Serine racemase: Activation by glutamate neurotransmission via glutamate receptor interacting protein and mediation of neuronal migration

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Serine racemase (SR), localized to astrocytic glia that ensheathe synapses, converts L-serine to D-serine, an endogenous ligand of the NMDA receptor. We report the activation of SR by glutamate neurotransmission involving α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors via glutamate receptor interacting protein (GRIP) and the physiologic regulation of cerebellar granule cell migration by SR. GRIP physiologically binds SR, augmenting SR activity and D-serine release. GRIP infection of neonatal mouse cerebellum *in vivo* enhances granule cell migration. Selective degradation of D-serine by D-amino acid oxidase and pharmacologic inhibition of SR impede migration, whereas D-serine activates the process. Thus, in neuronal migration, glutamate stimulates Bergmann glia to form and release D-serine, which, together with glutamate, activates NMDA receptors on granule neurons, chemokinetically enhancing migration.

α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor | D-serine

The NMDA receptor is activated by the neurotransmitter glutamate as well as a second agonist influencing a site that can be stimulated by glycine (1). Recent studies indicate that D-serine is a major, if not principal, ligand for this "glycine site" (2–5). D-serine is formed by serine racemase (SR) from L-serine (5). SR and D-serine are localized to a population of protoplasmic astrocytes that ensheath synapses and possess the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor. SR also occurs in radial glia such as Bergmann glia that guide cerebellar granule cells migrating from the external granule layer (EGL) to the internal granule layer (IGL) during cerebellar development (6, 7). This process, one of the best-characterized instances of neuronal migration, has been perplexing, because immature granule cells lack synaptic contacts. Moreover, whereas their migration is determined by glutamate acting via NMDA receptors, the cellular interactions that mediate the neuronal migration have not heretofore been identified (7).

We report that SR binds to the AMPA receptor binding protein GRIP (glutamate receptor interacting protein), leading to markedly enhanced SR activity and D-serine release from glia, actions that are elicited by AMPA receptor stimulation. Overexpression of GRIP in mice also augments NMDA receptor-mediated neuronal cell migration. In brain slices we demonstrate that D-serine is required for granule cell migration via chemokinetic influences on granule cells.

Materials and Methods

Yeast Two-Hybrid Screening. Yeast two-hybrid screening used Y190 yeast strains, which contain HIS3 and β-gal reporter genes. Full-length SR gene was subcloned into pPC97, containing GAL4 DNA binding domain. Rat hippocampus and cortex cDNA library was cloned into pPC86, containing GAL4 transactivation domain. More than one million clones were transformed and screened. All other constructs of SR and GRIP were subcloned into pPC97 or pPC86.

Protein Binding Assays. SR(WT) and SR(V339G) tagged with GST were transfected into human embryonic kidney (HEK)-293 cells by using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were harvested in lysis buffer [50 mM Tris·HCl, pH 7.4/50 mM NaCl/1 mM DTT/1 mM EDTA/1× PICS (protease inhibitor mixture) tablet (Roche, Basel, Switzerland)/1 mM PMSF]. Cell lysate was incubated with glutathione-Sepharose 4B beads (Amersham Pharmacia) at 4°C for 1 h, and the beads were washed with wash 1 (lysis buffer plus 0.1% Triton X-100) and wash 2 (250 mM NaCl, 1 mM EDTA in 50 mM Hepes, pH 7.4) three times.

Rat brains were homogenized in immunoprecipitation buffer (50 mM Tris·HCl, pH 7.4/50 mM NaCl/2 mM EDTA/1% Triton X-100/1 mM DTT/1 mM PMSF/1× PICS). The homogenate was centrifuged at 12,000 × *g* for 10 min, and the supernatant was incubated with SR antibody for 2 h at 4°C. Protein A/G-agarose beads were added to the mixture for 1 h at 4°C and washed with immunoprecipitation buffer (×3). Bound proteins were analyzed by Western blot with GRIP antibody.

Immunocytochemistry. Mixed glial culture cells were prepared and grown on poly-L-lysine (500 μg/ml)-coated glass coverslips, fixed with 4% paraformaldehyde in PBS for 5 min, partially trypsinized (only for anti-SR and anti-GRIP staining), permeabilized with 0.5% Triton X-100–PBS for 10 min, and blocked with 10% FBS–PBS for 10 min. Primary antibody incubations were conducted at 4°C overnight in 1% BSA–PBS. Mouse anti-glial fibrillary acidic protein (GFAP) (Research Diagnostics, Flanders, NJ) and rabbit anti-GluR2/3 (Chemicon) were used at 1 and 3 mg/ml, respectively. Rabbit anti-SR and anti-GRIP antibody were used at 1:100 and 1:200 dilutions, respectively. Rabbit secondary antibodies, FITC-conjugated anti-mouse, and Texas red-conjugated anti-rabbit (Jackson ImmunoResearch) were used at a 1:125 dilution for 1 h at 37°C. Cells were washed for 10 min (three times) with BSA–PBS after primary and secondary incubation and visualized with a confocal microscope.

D-Serine Synthesis Assay. SR and SR(V339G) cDNAs, subcloned into pTracer-CMV vector, were transfected into C6 glioma cells. After transfection (36 h), the cells were homogenized and dialyzed.

Abbreviations: GRIP, glutamate receptor interacting protein; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; SR, serine racemase; DAO, D-amino acid oxidase; EGL, external granule layer; IGL, internal granule layer; ML, molecular layer; HEK, human embryonic kidney; GFAP, glial fibrillary acidic protein; NBQX, 6-nitro-7-sulfamoylbenzo-(F)quinoxaline-2,3-dione; P*n*, postnatal day *n*; RT, room temperature; Phen, phenazine; Et-Phen, phenazine ethosulfate; Met-Phen, phenazine methosulfate.

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Cell lysates were incubated with 1 mM L-serine and 0.5 μM pyridoxal 5′phosphate at 37°C for 3 h. This reaction was performed three times. The reaction was stopped by adding trichloroacetic acid (5% final concentration), and the amino acids were ether-extracted. Samples were dried to completion and analyzed on HPLC (8).

GRIP PDZ-6 Recombinant Adenovirus Generation. GRIP PDZ-6 cDNA was subcloned into pADTrack-CMV vector. The recombinant virus expressing GRIP PDZ-6 protein was generated and amplified following the protocol of the AdEasy Adenovirus system (Quantum Technologies, Montreal).

Adenovirus Infection. Mixed glial cells were split equally and grown to 70% confluency in DMEM with 10% FBS, 5% glutamate, and 100 units of penicillin–streptomycin at 37°C in 5% CO_2 atmosphere. The cells were infected for 36 h with GFP or GRIP–PDZ6 recombinant adenovirus. The media were replaced with 0.1× Basal Media Eagle containing AMPA (1 mM) and 6-nitro-7-sulfamoylbenzo(F)quinoxaline-2,3-dione (NBQX) (2 mM) or PBS, as control. Incubation with the appropriate drug was conducted for 1 h at 37°C in 5% CO_2 atmosphere. Media were removed, and the cells were harvested for HPLC analysis. The experiment was replicated four times.

Mouse Adenoviral Infection and BrdUrd Labeling. At postnatal day 8 (P8), mice were injected in the brain with 10 μl of WT adenovirus or GRIP–PDZ-6 adenovirus. Mice were injected i.p. with 25 ml of 10 μg/ml BrdUrd (Roche Molecular Biochemicals) dissolved in saline solution, 0.007 M NaOH, and 0.9% NaCl. At P11, mice cerebella were removed to assay D-serine by HPLC, and the brains, perfused with 4% paraformaldehyde, were sectioned to 10-μm slices. The sections were washed with PBS for 5 min (three times), treated with 2 M HCl for 30 min at room temperature (RT), and neutralized with 0.1 M NaB_2O_7 for 10 min at RT. Sections were washed with PBS for 5 min (three times) and blocked with 3% BSA, 1% goat serum, and 0.3% Triton X-100 in PBS for 2 h at RT. Brain sections were stained with 1:100 BrdUrd antibody (Becton Dickinson) overnight at 4°C and washed with PBS for 5 min (three times). Anti-mouse secondary were added to sections for 1 h at RT and washed with PBS for 5 min (four times). The experiment was replicated three times.

Granule Cell Migration Assay. The granule cell migration assay was performed as described (6). The cerebellar slices (800 μm), sectioned sagittally, were placed in ACSF solution (125 mM NaCl/5 mM KCl/2 mM $CaCl_2$/1 mM $MgCl_2$/24 mM glucose/10 mM Hepes) and were preincubated in six-well plates with appropriate drugs or D-amino acid oxidase (DAO) (5 μg per well; Sigma-Aldrich) for 30 min. The slices were washed with ACSF three times and treated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (10 μg/ml, Molecular Probes) for 15 min. The cerebellar slices were washed (three times) and postincubated in appropriate conditions. The migration reaction was conducted in a 37°C, 5% CO_2 incubator for 4 h and was stopped by adding 4% paraformaldehyde. The cerebellar slices were cut sagittally into 200-μm slices, visualized by using a fluorescence microscope, and the migration distance was determined by using OPENLAB software (Improvision, Lexington, MA). Drug (Sigma) concentrations used were 10 μM D-serine, 10 μM AMPA, 100 μM NBQX, 50 μM phenazine ethosulfate (Et-Phen), 50 μM phenazine methosulfate (Met-Phen), and 100 μM phenazine (Phen).

Explant Culture Preparation. Cultures were prepared as described (9). P1–P3 mouse cerebella were isolated in Hanks' balanced salt. Cerebella were freed from meninges and sagittally sliced into 200- to 400-μm slices. The cerebellar cortices were isolated and sectioned into rectangular pieces. These pieces were then placed onto a poly-L-lysine/laminin-coated glass coverslip (≈5–10 explants per coverslip) and allowed to settle for 2–4 h. For solution bathing the explants were changed to minimum essential media (Invitrogen) supplemented with 30 mM glucose, 10% FCS, 90 units/ml penicillin, 90 μg/ml streptomycin, and 1.8 mM glutamine.

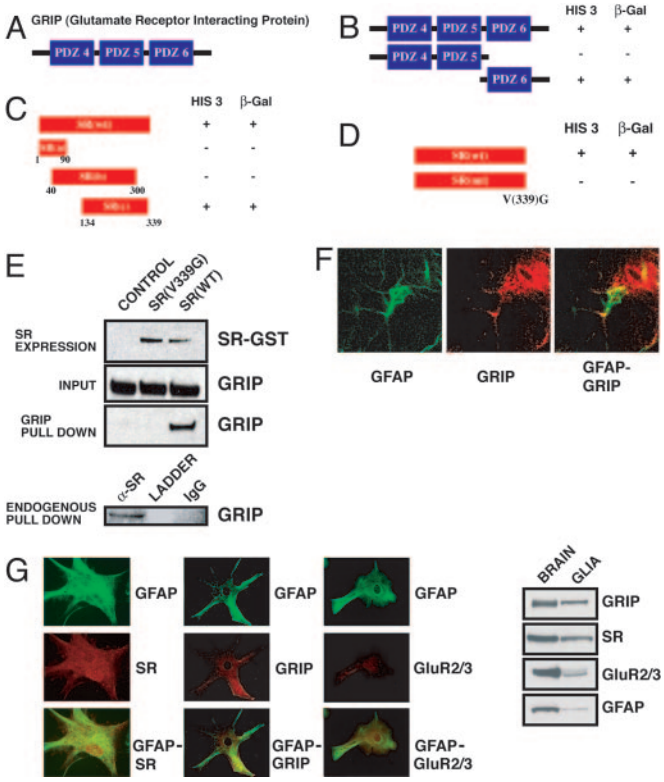

Fig. 1. GRIP binds SR in yeast two-hybrid analysis and by immunoprecipitation. (*A*) Of one million clones screened from rat hippocampus and cortex cDNA library, two clones were both His- and β-gal-positive. The two positive clones correspond to GRIP PDZ domains 4, 5, and 6. (*B*) Yeast two-hybrid analysis establishes selective binding of GRIP's PDZ domain 6 to SR. (*C* and *D*) Yeast two-hybrid analysis was used to determine which region of SR interacts with GRIP. The last amino acid of SR, Val-339, was mutated to glycine, which abolishes the interaction between SR and GRIP. (*E*) SR(WT) interacts with GRIP in HEK-293 cells, but SR(V339G) does not bind to GRIP. GRIP was coimmunoprecipitated from mouse brain with SR antibody. (*F*) Colocalization of GRIP and GFAP, a glial marker, in mouse brain. (*G*) Colocalization of GRIP, GluR2/3, and SR with GFAP. Primary mixed glial cultures were double-labeled by using anti-GRIP, anti-GluR2/3, anti-SR, and anti-GFAP. GRIP, SR, GluR2/3, and GRIP expression in primary glial culture is also shown by Western blot analysis.

Granule Cell Migration on SR-Transfected HEK-293 Cells. The explant cultures were placed on top of HEK-293 cells transfected with SR(WT), SR(K56G), and empty vector. HEK-293 cells were grown on poly-D-lysine-coated glass coverslips and cultured in 37°C, 5% CO_2 incubator for 2 days. Cultures were fixed with 4% paraformaldehyde in PBS for 5 min, permeabilized with 0.5% Triton X-100–PBS for 10 min, and blocked with 10% FBS–PBS for 10 min. Rabbit anti-NeuN antibody (1:1,000; Chemicon) diluted in 1% BSA–PBS was incubated with cultures at 4°C overnight. Rabbit secondary antibodies (Texas red-conjugated anti-rabbit) were used at 1:125 dilution for 1 h at 37°C. Explants were washed for 10 min (three times) with BSA–PBS after primary and secondary incubations. Granule cell migration was visualized and measured with a confocal microscope.

Calcium Imaging. Calcium imaging of spontaneous calcium transients was performed on cerebellar explant cultures after 2–3 days *in vitro*. The explants were loaded with calcium indicator

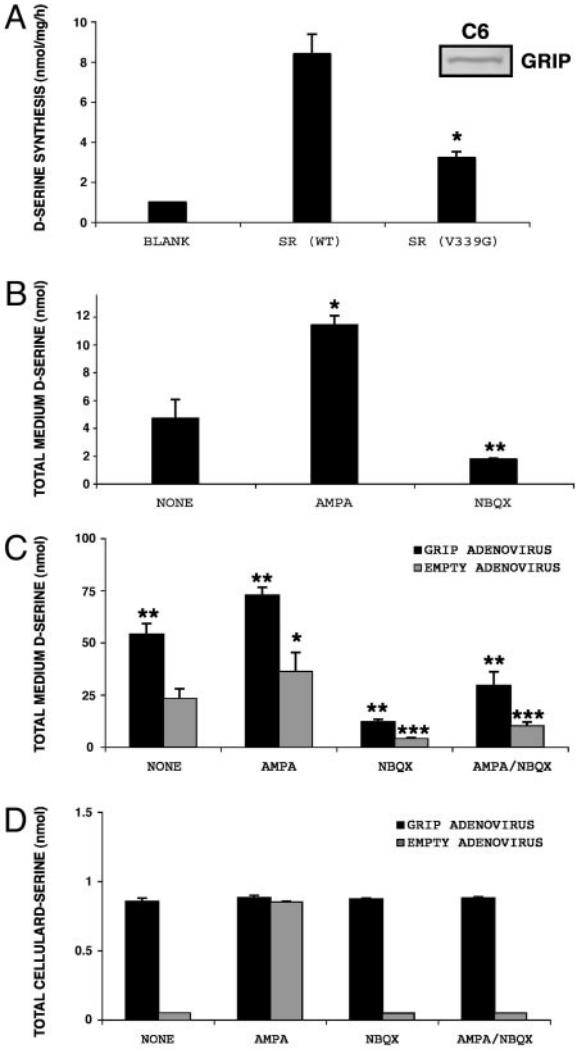

Fig. 2. SR interaction with GRIP increases D-serine synthesis and release. (*A*) Incubation of SR(WT)-transfected cell lysate with L-serine markedly augments D-serine synthesis, whereas incubation with cell lysate transfected with SR(V339G), which does not bind to GRIP, provides 65% less D-serine formation compared with SR(WT)-transfected cell lysate; *, $P < 0.005$. (*Inset*) C6 glioma cells express GRIP. (*B*) AMPA stimulation elicits 2.5 times more D-serine release than control; *, $P < 0.005$. D-serine release in control samples reflects endogenous AMPA receptor activation, as it is markedly reduced by the AMPA receptor antagonist NBQX; **, $P < 0.001$. (*C*) All GRIP virus-infected cultures display two to three times more D-serine release in the media than empty virus-infected cultures; **, $P < 0.001$. AMPA stimulates and NBQX inhibits D-serine release in GRIP and empty virus-infected cultures; *, $P < 0.005$ and ***, $P < 0.0005$. (*D*) With empty adenovirus, AMPA treatment increases cellular D-serine >10-fold, an effect blocked by NBQX. GRIP infection increases D-serine levels to the same values in all conditions. D-serine levels for control, NBQX-, and AMPA/NBQX-treated cell lysates were undetectable.

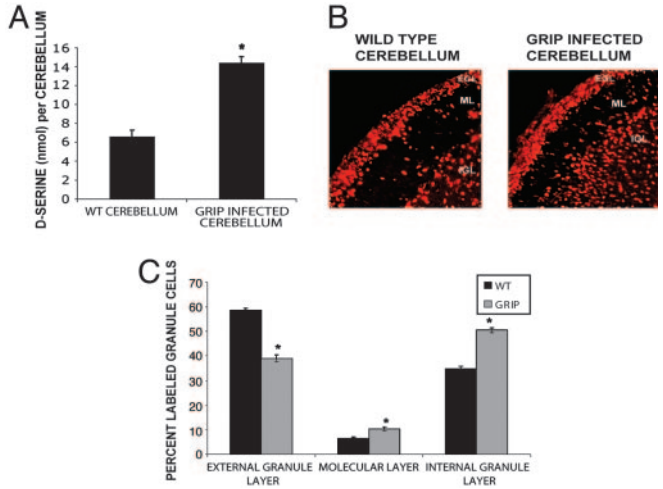

Fig. 3. GRIP augments D-serine cerebellar concentrations in intact mice and accelerates cerebellar granule cell migration. (*A*) GRIP adenoviral infection in P11 mice increases cerebellar D-serine levels. GRIP PDZ-6 adenovirus-infected cerebellum displays a 2-fold increase in D-serine levels compared with the empty adenoviral-infected cerebellum; *, $P < 0.001$. (*B*) BrdUrd staining of P11 cerebellum infected with WT and GRIP adenovirus. BrdUrd-labeled granule cells are stained and can be observed in the EGL, ML, and IGL. (*C*) There are two times more granule cells in the ML and 30% more granule cells in the IGL for GRIP-infected cerebellum compared with WT virus-infected cerebellum; *, $P < 0.001$. Granule cell density in the EGL is correspondingly reduced; *, $P < 0.001$.

Fura-2AM (Molecular Probes) by incubating in DMEM containing 2.5 μM Fura-2AM for 15–30 min at 37°C. The coverslips were washed (four times) with fresh DMEM and incubated at 37°C for 30 min to allow for deesterification of the dye. The coverslips were then mounted onto an Olympus BX51WI microscope stage and continuously perfused with ACSF at RT. Et-Phen stock solution was made fresh in ACSF. Images were obtained by using a ×60 LUMPlanFl objective (Olympus, Melville, NY, numerical aperture 0.90), Fura filter set (Chroma Technology, Rockingham, VT), and a charge-coupled device digital camera (C4749-98, Hamamatsu, Middlesex, NJ). Fluorescence images were obtained only for the 380-nm excitation wavelength to minimize UV exposure. Acquisition rates varied from 0.5 to 0.16 Hz, and exposure times ranged from 20 to 30 ms. Fluorescence images were analyzed by using OPENLAB software. Fluorescence data were normalized to baseline Fura 380 fluorescence levels and corrected for background. To ensure that fluorescence changes observed were not influenced by potential dye quenching, some explants were loaded with the calcium indicator Fluo-3AM (Molecular Probes) exactly as described for Fura-2AM, and a standard FITC filter cube set was used to acquire images. Data shown are with Fura-2AM.

Results

SR and GRIP Binding Interactions and Colocalization. Yeast two-hybrid analysis of full-length SR identifies GRIP containing PDZ domains 4, 5, and 6 as its interacting protein (Fig. 1*A*), and SR binds selectively to PDZ-6 but not PDZ-4 or PDZ-5 (Fig. 1*B*). PDZ domains interact with the three carboxyl-terminal amino acids of the partner proteins with a well characterized consensus sequence (10). The carboxyl-terminal portion of SR, -V-S-V, fits the PDZ domain binding ligand consensus. Yeast two-hybrid analysis of various portions of SR reveals that GRIP binds solely to the extreme carboxyl-terminal portion of SR (Fig. 1*C*). Mutation to glycine of the carboxyl-terminal valine (V339) of SR abolishes interactions with GRIP (Fig. 1*D*). Thus, like other interactors with PDZ domains, SR binds to GRIP via its carboxyl terminus. GST-tagged SR, transfected into HEK-293 cells, binds to endogenous GRIP, whereas GST-tagged SR(V339G) does not bind GRIP (Fig. 1*E*). These interactions also occur *in vivo*, as GRIP and SR coimmunoprecipitate from mouse brain extracts.

For GRIP, AMPA receptors, and SR to interact, they should exist in the same cells. AMPA receptors have been demonstrated in astrocytes biochemically and functionally, and GRIP has been identified in glia (11, 12). Our immunohistochemical staining of mouse brain and primary cultures of mixed glia confirms GRIP's localization to astrocytes, whose identity is verified by staining with GFAP (Fig. 1 *F* and *G*). Primary astrocytic cultures stain for the AMPA receptor subtypes GluR2 and GluR3 as well as SR.

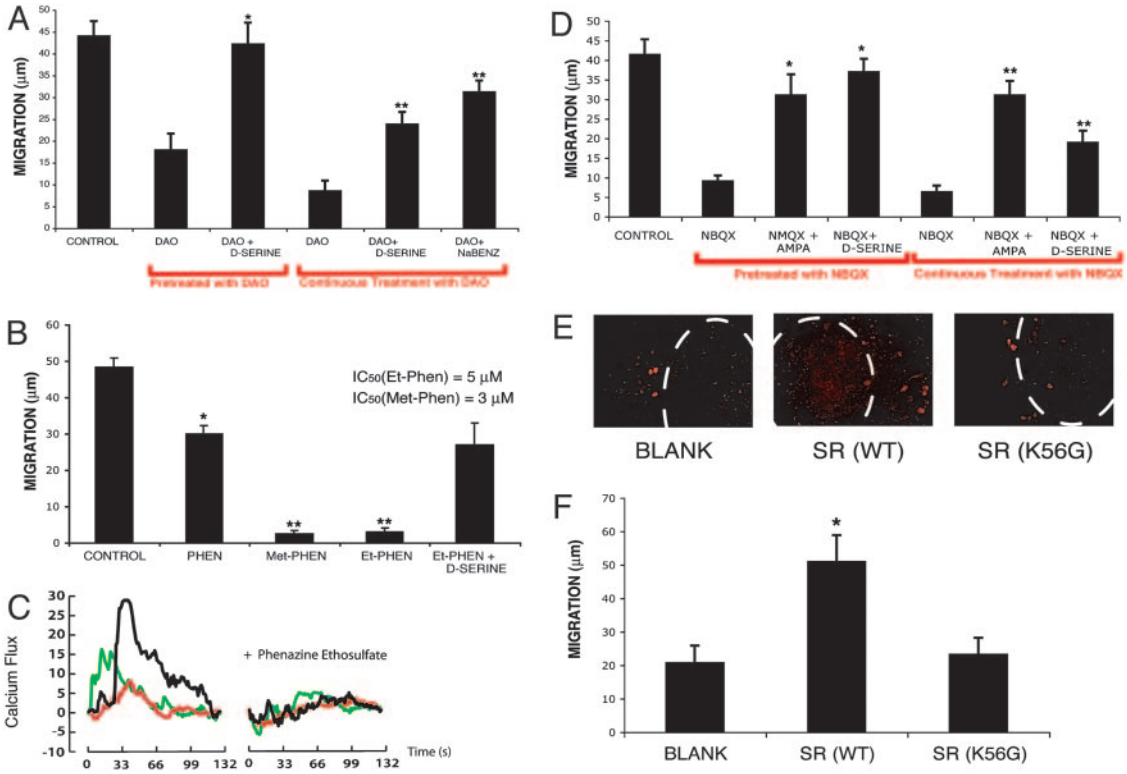

Fig. 4. D-serine increases neuronal migration.. (*A*) DAO and SR inhibitors decrease granule cell migration. Cerebellar slices were pretreated with DAO. When DAO is removed during the migration period, D-serine increases the granule cell migration distance by 2.5 times that of untreated slices; *, $P < 0.005$. Similar results were obtained for continuous treatment with DAO (**, $P < 0.005$) compared with DAO alone. Thus, D-serine or sodium benzoate (NaBENZ) rescue the granule cell migration from DAO's inhibitory effect. (*B*) Met-Phen and Et-Phen, inhibitors of SR block granule cell migration are shown; **, $P < 0.005$. Phen inhibits activity of SR only very weakly and exerts modest inhibitory effects on granule cell migration (*, $P < 0.05$) compared with control. Et-Phen's effects on granule cell migration are reversed by adding D-serine. (*C*) Inhibiting SR blocks granule cell calcium transients. Representative traces of spontaneous calcium transients in granule cells (3 days *in vitro*) that are inhibited by Et-Phen are shown. (*D*) AMPA receptor blockade by NBQX prevents granule cell migration. When NBQX was removed, granule cell migration could be rescued by adding AMPA or D-serine to the media (*, $P < 0.005$) compared with NBQX alone. Similar results were obtained with continuous treatment with NBQX during the migration period (**, $P < 0.005$) compared with NBQX alone. (*E*) D-serine increases granule cell migration in cerebellar explant cultures. Granule cells on HEK-293 cells transfected with empty plasmid and SR(K56G), which does not bind to pyridoxal 5′phosphate, do not migrate far from the cerebellar explant, whose border is shown by a dashed line. In SR(WT)-transfected condition, granule cells migrate further than control or SR(K56G) cells. Granule cells were stained with NeuN, a neuron specific marker. (*F*) Granule cells ($n = 30$) with SR(WT) transfection migrate more than twice the distance of control and SR(K56G) mutant preparations (*, $P < 0.005$).

Western blot analysis also reveals coexpression of GRIP, SR, GluR2/3, and GFAP in astrocytes (Fig. 1*G*).

AMPA Receptor Activation Stimulates SR and Releases D-Serine via GRIP. We used C6 glioma cells, a continuous cell line of astrocytes, to examine the influence of GRIP on SR activity. We transfected C6 cells, which contain endogenous GRIP, either with SR(WT) or SR(V339G) and monitored conversion of L-serine to D-serine in the transfected cell lysates (Fig. 2*A*). D-serine formation is reduced 65% in cells transfected with SR(V339G), suggesting that the enzyme activity depends on interactions of SR with GRIP.

To determine whether GRIP's known interactions with AMPA receptors regulate SR, we treated primary glial cultures with AMPA (Fig. 2*B*). In the absence of AMPA stimulation, levels of D-serine are ≈50-fold higher in the medium than in the cells, suggesting an ongoing release process. AMPA treatment triples the release of D-serine into the medium. Treatment with the AMPA receptor antagonist NBQX markedly reduces these levels, indicating that basal AMPA receptor activation by endogenous glutamate contributes to basal levels of D-serine.

To examine the role of GRIP in AMPA receptor-mediated D-serine release, we infected primary glial cultures with an adenovirus containing GRIP PDZ-6 with a bicistronic GFP gene. We were unable to compare effects of PDZ-6 with other PDZ domains of GRIP, because PDZ-4 and PDZ-5 bind directly to the receptor (13) and would cause interfering dominant negative effects, whereas PDZ-1, PDZ-2, PDZ-3, and PDZ-7 bind to other domains (14) that would interfere with SR studies. Virtually all cells are positive for GFP, indicating quantitative infection. With empty viral infection, D-serine levels in glial cells are only a few percent of levels in the medium. Thus, there is minimal storage of D-serine in glia with newly synthesized D-serine essentially all released into the medium (Fig. 2 *C* and *D*). NBQX treatment reduces basal D-serine levels in the medium by 80%, establishing that basal D-serine release is regulated by AMPA receptor activation (Fig. 2*C*). AMPA treatment elicits release of D-serine, which is prevented by NBQX. The ability of NBQX to block the AMPA–GRIP release of D-serine fits with the notion that AMPA receptor acts via GRIP to augment D-serine release. Under all GRIP viral infection conditions, D-serine release is twice that of empty viral infection, demonstrating that GRIP regulates D-serine formation and release in physiologic glial preparations. D-serine levels within glial cells are also markedly enhanced by GRIP viral infection with similar levels in control, AMPA-treated, NBQX-treated, and AMPA/NBQX-treated cells (Fig. 2*D*). In the cells with empty viral infection, only AMPA stimulation provides detectable levels of D-serine. Presumably, activation of SR by infected GRIP augments intracellular D-serine levels to the maximal amounts that can be maintained even in the

absence of AMPA treatment. Thus, AMPA receptor activation is crucial for D-serine release, and GRIP's binding to SR physiologically activates D-serine synthesis.

Viral Infection with GRIP Stimulates Neuronal Migration. Neuronal migration during development occurs along the processes of radial glia, which is best exemplified in the cerebellum. Migration of cerebellar granule cells from the EGL to the IGL uses Bergmann glia as a scaffold and is blocked by antagonists of the site of NMDA receptors that binds D-serine/glycine (7). We previously showed that SR is localized to Bergmann glia (5). To test the physiologic significance of SR and GRIP interactions, we explored granule cell migration along Bergmann glia. We infected P8 mice with WT and GRIP PDZ-6 adenovirus, which preferentially infects glial cells. Three days after viral infection, D-serine levels in GRIP adenovirus-infected cerebellum were twice those of WT adenovirus-infected cerebellum (Fig. 3*A*).

We labeled granule cells with BrdUrd to observe the influence of GRIP-SR interactions on granule cell migration (Fig. 3 *B* and *C*). In GRIP adenovirus-infected mice, the proportion of labeled cells is markedly reduced in EGL and comparably increased in the molecular layer (ML) and IGL, consistent with migration from EGL toward IGL.

Granule Cell Migration in Slices Requires D-Serine and Involves a Chemokinetic Mechanism. We used two strategies to demonstrate that D-serine is selectively required for cerebellar granule cell migration, one involving its degradation by DAO and selective inhibition of SR. DAO was identified by Krebs (15) as an enzyme that degrades D- but not L-amino acids. Under physiologic conditions, DAO selectively degrades D-serine (3). DAO treatment markedly reduces NMDA neurotransmission in brain cultures and slices, establishing D-serine as an endogenous ligand mediating NMDA neurotransmission (3). To ascertain a role for D-serine in granule cell migration, we treated mouse cerebellar slices at P10 with DAO under conditions in which we demonstrate major degradation of D-serine but no loss of glycine (data not shown). DAO treatment reduces granule cell migration ≈60% (Fig. 4*A*). Because DAO generates H_2O_2 that can be cytotoxic, we examined the reversibility of inhibition by treating preparations with D-serine, which restores migration. Similar effects of DAO and reversibility by D-serine are observed whether or not DAO is maintained in contact with slices during the 4 h in which granule cell migration is monitored. As an additional test of specificity, we treated slices with sodium benzoate, a DAO inhibitor (16), which reverses effects of DAO on granule cell migration.

We also lowered D-serine levels by treating slices with SR inhibitors (Fig. 4*B*). In assays of catalytic activity (17), Et-Phen and Met-Phen potently inhibit the enzyme with IC_{50} values of 5 and 3 μM, respectively, whereas Phen itself fails to inhibit enzyme activity at 100 μM, as observed also by others (C. Rojas, personal communication). Et-Phen and Met-Phen profoundly inhibit granule cell migration, whereas Phen elicits only a modest effect. The effect of Et-Phen is reduced by D-serine, ensuring that the drugs' influence on migration does not involve irreversible cytotoxic actions.

Granule cell migration depends on augmented intracellular calcium primarily derived from influx in response to NMDA receptor activation (9). We imaged intracellular calcium in migrating granule cell neurons of P2 mouse cerebellar explants (Fig. 4*C*). Treatment with Et-Phen markedly diminishes intracellular calcium, and this effect is reversed by removal of the drug (data not shown).

In mediating NMDA neurotransmission, D-serine is generated after activation by glutamate of AMPA receptors on astrocytes (4). Komuro and Rakic (7) did not find influences of 10 μM concentrations of the AMPA antagonist CNQX (6-cyano-7-nitroquinoxaline-2–3-dione) upon granule cell migration. We examined migration in the presence of the more potent AMPA antagonist NBQX at a higher concentration, 100 μM (Fig. 4*D*) (18). Presumably, 100 μM NBQX blocks AMPA receptors more completely than the 10 μM CNQX. The specificity of NBQX is evident by its reversal with added AMPA or D-serine. Drug effects are similar whether NBQX is continuously exposed to the granule cells during the 4 h of migration or removed before migration.

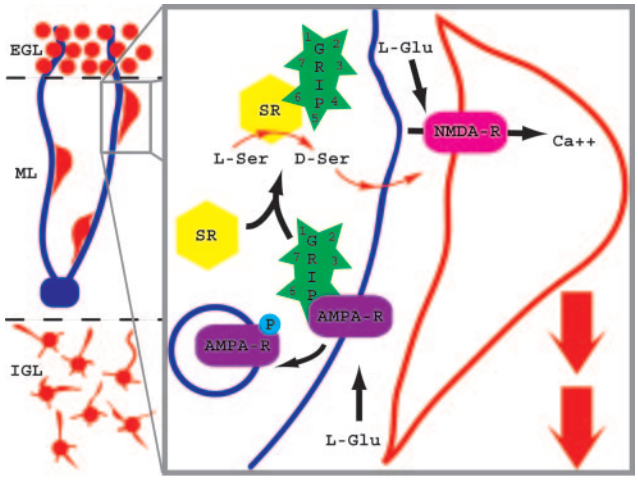

Fig. 5. Model for D-serine and glutamate regulation of cerebellar granule cell migration. Glutamate activates AMPA receptors (AMPA-R) on Bergmann glia, leading to receptor phosphorylation and dissociation of receptor-bound GRIP, which then binds to SR. GRIP activates SR with release of newly formed D-serine to join glutamate in stimulating NMDA receptor (NMDA-R) on granule cells, releasing intracellular calcium and facilitating migration from the EGL to the IGL.

How does D-serine influence granule cell migration? One possibility would be a chemokinetic influence on granule cells by increasing the activation of NMDA receptors. To examine this possibility, we evaluated granule cell migration in P2 cerebellar explants overlying HEK-293 cells transfected with SR, SR(K56G), or an empty vector (Fig. 4 *E* and *F*). In SR(K56G), the catalytic lysine is mutated to glycine, resulting in complete inactivation of the enzyme (19). Granule cell migration is markedly greater in preparations overlying SR-containing HEK-293 cells and over those with SR(K56G) or empty vector. Thus, D-serine appears to be a chemokinetic stimulus to granule cells.

Discussion

In the present study, we demonstrate a major role for GRIP in mediating activation by AMPA receptor neurotransmission of SR and D-serine release, findings consistent with the following model (Fig. 5). Released glutamate stimulates AMPA receptors on closely adjacent protoplasmic astrocytes, causing GRIP to bind to SR, enhancing the formation and release of D-serine. Our results suggest that GRIP, AMPA receptor, and SR do not form a ternary complex because the augmentation of D-serine release associated with GRIP transfection is the same in either the presence or absence of AMPA treatment. If GRIP enhances SR by linking AMPA receptors and SR, then SR should be stimulated by GRIP much more in the presence than in the absence of AMPA receptor activation. AMPA receptor activation triggers phosphorylation of the receptor at Ser-880, causing dissociation of GRIP (20) Such dissociated GRIP is presumably responsible for the physiological activation SR.

Activation of SR in our experiments led to much higher levels of D-serine in the media than the cells. This finding suggests some link between SR formation of D-serine and its release. Previously, we showed that AMPA receptor activation releases [^{3}H]D-serine from type II astrocytes labeled by the accumulation of the [^{3}H]amino acid (4). It is not clear whether accumulated [^{3}H]D-serine in those studies labeled endogenous stores with AMPA-

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induced release coupled to augmented synthesis. We do not know how D-serine is physiologically released, whether by a reversed transport mechanism or a vesicular process.

Calcium can stimulate recombinant SR purified from *Escherichia coli* (21), an action that may reflect endogenous regulation of SR by magnesium and ATP (17). We have examined the influence of calcium (0.1 mM) on SR activity in the presence of GRIP, but did not detect any change in catalytic activity (data not shown).

The regulation of SR by GRIP and its influence on D-serine release may have clinical implications. In vascular stroke, large amounts of glutamate are released and an impact on NMDA receptors is thought to be pathophysiologic, because drugs that block the glycine/D-serine site as well as the glutamate site of NMDA receptors reverse stroke damage (22). Conceivably, drugs that block the binding of GRIP to SR may also be therapeutic. Regulation of D-serine formation and release by GRIP may also be relevant to psychiatric disorders. Multiple recent studies have linked the gene for DAO to both schizophrenia and bipolar illness (23, 24). The gene for a protein designated G72, which augments DAO activity (23), is also implicated in these two psychiatric disorders. Additional evidence relating D-serine to schizophrenia includes the psychotomimetic effects of phencyclidine and other drugs that block NMDA receptors (25). The psychotomimetic actions of phencyclidine resemble schizophrenic symptoms more than those elicited by any other psychotogen. Additionally, oral administration of D-serine, D-cycloserine, and glycine alleviates schizophrenic symptoms in patients (26, 27). Thus, drugs that facilitate D-serine release by influencing interactions with GRIP might have therapeutic utility in schizophrenia.

We have also established a major physiologic role for D-serine and AMPA receptor–GRIP–SR interactions in neuronal migration. Thus, granule cell migration along Bergmann glia is blocked by degradation of D-serine or SR inhibition. The migration appears to involve GRIP influences on SR, because GRIP viral infection of intact mice augments granule cell migration. D-serine appears to influence granule cell migration in a chemokinetic mechanism, as migration in cerebellar explants is augmented by the application of HEK-293 cells transfected with active SR but not with catalytically inactive SR. Although the D-serine that activates granule cells derives from Bergmann glia, we don't know the source of the requisite glutamate that has been suggested to arise from parallel fibers of granule cells that have already reached the IGL (28).

D-serine may play different roles in the adult than in development, which may be mirrored in distinctive localizations at different ages. In most parts of the brain, D-serine's histochemical localizations match those of NMDA receptors much more closely than do those of glycine, consistent with a role for D-serine as the principal endogenous agonist for NMDA receptors, especially in the hippocampus where selective degradation of D-serine profoundly reduces NMDA neurotransmission (3). By contrast, in the adult cerebellum D-serine levels are very low, and localizations of glycine match those of NMDA receptors better than D-serine, suggesting that in the cerebellum glycine may be the principal endogenous agonist in the adult (29). In the neonatal cerebellum, however, D-serine levels are high, peaking at the time of granule cell migration, which suggests that the principal role of D-serine in the developing cerebellum is to serve as a coagonist for NMDA receptor-dependent granule cell migration.

Although multiple investigations support a role for NMDA receptors in neuronal migration (6, 7, 9, 30–33), a recent study (34) reported that NMDA receptor subtype I-deficient cerebellar granule cells can attain normal localization in intact mice. However, that study did not examine whether NMDA receptors influenced the rate of migration, as implied by our own and other studies.

Developing neurons do not make conventional synaptic connections, so one might not anticipate regulation of migration to involve neurotransmitters. Our findings, together with the earlier studies of Komuro and Rakic (6, 7, 9), establish a major role for glutamate and D-serine in migration, which does not use conventional synaptic transmission but instead uses glial release of D-serine as a neuromodulator. Our evidence that D-serine acts as a chemokinetic stimulant for granule cells reflects a novel action for neurotransmitter/neuromodulator substances.

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