Glia-Derived D-Serine Controls NMDA Receptor Activity and Synaptic Memory

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SUMMARY

The NMDA receptor is a key player in excitatory transmission and synaptic plasticity in the central nervous system. Its activation requires the binding of both glutamate and a coagonist like D-serine to its glycine site. As D-serine is released exclusively by astrocytes, we studied the physiological impact of the glial environment on NMDA receptor-dependent activity and plasticity. To this end, we took advantage of the changing astrocytic ensheathing of neurons occurring in the supraoptic nucleus during lactation. We provide direct evidence that in this hypothalamic structure the endogenous coagonist of NMDA receptors is D-serine and not glycine. Consequently, the degree of astrocytic coverage of neurons governs the level of glycine site occupancy on the NMDA receptor, thereby affecting their availability for activation and thus the activity dependence of long-term synaptic changes. Such a contribution of astrocytes to synaptic metaplasticity fuels the emerging concept that astrocytes are dynamic partners of brain signaling.

INTRODUCTION

There is strong evidence for reciprocal communication between neurons and glia (Fields and Stevens-Graham, 2002; Haydon, 2001; Volterra and Meldolesi, 2005). Astrocytes detect changes in the activity of their adjacent neurons through ion channels, receptors, and transporters expressed on their surface and, in turn, respond by releasing a variety of neuroactive substances known as gliotransmitters. Although the transmitters mediating such glia-to-neuron signaling are generally considered to be glutamate (Angulo et al., 2004; Fellin et al., 2004) or ATP (Gordon et al., 2005; Newman, 2003; Zhang et al., 2003), it is now obvious that another important gliotransmitter is the amino acid D-serine (Mothet et al., 2000), which serves as a ligand at the strychnine-insensitive glycine site of NMDA receptors (NMDARs; Johnson and Ascher, 1987). D-serine is synthesized in astrocytes (Stevens et al., 2003; Wolosker et al., 1999) and is released by glutamate receptor stimulation through a calcium- and SNARE-dependent exocytotic pathway (Mothet et al., 2005). Its high degree of colocalization with NMDARs (Schell et al., 1995, 1997), the reduction of NMDA-mediated responses, and the abolishment of NMDA-induced neurotoxicity in brain slices and cell cultures following its enzymatic degradation (Mothet et al., 2000; Stevens et al., 2003; Yang et al., 2003; Shleper et al., 2005) has led to the conclusion that D-serine is an endogenous ligand for NMDARs in the brain.

In many brain areas, astrocytic processes are closely apposed to synapses, including those utilizing glutamate as a neurotransmitter. Because of this anatomical relationship, it is highly likely that they can influence, via the release of D-serine, NMDAR activity and NMDAR-dependent processes, including glutamatergic transmission, regenerative firing, and synaptic plasticity. To address this issue in a physiological context, we took advantage of the hypothalamic supraoptic nucleus (SON), a homogeneous neuronal center composed of oxytocin (OT) and vasopressin (VP) neurons, known to undergo an extensive reduction of astrocytic ensheathing of its neurons and synapses under conditions like lactation (Hatton, 1997; Theodosis and Poulain, 1993). We previously showed that this neuronal-glial remodeling modified glutamate clearance and diffusion, thereby affecting synaptic efficacy at glutamatergic and adjacent GABAergic synapses (Oliet et al., 2001; Piet et al., 2004).

Using different experimental approaches, including specific enzymatic degradation, we here demonstrate that there are high levels of D-serine in the rat SON and that it is the only endogenous ligand of NMDARs in this area of the hypothalamus. By comparing NMDAR activity in SON neurons of virgin and lactating animals, we show that D-serine concentrations within the synaptic cleft, and thus the level of occupancy of the glycine site at synaptic NMDARs, depend on the degree of astrocytic coverage of synapses. This has direct consequences on long-term
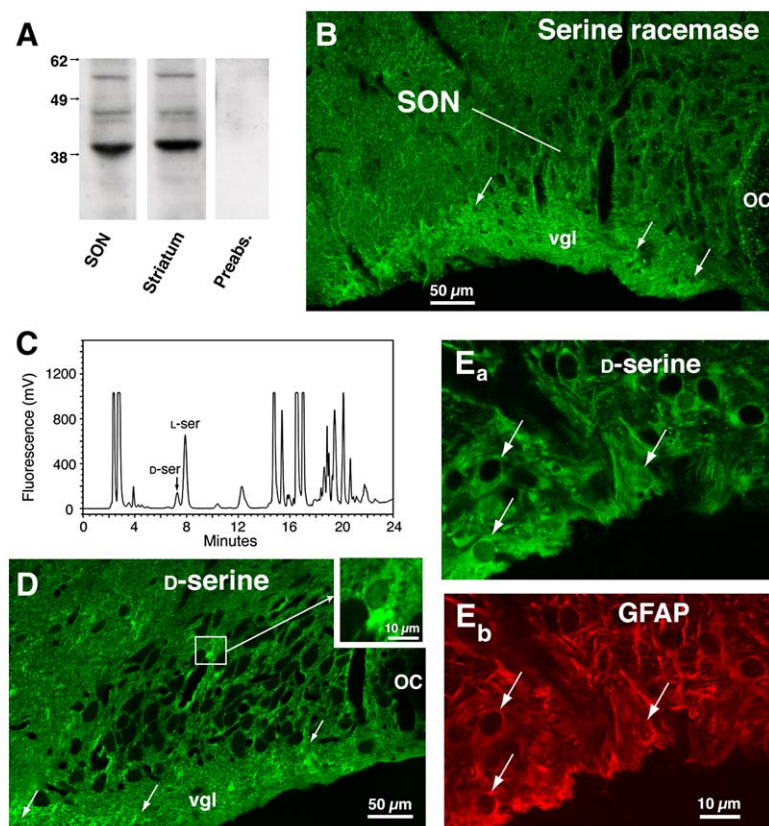
Figure 1. Localization of Serine Racemase and D-Serine in the Rat SON

(A) Immunoblotting of SON extracts revealed high levels of serine racemase, as in control extracts of striatum. Molecular weights are indicated on the left. All reaction disappeared in extracts when the antibody was preabsorbed with blocking peptide (preabs.).

(B) Immunofluorescence revealed a strong reaction for serine racemase throughout the SON, localized in large processes along blood vessels and in the neuropile surrounding immunonegative magnocellular somata. The reaction was particularly high in the ventral limits of the nucleus (vgl) where cell bodies of SON astrocytes (arrows) accumulate.

(C) Detection of D- and L-serine (D-ser and L-ser, respectively) in SON extracts with HPLC. Retention times for D- and L-serine were 6.802 ± 0.1455 min and 7.248 ± 0.143 min, respectively.

(D) Immunofluorescence for D-serine showed a similar distribution to that of serine racemase. Strong labeling was visible in all astrocytes, in radial types with cell bodies in the vgl (arrows), and in stellate ones scattered in the nucleus proper (and at higher magnification in the inset). Double immunofluorescence for D-serine (Ea) and the astrocytic marker GFAP (Eb) confirmed the presence of the amino acid in cell bodies (arrows) and processes of all SON astrocytes. oc, optic chiasma.

synaptic plasticity whose dependence on neuronal activity is a function of the number of NMDARs activated during the induction period. Our findings thus provide a clear example of metaplasticity of synaptic transmission mediated by astrocytes through the release of D-serine.

RESULTS

D-Serine Is Synthesized by SON Astrocytes

We used several approaches to analyze the presence of D-serine in the rat SON. First, immunoblot analysis revealed that this hypothalamic nucleus is enriched in serine racemase, the enzyme converting L-serine to D-serine (Wolosker et al., 1999). Serine racemase levels in SON extracts were similar to those in the striatum, another D-serine enriched-region (Hashimoto and Oka, 1997; Figure 1A). Secondly, immunocytochemistry showed specific immunolabeling for serine racemase in all astrocytic cell bodies and processes throughout the nucleus (Figure 1B). Third, HPLC analyses revealed D-serine levels in the SON (30.58 ± 3.54 nmol/mg; n = 4) equivalent to those measured in the striatum, with a ratio of D- to L-serine of 0.201 (Figure 1C). Finally, single and double immunolabelings clearly showed that D-serine immunoreactivity occurred in all SON astrocytes, in a manner similar to that for serine racemase (Figures 1D and 1E), whereas it was absent from oxytocinergic and vasopressinergic somata

and fibers (see Figure S1 in the Supplemental Data available with this article online).

D-Serine Is the Endogenous Ligand of NMDARs in the SON

SON neurons are driven by excitatory glutamatergic afferents, acting via postsynaptic AMPARs and NMDARs (Stern et al., 1999). To determine the contribution of NMDARs to evoked excitatory postsynaptic currents (EPSCs), we first evaluated the AMPA/NMDA ratio in slices from virgin rats, where astrocytic coverage of SON neurons and their synapses is maximal (Hatton, 1997; Theodosis and Poulain, 1993; Figures 2A and 2B). If D-serine is the endogenous coagonist of NMDARs, its specific enzymatic degradation by D-amino acid oxidase (DAAO; 0.17 U/ml; D'Aniello et al., 1993) should reduce the amplitude of NMDAR-mediated EPSCs (Mothet et al., 2000; Stevens et al., 2003; Yang et al., 2003). As seen with whole-cell patch clamp recordings of SON neurons, the NMDA component of the EPSCs was reduced significantly in DAAO-treated slices, with an AMPA/NMDA ratio (4.38 ± 0.85; n = 9) larger than that measured in the absence of the enzyme (1.7 ± 0.4; n = 31; $p < 0.05$; Figures 2A and 2B). Such a reduction was not due to a deleterious action of D-serine oxidation byproducts on NMDARs since acute application of D-serine (100 μM) restored the AMPA/NMDA ratio to values measured in the absence of the enzyme (1.98 ± 0.69; n = 6), indicating that NMDARs
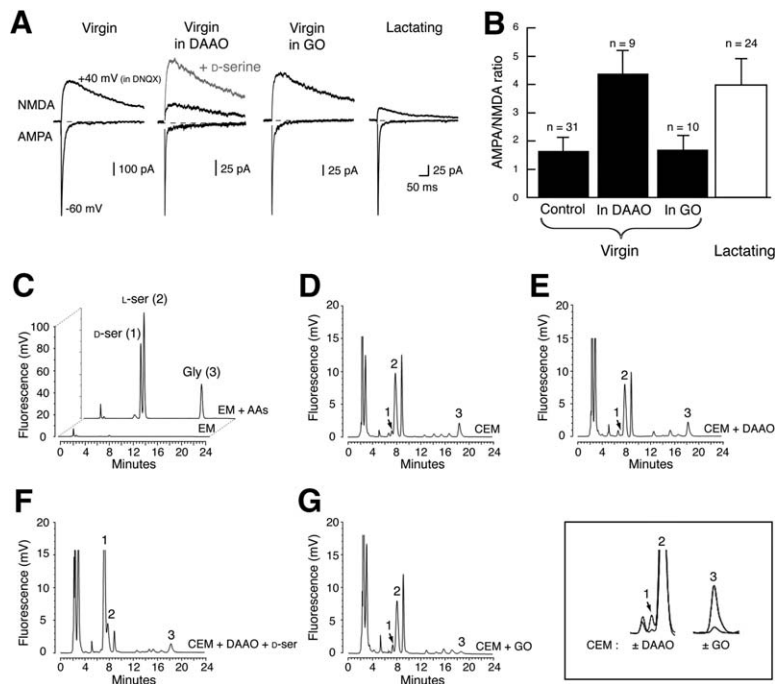
Figure 2. D-Serine Is the Endogenous Coagonist of Synaptic NMDARs in the SON

(A) Example of the contribution of AMPAR and NMDAR to evoked EPSCs and corresponding summary histogram of the AMPA/NMDA ratio (B) in SON neurons of virgin and lactating rats and after D-amino acid oxidase (DAAO) or glycine oxidase (GO) treatment in virgin rats. All recordings were carried out in the presence of bicuculline. The ratio was obtained by measuring the peak amplitude of AMPAR-mediated EPSCs at −60 mV and of NMDAR-mediated EPSCs at +40 mV in the presence of DNQX. Note that the AMPA/NMDA ratio was enhanced significantly in DAAO-treated-slices from virgin rats and in untreated slices from lactating rats, whereas GO had no effect. Data are reported as means ± SEM.

(C–G) Specificity of DAAO and GO activities. Before slice incubation, the extracellular medium (EM) did not contain any amino acids (AAs; lower trace). D-serine, L-serine, and glycine (D-ser, L-ser, and gly, respectively) were recovered (upper trace) after addition and derivatization of a mixture of standards (100 pmol each) with respective retention times: 7.594 ± 0.029 min, 8.153 ± 0.054 min, and 18.230 ± 0.60 min (C).

(D) On the other hand, extracellular medium exposed to SON slices (conditioned medium, CEM) for 45 min displayed complex chromatogram profiles corresponding to D-serine, L-serine, and glycine. When DAAO was included in the medium (E), ambient levels of D-serine were reduced while L-serine or glycine levels remained unaffected. Addition of 50 pmol D-serine to the DAAO-treated medium confirmed the identity of the D-serine peak (F). When GO was included in the bath (G), ambient levels of glycine were reduced, while those of D- or L-serine were not. The inset illustrates peaks for D-serine, L-serine, and glycine in control and DAAO- or GO-treated conditioned extracellular medium at higher magnification. The chromatograms are representative of at least three different experiments.

were still functional. The possibility remained that the DAAO solution contained a contaminant that could act as a competitive antagonist of the glycine site. To test this hypothesis, we recorded NMDA responses in acutely isolated SON neurons (see Supplemental Experimental Procedures). In the presence of nonsaturating concentrations of glycine (2 μM), DAAO did not significantly affect responses mediated by application of 500 μM NMDA (101.6 ± 7.6% of control; n = 4; Figure S2) thus ruling out an action from a competitive antagonist contaminant.

Interestingly, NMDAR-mediated EPSCs were still detectable in the presence of DAAO. Although this may be due to incomplete degradation of D-serine in slices, in agreement with HPLC analysis that revealed the presence of residual D-serine levels in DAAO-treated slices (28.2 ± 4.6% of control values; n = 4; data shown), it is also possible that glycine, which is a very poor substrate of DAAO (see Experimental Procedures), may act as an endogenous coagonist for a subpopulation of NMDARs in the SON. We therefore examined the effect of glycine oxidase (GO), an enzyme that degrades specifically glycine (Job et al., 2002a; 2002b; Mortl et al., 2004), on NMDAR-mediated EPSCs. After incubation of hypothalamic slices with GO (0.17 U/ml), the AMPA/NMDA ratio was unchanged (1.52 ± 0.41; n = 10; Figures 2A and 2B)

indicating that glycine is not an endogenous ligand of NMDARs in the SON.

To ensure that GO was effectively degrading glycine in our slices and to confirm the specificity of DAAO in degrading D-serine (the kinetic efficiency of DAAO and GO on D-serine and glycine is reported in the Experimental Procedures), we characterized further the activity of these enzymes with HPLC analysis. Neither L-serine, D-serine, nor glycine were detected in the extracellular medium before slice incubation (Figure 2C). On the other hand, significant levels of the three amino acids were detected (D-serine, 43.2 ±13.1 nM; L-serine, 1055.8 ± 228.5 nM; glycine, 223 ± 46.9 nM; n = 3) in the medium bathing slices for 45 min (conditioned extracellular medium; Figure 2D). DAAO degraded specifically most D-serine (6.2 ± 0.5% of control values; n = 3) without affecting significantly glycine (90.0 ± 8.7%; n = 3) or L-serine (95.6 ± 12.7%, n = 3) (Figures 2E and 2F). Conversely, GO reduced glycine concentrations dramatically (9.3 ± 3.4%, n = 3), leaving L- and D-serine levels unaffected (97.4 ± 9.2% and 98.4 ± 8.1%, respectively; n = 3; Figure 2G). Finally, to ensure that GO efficiently degraded glycine within the slices, we performed control recordings in the nucleus of the tractus solitarius (NTS), a brain stem structure where endogenous glycine is likely to play a physiological role (Kubo and Kihara,
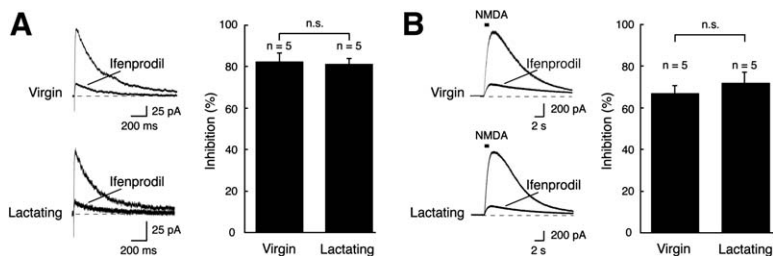
1987; Miazaki et al., 1999). Incubation of brain stem slices (see Supplemental Experimental Procedures) with GO significantly increased the AMPA/NMDA ratio measured in NTS neurons (from 2.1 ± 0.2, n = 11, to 3.3 ± 0.3, n = 8; p < 0.05; Figure S3), indicating that ambient glycine modulates NMDARs in the NTS. These findings, confirming the high specificity of the two employed flavoproteins (see Experimental Procedures) under the experimental conditions used, show that GO is indeed entering the slices and degrading glycine efficiently, thus demonstrating that D-serine is the major, if not the only, endogenous ligand of NMDARs in the SON.

Astrocytes Control the Activity of Synaptic NMDARs

Since D-serine is released from astrocytes (Mothet et al., 2005; Schell et al., 1995), reduction of glial ensheathment of glutamatergic synapses, as it occurs in the SON of lactating rats (Oliet et al., 2001; Theodosis and Poulain, 1993), should lead to diminished D-serine concentrations in the synaptic cleft and, therefore, to a reduction of NMDAR activity. Indeed, the AMPA/NMDA ratio measured in the SON of lactating rats (4.2 ± 0.9; n = 24) was significantly larger than that measured in the SON of virgin animals (p < 0.05) whereas it was not statistically different from that observed in DAAO-treated virgin slices (p > 0.05; Figures 2A and 2B). Since AMPAR-mediated responses in SON neurons are not altered by lactation (Oliet et al., 2001; Stern et al., 2000), such a change in the ratio implies a change in NMDAR-mediated EPSCs.

Because different NR2 subunits confer distinct biophysical properties to NMDARs (Laurie and Seeburg, 1994), we then tested whether the change in AMPA/NMDA ratio during lactation was due to a switch in NMDAR subunits. This was done by comparing the kinetics of NMDAR-mediated EPSCs and by assessing the effect of the selective NR2B antagonist, ifenprodil (Williams, 1993), on SON neurons from virgin and lactating rats. NMDA EPSC decay measured in lactating rats (n = 18) was not statistically different (p > 0.05) from that measured in virgin animals (n = 25), suggesting a similar subunit composition at synaptic NMDARs. Furthermore, in both groups of animals, ifenprodil (10 μM) reduced most of the evoked NMDAR-mediated EPSC amplitude to the same extent (Figure 3A), as well as the responses elicited by local application of NMDA (50 μM; Figure 3B) that activates both synaptic and extrasynaptic NMDARs. Since the majority of NMDARs on SON neurons of virgin and lactating rats are NR2B-containing receptors, a switch in subunit composi-

tion cannot account for the reduced NMDAR-mediated EPSCs in lactating rats.

If the reduction in NMDAR-mediated EPSCs responses in lactating rats reflects lower concentrations of the endogenous coagonist around synapses, then application of D-serine at saturating concentrations should have a weaker effect on SON neurons in virgin than in lactating rats where it should restore most of NMDA responses. Indeed, D-serine (100 μM) increased the peak amplitude of NMDAR-mediated EPSCs by 27.2 ± 4.4% (n = 10) in SON neurons from virgin rats, thereby diminishing the AMPA/NMDA ratio from 1.14 ± 0.25 to 0.89 ± 0.20, while it greatly enhanced NMDAR-mediated EPSCs in lactating rats (+143.7 ± 40.0%; n = 13), reducing the AMPA/NMDA ratio from 3.50 ± 0.81 to 1.52 ± 0.30, a value similar to that measured in virgin rats (p > 0.05; Figure 4A). These observations indicate, therefore, that a reduced concentration of D-serine in the synaptic cleft is responsible for the impairment of NMDAR-mediated EPSCs and that the level of occupancy of the glycine site of synaptic NMDARs is critically controlled by the extent of astrocytic coverage of the neuron.

It is noteworthy that treatment of hypothalamic slices from lactating rats with DAAO did not affect the AMPA/NMDA ratio significantly (3.65 ± 0.82, n = 5; p > 0.05; data not shown). This ratio is similar to that measured in virgin rats in the presence of DAAO and probably reflects the incomplete degradation within the synaptic cleft of D-serine whose concentration may still be sufficient to activate a small number of NMDARs.

D-Serine Is Released Preferentially at Synapses

NMDARs exist at synaptic and extrasynaptic sites where they ensure specific functions (Hardingham et al., 2002). To assess whether the astrocytic environment regulates the level of occupancy of the glycine site of extrasynaptic and synaptic NMDARs to the same extent, we monitored the activity of all NMDARs by applying puffs of NMDA (50 μM) directly on SON neurons. The responses elicited by these local applications of the agonist and that reflected activation mainly of extrasynaptic NMDARs, were enhanced considerably by D-serine and to a similar extent in slices from virgin (+177.0 ± 29.8%; n = 8) and lactating (+164.1 ± 31.1; n = 12) rats (Figures 4B and 4C). To make sure that puff applications of NMDA did not blow away ambient levels of D-serine and/or glycine, we performed a set of experiments in which NMDA (50 μM) was bath-applied. In the four neurons tested, D-serine increased
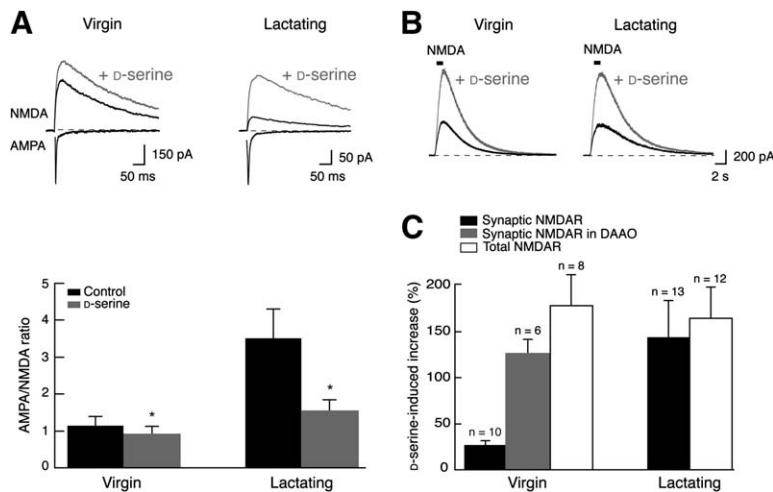
(A) Effect of D-serine application (100 μM) on the AMPA/NMDA ratio in slices from virgin and lactating rats. Top: examples of the AMPA and NMDA components of the evoked EPSCs (black traces) and of the NMDA component during D-serine application (gray traces). Bottom: histogram summarizing mean AMPA/NMDA ratios in the presence (gray bars) and absence (black bars) of D-serine. In the presence of D-serine, the AMPA/NMDA ratio in lactating rats is not statistically different from that measured in virgin slices.
(B) D-serine enhanced the amplitude of responses elicited by local puffs of NMDA (50 μM) in virgin and lactating rats.
(C) Summary histogram showing the increase in amplitude of the NMDA component of evoked EPSCs (filled bars) and of the responses elicited by local NMDA puffs (empty bars) induced by D-serine in virgin and lactating animals. The effect of D-serine on evoked EPSCs in DAAO-treated virgin slices is also illustrated (gray bar). Data are reported as means ± SEM.

NMDA responses by 230.2 ± 40.3% (p < 0.05; Figure S4), a value not different from that obtained with puff applications (p > 0.05). These results indicate that the level of occupancy of the glycine site at extrasynaptic NMDARs is far from saturated and that it is not affected by the relative absence of astrocytic processes. Such a discrepancy in the level of occupancy of the glycine binding site between extrasynaptic and synaptic NMDARs is in accord with observations reported earlier (Mothet et al., 2000) and suggests that D-serine release from astrocytes occurs preferentially at the synapse.

Astrocytes Control Long-Term Synaptic Plasticity
Long-term potentiation (LTP) and long-term depression (LTD) induction relies on Ca^{2+} entry through NMDARs (Malenka and Nicoll, 1999). In view of the active participation of astrocytes in the regulation of synaptic NMDAR activity, we then examined whether such phenomena were sensitive to the glial environment of neurons. In the SON of virgin rats, membrane depolarization to 0 mV paired with afferent stimulation at 2 Hz for 45 s resulted in a significant persistent potentiation of the amplitude of evoked EPSCs (194.1 ± 19.5% of control after 30 min, n = 7; Figure 5A). This LTP required NMDAR activation since it was blocked (102.0 ± 6.8%; n = 7; Figure 5B) by bath application of the NMDAR antagonist, D-AP5 (50 μM). In contrast, the same pairing protocol in SON neurons of lactating rats induced LTD (71.5 ± 13.6%; n = 10; Figure 5C), which was also NMDA-dependent since it was inhibited by D-AP5 (94.0 ± 16.7%; n = 10; Figure 5D). Moreover, LTP switched to LTD (75.3 ± 14.2%; n = 11; Figure 5E) in response to the same pairing protocol in virgin rats when concentrations of D-serine were reduced experimentally by DAAO. Conversely, when D-serine was applied to

slices of lactating rats, this protocol induced LTP (221.6 ± 29.6%; n = 7; Figure 5F). It is obvious then that a diminished concentration of D-serine in the synaptic cleft reduces the number of NMDARs available for activation and, therefore, Ca^{2+} entry in the neurons. This reduced Ca^{2+} entry, while not sufficient to induce LTP, triggers LTD. Conversely, under conditions where NMDARs are fully available for activation, Ca^{2+} entry leads to LTP in both virgin and lactating animals (Figures 5A and 5F). Our findings are thus consistent with those obtained previously in the hippocampus where a protocol normally inducing LTP produced LTD when the number of NMDARs activated during the induction period was attenuated by low concentrations of D-AP5 (Cummings et al., 1996).

Moreover, a stimulation protocol (Oliet et al., 1997) that induced NMDAR-dependent LTD in the SON of virgin animals (56.4 ± 9.0%; n = 11; Figures 6A and 6B) was ineffective in lactating rats (89.2 ± 10.4%; n = 9; Figure 6C), except when D-serine was present in the bathing solution (66.1 ± 7.9%; n = 8; Figure 6D). These findings fit with the Bienenstock, Cooper, and Munro (BCM) model for variation in the threshold for LTP (θ_M; Figure 7A), from which one can predict that reduced D-serine concentrations will shift θ_M toward higher values, thereby requiring more absolute activity to generate LTP (Abraham and Bear, 1996; Bienenstock et al., 1982). This prediction was confirmed using high frequency stimulation (HFS) of afferent inputs that caused NMDAR-dependent LTP in the SON of both virgin (192.5 ± 21.1%; n = 12; Figure 6E) and lactating (165.6 ± 20.5%; n = 14; Figure 6F) rats. It is possible then to potentiate glutamatergic transmission in the SON of lactating rats, provided that synapses are stimulated strongly enough. Such a shift in θ_M (Figures 7A and 7B) reflects a higher order of plasticity of synaptic

Figure 5 (left panels):

A — Virgin — Control — Normalized amplitude (%) — Pairing — n = 7

B — D-AP5 — Normalized amplitude (%) — n = 7

C — Lactating — Control — Normalized amplitude (%) — n = 10

D — D-AP5 — Normalized amplitude (%) — n = 10

E — DAAO — Normalized amplitude (%) — n = 11 — Time (min)

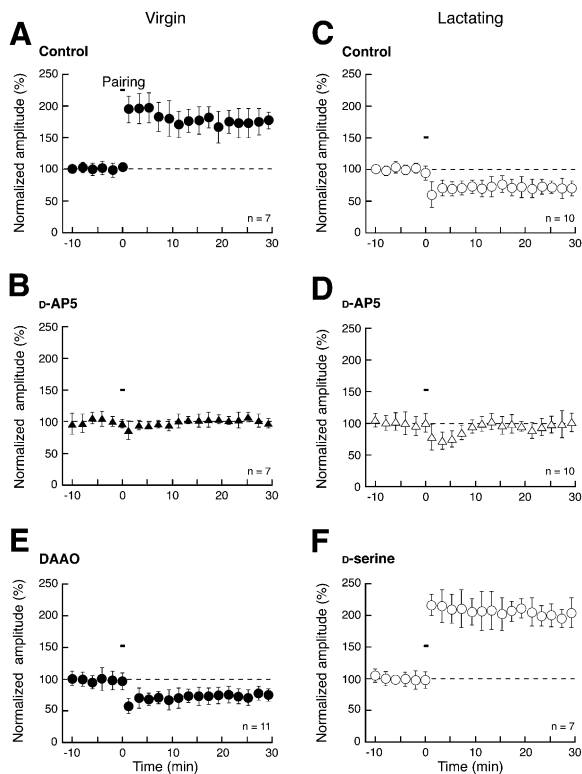F — D-serine — Normalized amplitude (%) — n = 7 — Time (min)

Figure 5. Astrocytes Influence Pairing LTP
(A) Pairing membrane depolarization with afferent input stimulation at 2 Hz for 45 s (bar) potentiated the amplitude of evoked EPSCs in SON neurons of virgin rats.
(B) LTP induction was dependent on NMDAR activation since it was blocked by D-AP5.
(C) In slices from lactating rats, where the NMDA component of the EPSCs was largely reduced, the pairing protocol induced a persistent depression of the evoked EPSC amplitude.
(D) This LTD was also NMDAR dependent since it was blocked by D-AP5.
(E) In slices from virgin rats treated with DAAO to reduce endogenous concentrations of D-serine, a similar LTD was observed.
(F) When all the synaptic NMDA receptors were made available for activation by including D-serine in the solution bathing slices from lactating rats, the pairing protocol induced LTP instead of LTD. Data are reported as means ± SEM.

Figure 6 (right panels):

A — Virgin — Control — Normalized amplitude (%) — 5 Hz/3min — n = 11

B — D-AP5 — Normalized amplitude (%) — n = 11

C — Lactating — Control — Normalized amplitude (%) — n = 9

D — D-serine — Normalized amplitude (%) — n = 8

E — Normalized amplitude (%) — HFS — ● Control ▲ D-AP5 — n = 12 — n = 8 — Time (min)

F — Control — Normalized amplitude (%) — n = 14 — Time (min)

Figure 6. Astrocytic-Dependent Metaplasticity
(A) Stimulating afferent fibers at 5 Hz for 3 min caused LTD in virgin rat SON.
(B) LTD induction required NMDAR activation since it was prevented by D-AP5. In the SON of lactating rats, although LTD generated by the same paradigm of stimulation was largely impaired (C), it was restored by D-serine (D). High-frequency stimulation (HFS) induced NMDAR-dependent LTP in the SON of both virgin (E) and lactating (F) rats. Data are reported as means ± SEM.

transmission, also referred to as metaplasticity (Abraham and Bear, 1996).

DISCUSSION

Data accumulated over the last 10 years have clearly established that astrocytes, in addition to scaffolding and metabolic functions, contribute to the control of synaptic transmission (Araque et al., 1999; Fields and Stevens-Graham, 2002; Haydon, 2001; Volterra and Meldolesi, 2005). We ourselves demonstrated that astrocytes in the SON regulate synaptic strength by controlling glutamate concentration and diffusion in the extracellular space, thereby affecting the activity of presynaptic metabotropic
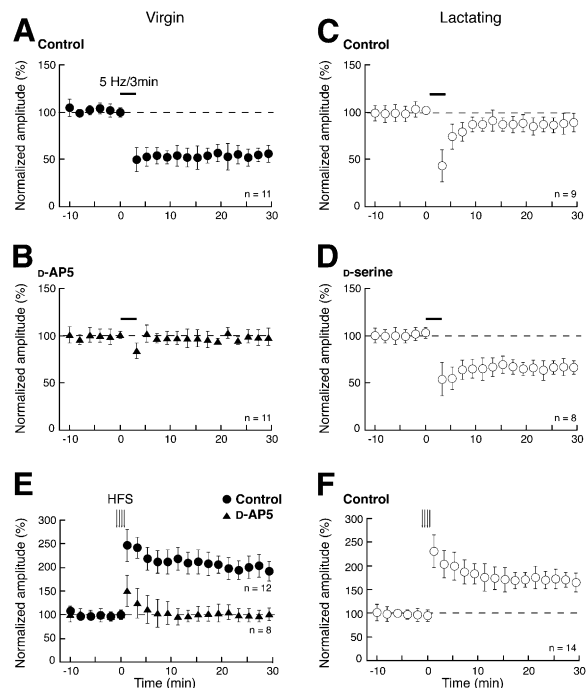
glutamate receptors (Oliet et al., 2001; Piet et al., 2004). As we show here, because of their intimate anatomical relationship with synapses (Oliet et al., 2001; Theodosis and Poulain, 1993) and through their release of D-serine (Mothet et al., 2005; Schell et al., 1995), astrocytes regulate activation of synaptic NMDARs and therefore glutamatergic transmission at the postsynaptic level as well. Furthermore, changes in the extent of astrocytic enwrapping of synapses will modify D-serine levels and therefore strongly influence NMDAR-dependent long-term synaptic changes, whose direction and magnitude depend on the number of receptors activated during afferent input stimulation (Cummings et al., 1996; Malenka and Nicoll, 1999).

Our biochemical analyses revealed high concentrations of D-serine and of its biosynthetic enzyme, serine racemase, in the SON. As clearly shown with immunocytochemistry, both were localized in all SON astrocytes and D-serine was absent from neuronal elements. When D-serine was specifically degraded with DAAO, a condition that was confirmed by HPLC analyses, NMDAR-mediated EPSCs were greatly reduced whereas equivalent degradation of glycine with GO did not affect NMDAR activity. Taken together, our biochemical, anatomical, and electrophysiological observations unambiguously demonstrate
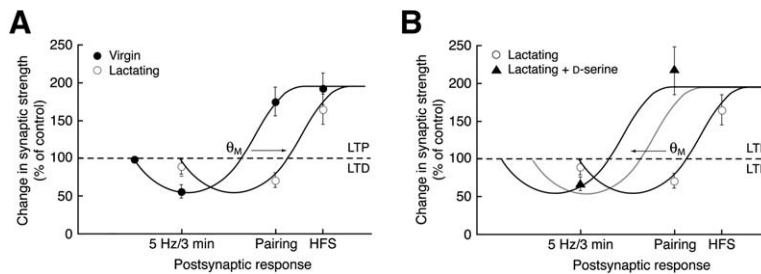
Figure 7. Bienenstock, Cooper, and Munro Model for Modification of LTP Threshold

(A) Points represent percent changes in EPSC amplitude measured 30 min after the induction of LTD, pairing LTP and HFS-LTP, respectively. These points were positioned arbitrarily on the x axis. A curve was drawn to fit the data obtained in virgin rats (filled circles) and then shifted to fit the points obtained in lactating animals (empty circles). Note that the threshold for LTP induction (θ_M) is shifted toward higher activity values in lactating rats.

(B) The relationship obtained in lactating rats is shifted toward lower activity values in the presence of D-serine (filled triangles). Moreover, it is shifted to the left of the curve obtained in virgin animals (gray curve taken from [A]), probably because all NMDARs are available for activation in the presence of saturating concentration of D-serine. Data are reported as means ± SEM.

that D-serine, and not glycine, is the endogenous ligand of NMDARs in the SON. This is the first time that the respective roles of endogenous D-serine and glycine on NMDAR activity are assessed in a brain region. That glycine does not play a role in NMDAR function in the SON is in agreement with the paucity of glycinergic fibers in this region (Rampon et al., 1996) and with the observation that taurine, and not glycine, is the endogenous ligand of strychnine-sensitive glycinergic receptors expressed on SON neurons (Hussy et al., 1997).

Our comparative analysis of a neuronal structure like the SON, which presents different astrocytic environment under distinct physiological conditions (Hatton, 1997; Theodosis and Poulain, 1993), allowed us to demonstrate that synaptic NMDAR-mediated responses were strongly influenced by astrocytes. Changes in NMDAR activity can result from a number of causes including the following: (1) a switch in the expression NR2 subunits, (2) modified coagonist concentrations, (3) desensitization, and/or (4) removal of receptors from the membrane. To test the first scenario, we applied ifenprodil, a specific antagonist of NR2B-containing NMDARs. This antagonist greatly reduced synaptic and extrasynaptic NMDAR-mediated responses and to the same extent in virgin and lactating rats. This result indicates that, unlike GABA-A receptors (Brussaard et al., 1997), there are no adaptative changes in NMDAR subunit composition in SON neurons during lactation. Because glial-derived D-serine is the endogenous ligand of NMDARs in the SON, the second scenario appears more plausible. Application of saturating concentrations of D-serine in the SON of virgin animals induced a small but significant increase in NMDA EPSCs amplitude, indicating that the glycine site was close to saturation when glial coverage was maximal. On the other hand, in lactating rats, D-serine enhanced NMDAR-mediated responses dramatically, revealing a low level of glycine site occupancy under conditions of reduced astrocytic coverage. Interestingly, NMDAR responses obtained in the presence of D-serine in lactating rats were no different from those measured in virgin rats. This rules out the possibility that our results are due to receptor desensitization or removal of receptors from the membrane. It is clear then that the level of occupancy

of the NMDAR glycine binding site closely depends on the degree of astrocytic ensheathing of synapses and that the reduced NMDAR activity in lactating rats can be entirely explained by diminished concentrations of D-serine within the synaptic cleft.

Our observations also show that astrocytes control the level of activity of NMDARs at synaptic but not extrasynaptic sites. Applying saturating concentrations of D-serine in virgin and lactating rats indicated that glycine site occupancy at extrasynaptic NMDARs was low and independent of the astrocytic environment. These findings suggest that D-serine is released and/or accumulates preferentially at glutamatergic synapses, which fits well with the observation that D-serine is released from glial cells following activation of astrocytic glutamatergic receptors (Kim et al., 2005; Mothet et al., 2005; Schell et al., 1995). Spontaneous and evoked transmitter release at glutamatergic inputs could provide an excitatory drive to astrocytes inducing the release of D-serine close to synaptic NMDARs. This would explain why occupancy of the glycine site is high at synaptic sites, whereas it is low at extrasynaptic sites. The conditions and the mechanisms by which occupancy of the glycine site at extrasynaptic NMDARs can be increased remain to be determined. One possibility is astrocytic release of vesicles containing both glutamate and D-serine at extrasynaptic sites (Mothet et al., 2005).

In addition to fast excitatory neurotransmission, NMDARs actively participate to synaptic plasticity, like LTP and LTD, the cellular substrates for learning and memory (Malenka and Nicoll, 1999). Ca^{2+} entry during NMDAR stimulation can trigger intracellular second messenger pathways leading to persistent changes of EPSC amplitude due to the insertion or removal of AMPARs from synapses (Luscher et al., 2000; Nicoll, 2003). Our data indicate that reduced glial coverage of neurons and their synapses shifts the activity-dependence of long-term synaptic changes toward higher activity values. This finding is in agreement with other studies (Cummings et al., 1996) showing that the number of NMDARs activated during afferent stimulation governs the direction and the magnitude of synaptic plasticity. Indeed, the physiological reduction of D-serine concentrations at

glutamatergic synapses in the SON of lactating rats affects synaptic plasticity in a manner closely similar to that produced by partial blockade of NMDARs obtained via pharmacological means.

The resulting shift in θ_M in SON neurons of lactating rats should favor potentiation at glutamatergic inputs displaying high activity, like those transmitting suckling information to oxytocin SON neurons during lactation (Jourdain et al., 1998), compared to synapses exhibiting lower activity that would be depressed rather than potentiated (Figure 7A). Such a shift in the activity dependence of synaptic plasticity is completely reversed in the presence of saturating concentrations of D-serine (Figure 7B). Our data establish, therefore, that variations in the astrocytic environment of neurons and synapses that can take place in response to different physiological and pathological conditions (Hatton, 1997; Hirrlinger et al., 2004; Iino et al., 2001; Laming et al., 2000; Theodosis and Poulain, 1993) directly contribute to the postsynaptic control of excitatory neurotransmission and to its bidirectional plasticity.

EXPERIMENTAL PROCEDURES

Slice Preparation and Electrophysiology

Hypothalamic coronal slices (300 μm) that included the SON were obtained from female virgin (1–3 months old) and day 7–10 lactating Wistar rats (Oliet and Poulain, 1999). Briefly, rats were anaesthetized with isoflurane and decapitated. After dissection, the brain was placed in ice-cold artificial cerebrospinal fluid (ACSF) saturated with 95% O_2 and 5% CO_2 and frontal slices cut on a vibratome (Leica). ACSF contained 123 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 26.2 mM $NaHCO_3$, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 10 mM glucose (pH 7.4; 295 mosmol/kg). The slices were hemisected along the midline and maintained at 30°C–34°C for at least 1 hr before being transferred to a recording chamber where they were perfused with ACSF containing 20 μM bicuculline. Magnocellular neurons were identified with differential interference contrast microscopy (Olympus BX50), and membrane currents were recorded with a Multiclamp 700A amplifier (Axon Instruments, Inc.) using patch-clamp pipettes (3–5 MΩ) filled with an intracellular solution containing 130 mM CsCl, 10 mM NaCl, 10 mM HEPES, 1 mM EGTA, 0.1 mM $CaCl_2$, and 5 mM QX-314 (pH 7.1–7.2 adjusted with CsOH; 295 mosmol/kg). For LTD and HFS-LTP experiments, patch pipettes were filled with 120 mM K-gluconate, 1.3 mM $MgCl_2$, 1 mM EGTA, 10 mM HEPES, 0.1 mM $CaCl_2$ (pH 7.1–7.2 adjusted with KOH; 295 mosmol/kg). Signals were filtered at 2 kHz and digitized at 5 kHz via a DigiData 1322 interface (Axon Instruments, Inc.). Series resistance (6–20 MΩ) was monitored online, and cells were excluded from data analysis if more than a 15% change occurred during the course of the experiment. Data were collected and analyzed online using pClamp 9 software (Axon Instruments, Inc.). The stimulating electrode was placed in an area dorsal to the recording site outside the SON; EPSCs were evoked at 0.066 Hz.

The AMPA/NMDA ratio corresponds to the ratio of the peak amplitude of the EPSCs recorded at −60 mV over the peak amplitude of the EPSCs recorded at +40 mV in the presence of DNQX (10 μM). LTP was induced by applying a pairing protocol consisting of simultaneous depolarization of the neuron to 0 mV and afferent stimulation at 2 Hz for 45 s or a high-frequency stimulation (HFS) protocol consisting of a 100 Hz train of stimuli for 1 s repeated four times at 15 s intervals in current clamp mode. To induce LTD we applied a 5 Hz/3 min train of stimuli in current clamp mode.

All drugs were bath applied, except NMDA (50 μM) that was applied locally as a puff (1 s) using a glass electrode connected to a picospritzer

and positioned within 100 μm of the recorded neuron. Appropriate stock solutions were made and diluted with ACSF just before application. Drugs included bicuculline methobromide, DNQX, D-AP5, ifenprodil (Tocris), D-serine (Sigma). Recombinant *R. gracilis* D-amino acid oxidase (DAAO, EC 1.4.3.3) was overexpressed in *E. coli* cells and purified as reported earlier (Molla et al., 1998); the final enzyme preparation had a specific activity of 55 U/mg protein on D-serine as substrate. Recombinant *B. subtilis* glycine oxidase (GO, EC 1.4.3.19) was overexpressed in *E. coli* cells (Job et al., 2002b); the final enzyme preparation had a specific activity of 0.5 U/mg protein on glycine as substrate. The higher specificity of the reaction of DAAO for D-serine and of GO for glycine is well demonstrated by the apparent kinetic efficiency (k_{cat}/K_m ratio) for these two substrates: k_{cat}/K_m ratios of 3.0 and 0.058 $mM^{-1}s^{-1}$ were determined for DAAO on D-serine and glycine, respectively, while the k_{cat}/K_m ratios determined for GO is 0.00025 and 0.867 $mM^{-1}s^{-1}$ on D-serine and glycine, respectively. To degrade D-serine or glycine, slices were incubated for at least 45 min and then continuously perfused with ACSF containing DAAO (0.17 U/ml) or GO (0.17 U/ml), respectively. Data, reported as means ± SEM., were compared using the nonparametric Mann-Whitney U test and the parametric paired or unpaired Student's t test. Significance was assessed at $p < 0.05$.

Immunoblot Analysis

This analysis was performed as described earlier (Mothet et al., 2005) on tissue extracts obtained from five 2-month-old virgin female rats. The animals were decapitated under isoflurane anesthesia, their brains quickly removed and placed in ice-cold ACSF. The two bilateral SON were dissected under microscopic control from frontal slices (300 μm) cut with a vibratome, frozen on dry ice, and pooled in Eppendorf tubes. Pieces of striatum were obtained concurrently. Tissues were homogenized in ice-cold 50 mM Tris-HCl pH 6.8 containing 2 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS), and a protease inhibitor cocktail (Sigma). Each extract (40 μg/lane) was then subjected to denaturing 12% SDS-PAGE electrophoresis and electroblotted onto a PVDF membrane (Immobilon-P, Millipore). Transfers were performed overnight (50 mA; 4°C) in a solution containing 20 mM Tris, 150 mM glycine, 20% methanol, and 0.01% SDS. The membranes were incubated for 1 hr in a blocking solution that included horse serum in Tris-buffered saline (TBS) (50 mM Tris-HCl [pH 8.0], 500 mM NaCl) and 0.05% Tween (TTBS). Serine racemase was probed with affinity-purified polyclonal goat antibodies raised against the N terminus of the protein (diluted 1/200; Santa Cruz Biotechnology). Membranes were washed and then incubated for 1 hr at room temp with peroxidase-conjugated anti-goat immunoglobulins (Igs; 1/2000; Vector Laboratories). Immunoblots were washed three times for 5 min each in TTBS and then for 5 min in TBS; immunoreactivity was revealed finally by enhanced chemiluminescence (Amersham Pharmacia Biotech). Molecular sizes were estimated by separating the SeeBlue Plus 2 prestained molecular weight markers (4–250 kDa) in parallel (Invitrogen). Protein content was determined by the Lowry method using the DC protein Bio-Rad assay. Controls included incubation with diluted serum preabsorbed with blocking peptide (Santa Cruz Biotechnology).

Immunohistochemistry

Under urethane anesthesia, two adult male, seven virgin female, and four lactating rats were injected intracardially with heparin and then perfused with a solution containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 1% sodium metabisulfite in sodium phosphate buffer (0.1 M [pH 7.2–7.4], 20 min, room temperature). Blocks containing the hypothalamus were removed and left in the same fixative (2 hr minimum; 4°C). Freely floating frontal sections (ca 50 μm), cut on a vibratome, then underwent immunofluorescence. Before immunostaining, sections were incubated for 10 min in PBS with 0.1 M sodium borohydride. For single immunolabeling, sections were incubated with polyclonal goat Igs against serine racemase (diluted 1/100; 5 days at

4°C; Santa Cruz Biotechnology) or rabbit Igs against D-serine (diluted 1/2000; 48 hr at 4°C; Gemac). Immunoreactivities were revealed with affinity-purified CY2-conjugated anti-goat (Jackson Labs) or FITC-conjugated anti-rabbit Igs (diluted 1/400 and 1/800, respectively; Biosys). For double immunolabeling, sections were incubated in mixtures of anti-D-serine (diluted 1/2000) and a monoclonal mouse antibody against glial fibrillary acidic protein (GFAP; diluted 1/500; Sigma); mixtures of FITC-conjugated anti-rabbit and Texas red-conjugated anti-mouse Igs (diluted 1/800) served as immunolabels. All antibody solutions contained 0.2 metabisulfite. Observations were made with epifluorescence with a Leica DMR microscope or with a Leica confocal scanning microscope using appropriate filters. Controls included omission of primary antibodies and incubation in normal rabbit serum. These sections showed no specific immunolabeling.

HPLC Analysis
Fragments of the SON and striatum from four virgin rats were pooled and sonicated in 5 vol of ice-cold 10% trichloroacetic acid and centrifuged (20 min; 13,000 rpm) to remove protein. Soluble fractions were extracted with water-saturated ether and neutralized with NaOH before precolumn derivatization with o-phthaldialdehyde/N-acetyl-L-cysteine in borate buffer (0.01M; pH 10.4). Diastereoisomer derivatives were then resolved by HPLC analysis on a 4.9 μm Waters Novapack C18 (3.9 × 150 mm) reversed-phase column in isocratic conditions. The mobile phase consisted in sodium acetate buffer (50 mM; pH 6.2) plus 1.2% tetrahydrofluoran flowing at a rate of 0.6 ml/min. Identification and quantification of the compounds was obtained on the basis of retention times and peak heights and areas, compared to those associated with D-serine, L-serine, and glycine external standards added to the samples. The standards were prepared as 100 mM stock solution in distilled water. Linearity was determined by injecting increasing concentrations of amino acids (20–100 picomoles), and calibration curves and equations were used to determine the amount of amino acids in the experimental samples. Extracellular media were first centrifuged to precipitate proteins and the supernatant (amino acids phase) were derivatized before HPLC analysis as described above. Twenty microliter samples were injected into the column for HPLC analysis. Three animals were used in each condition.

Supplemental Data
Supplemental Data include four figures and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/cgi/content/full/125/4/775/DC1/.

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