimulus intensity (30% in average in 10 of 15 tested slices) (Fig. 9*D*). This suggests that a significant fraction of the D-Ile-mediated enhancement of NMDAR-mediated potentials could reflect the release of endogenous glycine as well. The data are consistent with the notion that Asc-1 also releases glycine from acute slices by antiporter activity (Fig. 4*C*).

Additional evidence that Asc-1 contributes to glycine dynamics comes from experiments with SR-KO mice (Fig. 10). We found that these mice exhibit normal NMDAR fEPSPs, suggesting a possible compensatory mechanism by endogenous glycine, because their D-serine levels are only 10% of the WT (Fig. 10*A*). Furthermore, we found that D-Ile significantly stimulated NMDAR fEPSPs by ~40% in SR-KO mice (10 of 12 tested slices), strengthening the notion that Asc-1 also releases glycine (Fig. 10*B*). Exogenously added D-serine stimulated NMDAR fEPSPs by 40%, in both WT (9 of 10 tested slices) and SR-KO (8 of 10 tested slices) mice, indicating similar occupancy of the coagonist binding site in the two groups of animals (Fig. 10*C*). These effects were specific for NMDARs, because the AMPAR fEPSPs were similar in WT and SR-KO (Fig. 11*A*) mice and unaffected by D-Ile (Fig. 11*B*,*C*).

Discussion

The relative contribution of neurons versus astrocytes in D-serine signaling has been unclear. Recent reports have demonstrated that SR is predominantly expressed by glutamatergic neurons (Kartvelishvily et al., 2006; Ito et al., 2007; Yoshikawa et al., 2007; Miya et al., 2008; Balu and Coyle, 2012; Benneyworth et al., 2012). Our study now demonstrates that Asc-1, a neuronal specific transporter, releases D-serine to regulate NMDAR-dependent synaptic activity. Furthermore, our data disclose a role of Asc-1 in mediating concurrent release of neuronal glycine, which also plays a role in activating NMDARs, especially at low-frequency stimulation.

To investigate the role of neuronal D-serine, we looked for compounds that specifically affect neuronal D-serine dynamics without acting on D-serine release by astrocytes. We found that D-Ile interacts with Asc-1 in transfected cells, primary neuronal cultures, and acute hippocampal slices, enhancing the neuronal release of D-serine via D-Ile/D-serine exchange.

Because the K_i of D-Ile for Asc-1 is quite high, a key issue was to evaluate its selectivity against additional targets that may affect D-serine or glycine dynamics. We found that D-Ile induces D-serine release from neurons but not astrocytes, which is in agreement with Asc-1 localization in neuronal structures (Helboe et al., 2003; Matsuo et al., 2004). In addition, D-Ile interacts with Asc-1 in transfected cells but does not affect Na^+-dependent D-serine transporters such as ASCT1 and ASCT2. We also found that D-Ile does not bind to the coagonist site of NMDARs, nor does it affect GlyT1, GlyT2, glutamate, GABA, and dopamine transporters.

Targeted deletion of Asc-1 gene in mice indicates that Asc-1 is responsible for >90% of the Na^+-independent transport of D-serine (Rutter et al., 2007). To investigate whether Asc-1 is the main target of D-Ile, we monitored its effects in the absence of Na^+, which allows near complete isolation of the Asc-1 component of D-serine transport. We found that D-Ile effects in both neuronal cultures and slices were independent on Na^+. Thus,

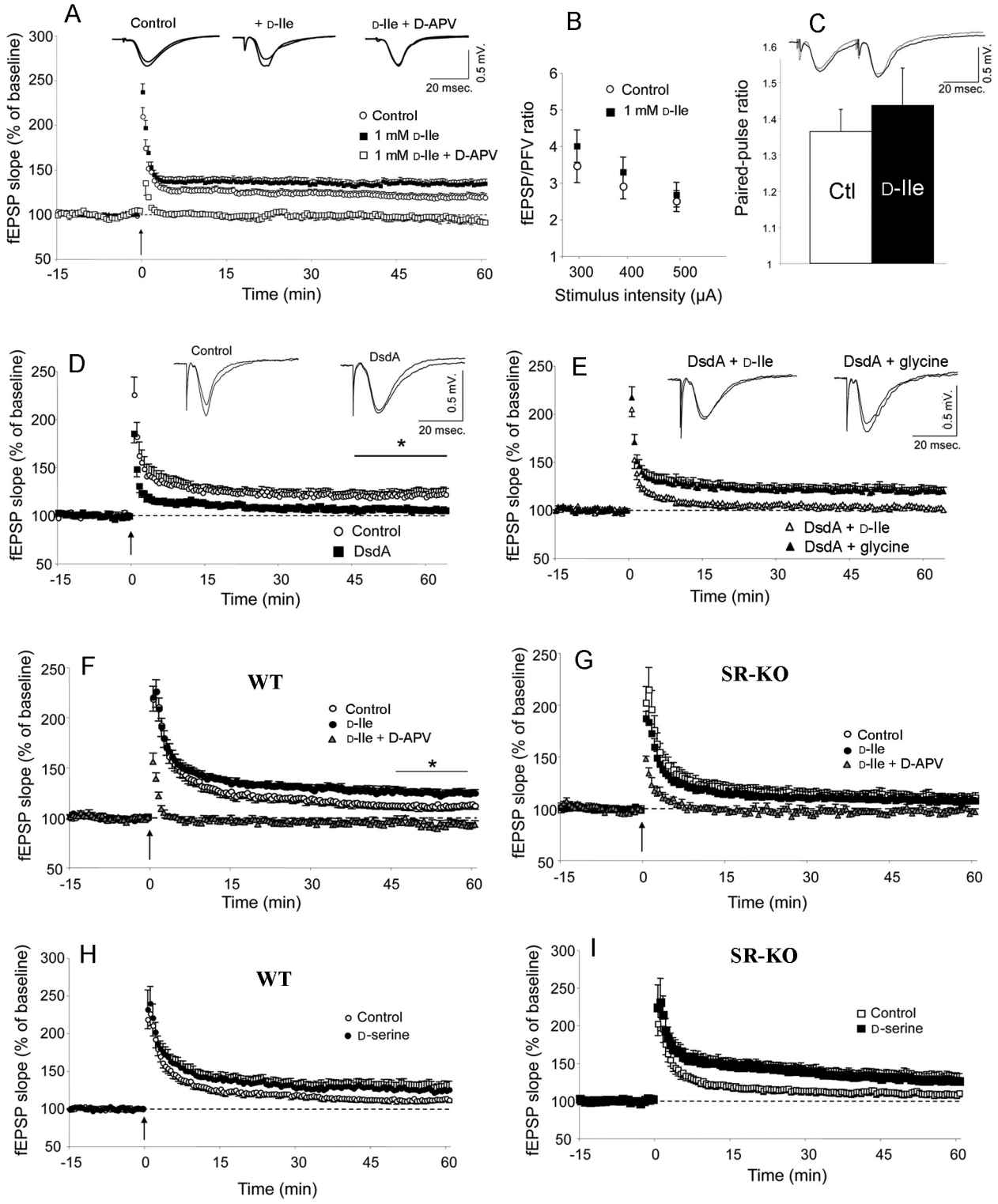

Figure 8. D-Ile enhances NMDAR-dependent LTP in hippocampal slices through D-serine release. ***A***, LTP induced by TBS in the hippocampal CA1 area of young adult rats is significantly enhanced by 1 mM D-Ile (■, $n = 13$) when compared with control slices (○, $n = 12$). LTP mediated by D-Ile is dependent on NMDARs, because it is abolished in the presence of 80 μM D-APV (□, $n = 10$). The inset depicts representative traces of fEPSPs recorded before and 60 min after TBS in a control slice and in the presence of D-Ile with or without D-APV. ***B***, Basal synaptic transmission is not affected by D-Ile. AMPAR fEPSP/PFV ratio was calculated at increasing stimuli intensity before (open symbols) and 15 min after application of 1 mM D-Ile (filled symbols). The values are mean ± SEM of recordings from 12 slices. Ctl, Control. ***C***, D-Ile does not affect the paired-pulse facilitation determined at 30 ms stimulation interval in slices from adult Sprague Dawley rats. The values are mean ± SEM of eight experiments. The inset depicts representative traces of PPF recordings before (solid line) and after (dashed line) D-Ile application. ***D***, D-serine is critical for the expression of synaptic plasticity. TBS-induced LTP as shown in control slices ($n = 11$) was abolished in slices incubated for at least 90 min with recombinant DsdA enzyme to deplete endogenous D-serine ($n = 13$). ***E***, D-Ile-mediated LTP increase requires endogenous D-serine. LTP expression in DsdA-pretreated slices is not enhanced by adding 1 mM D-Ile ($n = 13$ slices) but is rescued by exogenous 500 μM glycine ($n = 12$). ***F***, D-Ile also enhances LTP expression in WT mice. Effects of 1 mM D-Ile ($n = 14$ slices) on D-APV-sensitive LTP induced by one train of 100 Hz for 1 s in CA1 area compared with control conditions ($n = 17$). ***G***, D-Ile improvement of LTP requires D-serine. TBS-induced LTP generated in slices from SR-KO mice ($n = 17$) is not enhanced by D-Ile ($n = 14$). ***H***, ***I***, LTP expression is enhanced by saturating the NMDAR binding sites with 100 μM D-serine in both WT (***H***) and SR-KO (***I***) mice. *$p < 0.05$, different from control.

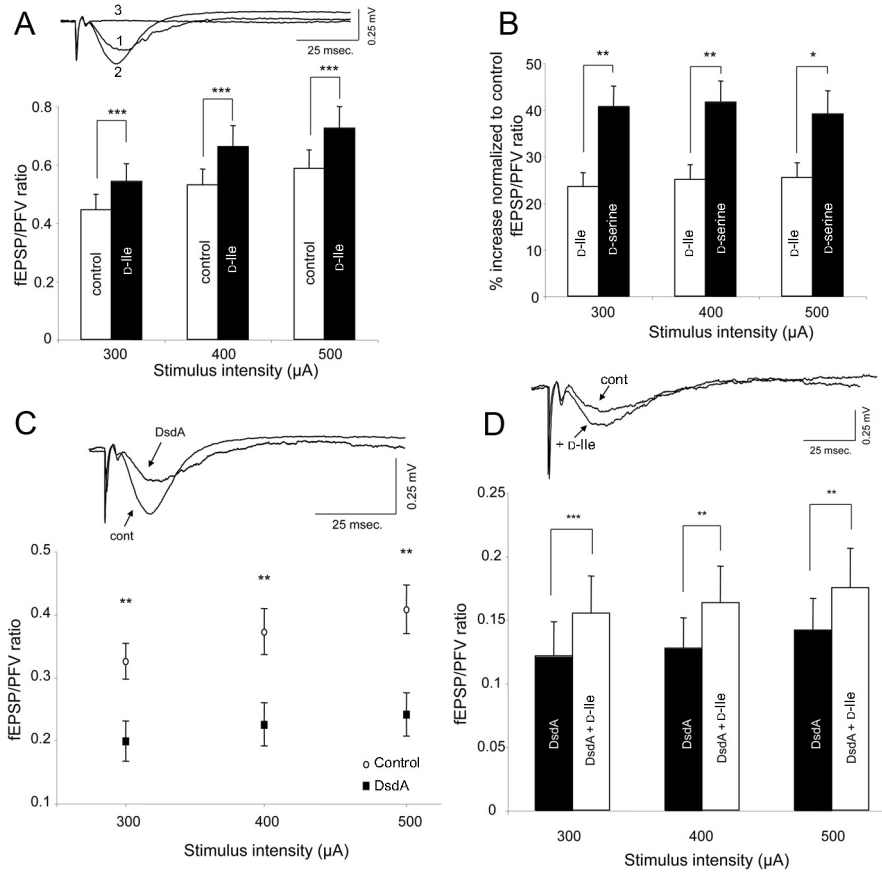

Figure 9. D-Ile enhances NMDAR synaptic potentials in hippocampal slices via D-serine and glycine release. ***A***, D-Ile increases NMDAR potentials isolated in low-magnesium medium supplemented with NBQX. fEPSP/PFV ratios were monitored at increasing stimuli intensity before (open bars) and after (filled bars, $n = 11$) addition of 1 mM D-Ile. The inset depicts representative traces of NMDAR fEPSPs recorded before (***1***) and after (***2***) the addition of D-Ile or D-Ile plus 30 μM D-APV (***3***). ***B***, Percentage increase in NMDAR fEPSP/PFV ratio induced by either 1 mM D-Ile or 100 μM D-serine ($n = 12$) at different stimuli intensity. ***C***, Endogenous D-serine is required for NMDAR potentials. When compared with control slices (○, $n = 32$), the fEPSP/PFV ratio is significantly decreased in slices pretreated for at least 90 min with 20 μg/ml DsdA (■, $n = 18$) to deplete endogenous D-serine. ***D***, D-Ile still increases NMDAR potentials in D-serine-depleted slices. fEPSP/PFV ratio in slices incubated with DsdA before (filled bars) and after (open bars, $n = 10$ slices) 1 mM D-Ile. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$, different from control.

D-Ile-induced D-serine release from slices perfused without Na^+ was at least as high as that observed under normal Na^+ concentration. Furthermore, D-Ile effects were also inhibited by D-alanine, an Asc-1 substrate whose transport is disrupted in Asc-1 KO mice (Rutter et al., 2007). We found that D-alanine blocks D-[^{14}C]Ile transport in neurons and inhibits D-Ile-induced D-serine release from slices when added before D-Ile.

Together, these results suggest that D-Ile is quite selective for Asc-1. However, they do not completely rule out the involvement of additional transporters. Indeed, it is possible that a small fraction of D-serine can also be released by another carrier whose kinetics is indistinguishable from Asc-1. Alternatively, Asc-1 antiporter activity may trigger D-serine release from other pathways via the release of an unidentified mediator.

We also considered the possibility that D-Ile directly opens channels that release both glutamate and D-serine, such as volume-regulated channels (Takano et al., 2005; Rosenberg et al., 2010) or connexin 43 hemichannels (Stehberg et al., 2012). However, D-Ile does not affect endogenous glutamate release from slices, nor does it change the paired-pulse facilitation, an indicator of presynaptic glutamate release. In addition, D-Ile does not affect AMPAR transmission, indicating that it does not activate nonselective channels that are expected to release glutamate as well.

We demonstrate that D-Ile affects synaptic plasticity in CA1 neuronal networks via enhancement of NMDAR-dependent LTP, suggesting a role for neuronal D-serine release. However, it is conceivable that D-Ile, especially at high concentrations, enhances LTP by mechanisms unrelated to D-serine release. To investigate this possibility, we used several controls using slices from SR-KO mice or slices in which endogenous D-serine was depleted by treating with DsdA, an enzyme that selectively destroys D-serine (Shleper et al., 2005). We found that D-Ile does not enhance LTP in either of the D-serine depletion settings, supporting the notion that D-Ile effects are mediated by endogenous neuronal D-serine.

We also considered the possibility that Asc-1 hetero-exchange by D-Ile does not reflect a physiological condition. However, in normal brain, the extracellular concentrations of Asc-1 amino acid substrates are enough to saturate Asc-1 antiporter activity (Lindroth et al., 1985; Fukasawa et al., 2000). This contrasts with the very low extracellular concentration of the Asc-1 substrates detected in the slice perfusates of our *in vitro* experiments, presumably caused by the washout of the extracellular medium during perfusion. Supplementing the perfusion buffer with extracellular Asc-1 substrates at physiological levels increases the rate of D-serine release to levels similar to those observed with D-Ile alone. Thus, D-Ile appears to restore the physiologic activity of Asc-1.

Although we describe a role of Asc-1 in mediating D-serine release, its relative contribution for physiological NMDAR activation is not warranted by the effects of D-Ile. Indeed, Mothet et al. (2005) demonstrated vesicular D-serine release from astrocytes, and this pathway is required for hippocampal LTP (Henneberger et al., 2010). A major role of glia in D-serine release has also been demonstrated in the hypothalamus and prefrontal cortex, as well (Panatier et al., 2006; Fossat et al., 2012). In addition, our data do not discard a role for ASCT1 and ASCT2 in mediating D-serine release. Although these transporters are insensitive to D-Ile, they likely play a role in D-serine dynamics, especially in the retina, which lacks Asc-1-mediated D-serine transport (O'Brien et al., 2005). These pathways are presumably regulated by different mechanisms and operate at different timescales in distinct synaptic and extrasynaptic microenvironments.

Another aspect of our study concerns the role of Asc-1 in glycine dynamics. The glycine transporters GlyT1 and GlyT2 are generally considered the sole regulators of extracellular glycine concentrations, whereas Asc-1 was not previously thought to affect glycine levels. We found that Asc-1 also plays a role as a pathway for glycine release along with D-serine. This provides a mechanism to release NMDAR coagonists from neurons, indicating that the dynamics of these two coagonists are connected.

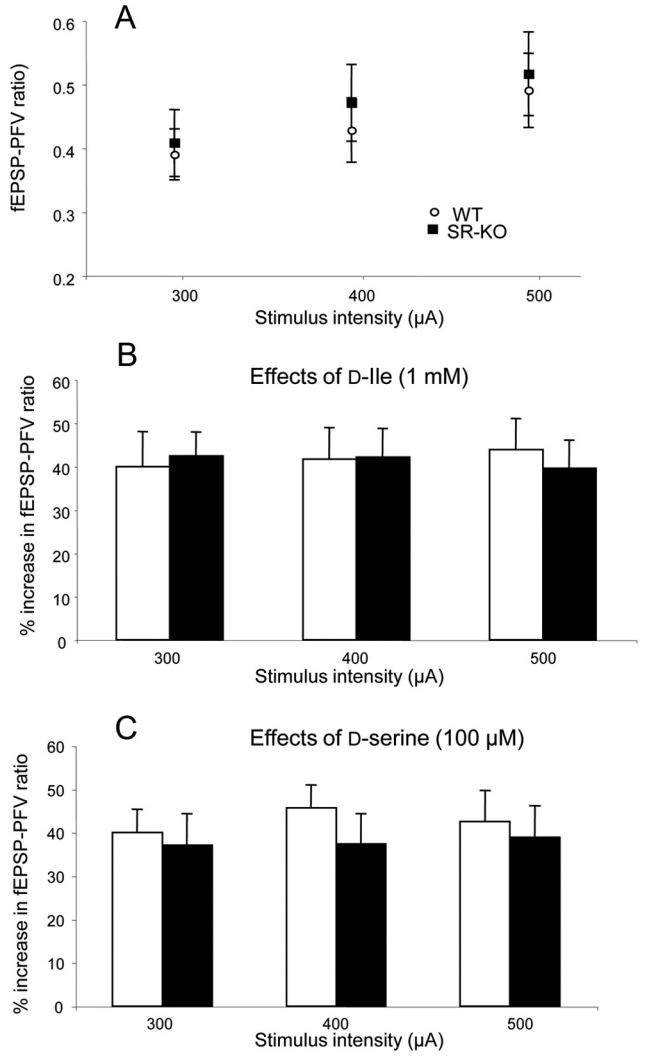

Figure 10. D-Ile increases NMDAR potentials in hippocampal slices from WT and SR-KO mice. ***A***, NMDAR synaptic transmission is preserved in SR-KO adult mice. fEPSP/PFV ratios of isolated NMDAR potentials determined with increasing stimuli intensities in slices from WT (○, $n = 22$) and SR-KO (■, $n = 22$) mice. ***B***, NMDAR fEPSPs are equally enhanced by D-Ile in slices from WT and SR-KO mice. Bars depict a 40% increase in the NMDAR potentials during application of 1 mM D-Ile onto slices from both WT (open bars, $n = 12$) and SR-KO (filled bars, $n = 10$) mice. ***C***, D-serine equally stimulates NMDAR in both groups of mice. Effect of exogenous application of 100 μM D-serine in slices from WT (open bars, $n = 9$) and SR-KO (filled bars, $n = 8$) mice.

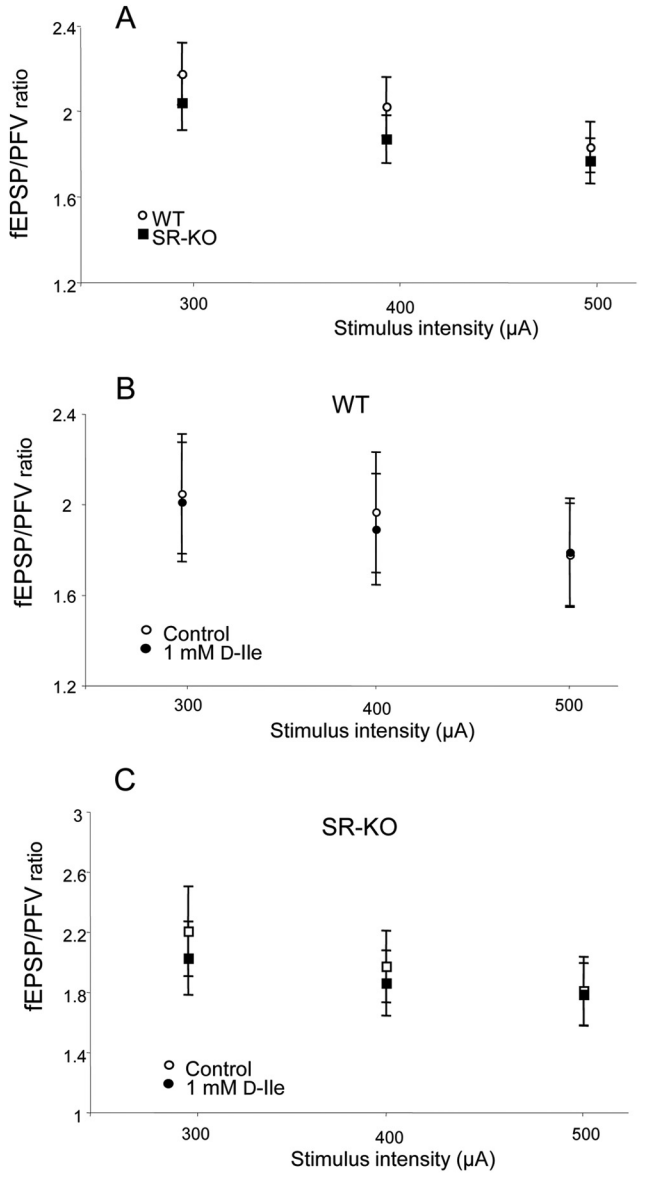

Figure 11. D-Ile does not affect basal glutamatergic transmission. ***A***, AMPAR synaptic transmission in hippocampal CA1 is unchanged in slices from SR-KO compared with WT mice. fEPSP/PFV ratios of AMPAR potentials were determined with increasing stimuli intensities in slices from WT (○, $n = 59$) and SR-KO (■, $n = 59$) mice. ***B***, AMPAR fEPSPs are not altered by D-Ile. fEPSP/PFV ratios of AMPAR potentials induced at CA3/CA1 synapses showing the absence of significant effects of 1 mM D-Ile in slices from WT mice ($n = 17$). ***C***, D-Ile has no effect on AMPAR potentials in slices from SR-KO mice ($n = 15$).

The role of Asc-1 in glycine release was further defined by the electrophysiological experiments. We found that D-serine was not the sole coagonist for low-frequency-activated NMDAR potentials, contrasting with its critical role in the high-frequency NMDAR activation required for LTP. Thus, although depletion of endogenous D-serine by DsdA drastically decreased the NMDAR fEPSPs confirming the major role of D-serine in modulating NMDARs, it did not affect the stimulatory effect of D-Ile. This suggests an effect of D-Ile on glycine release as also revealed in our biochemical experiments. Furthermore, D-Ile robustly increased the NMDAR fEPSPs in adult SR-KO mice, likely reflecting glycine release through Asc-1. These results are compatible with the notion that glycine compensates for D-serine deficits, at least under moderate NMDAR activation, such as those induced by low-frequency stimulation of glutamatergic afferents.

Papouin et al. (2012) recently reported that D-serine is the sole coagonist at the synaptic NMDARs, whereas glycine is only required for extrasynaptic NMDAR activation. Conversely, our observation that the synaptic NMDAR responses are essentially unaltered in adult SR-KOs suggests that D-serine is not the sole coagonist at the synapse. Our SR-KO mice do not exhibit changes in the expression of NMDAR subunits (Inoue et al., 2008), and their extracellular levels of glycine, glutamate, and glutamine monitored by *in vivo* hippocampal microdialysis are the same as the WT mice (Horio et al., 2011). Like Papouin et al. (2012), we observed that acute enzymatic depletion of D-serine abolishes LTP and strongly diminishes the synaptic NMDAR responses by 60–70%. A parsimonious explanation for these findings is that D-serine is the main synaptic NMDAR coagonist under normal conditions, but there is still substantial overlap with glycine at synaptic NMDARs. Glycine effects become more evident when its release is enhanced by D-Ile or by deleting SR gene.

The effects of D-serine in synaptic plasticity have been attributed to its role as a gliotransmitter (Mothet et al., 2005; Panatier et al., 2006; Henneberger et al., 2010; Papouin et al., 2012), but the role of gliotransmission is still under debate (Agulhon et al., 2010, 2012; Nedergaard and Verkhratsky, 2012). Our study now suggests a role of neuronal D-serine released by Asc-1 in modulating LTP and synaptic NMDAR responses. Our data also indicate a role of neuronal glycine release in activating synaptic NMDARs. In this framework, Asc-1 provides a novel pathway for concurrent non-vesicular release of D-serine and glycine from neurons. Blockers of Asc-1 may therefore provide a new strategy to decrease NMDAR coactivation, whereas activators of the Asc-1 antiporter may be useful for conditions in which NMDAR function may be decreased, such as normal aging and schizophrenia.

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