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Glycine binding primes NMDA receptor internalization

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NMDA (*N*-methyl-D-aspartate) receptors (NMDARs) are a principal subtype of excitatory ligand-gated ion channel with prominent roles in physiological and disease processes in the central nervous system[1]. Recognition that glycine potentiates NMDAR-mediated currents[2] as well as being a requisite co-agonist of the NMDAR subtype of 'glutamate' receptor[3] profoundly changed our understanding of chemical synaptic communication in the central nervous system. The binding of both glycine and glutamate is necessary to cause opening of the NMDAR conductance pore[1]. Although binding of either agonist alone is insufficient to cause current flow through the channel, we report here that stimulation of the glycine site initiates signalling through the NMDAR complex, priming the receptors for clathrin-dependent endocytosis. Glycine binding alone does not cause the receptor to be endocytosed; this requires both glycine and glutamate site activation of NMDARs. The priming effect of glycine is mimicked by the NMDAR glycine site agonist D-serine, and is blocked by competitive glycine site antagonists. Synaptic as well as extrasynaptic NMDARs are primed for internalization by glycine site stimulation. Our results demonstrate transmembrane signal transduction through activating the glycine site of NMDARs, and elucidate a model for modulating cell–cell communication in the central nervous system.

The turnover of cell surface NMDARs is much slower than that of other ligand-gated ion channels, such as AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)[4–9] and $GABA_A$ (γ-aminobutyric acid A)[10] receptors, and NMDARs are often considered 'stable' in the plasma membrane. However, recent evidence has raised the possibility that NMDARs may undergo regulated transport to and from the cell surface[11–13]. Here, we recorded whole-cell currents from neurons acutely isolated from the CA1 region of the hippocampus. We probed NMDAR activity with regularly timed test applications of NMDA (50 μM) plus glycine (1 μM), and observed that the NMDAR-mediated currents were stable for recording periods of 45 min or longer (Fig. 1a). In contrast, on administering a conditioning stimulus for NMDARs (1 mM NMDA plus 100 μM glycine; 2–3 min duration) there was a sharp decline in the NMDAR currents followed by a progressive decrease to a level 57 ± 3% (mean ± s.e.m.; $n = 6$ cells) of the control NMDAR current amplitude (Fig. 1b). The depression of NMDAR currents was stable and the currents did not recover even during the longest recordings, up to 1 h. The depression of NMDAR currents was associated with no change in reversal potential, no loss of neuronal integrity and no alteration in single channel conductance or mean open time (see Supplementary Information 1).

It is possible that the decline in NMDAR currents was due to a decrease in the number of cell-surface receptors. Thus, we determined whether the depression of NMDAR currents might be due to clathrin-dependent receptor internalization. For this we used an amphiphysin SH3 domain recombinant protein[6,14], which prevents recruitment of dynamin to clathrin-coated pits and thereby blocks endocytosis. We found that when wild-type amphiphysin SH3 domain protein was administered intracellularly during whole-cell recording, the co-agonist conditioning stimulation produced a small, transient decline in NMDAR current amplitude but no sustained decrease in the currents (Fig. 1c). In contrast, when we administered an amphiphysin SH3 domain protein with a double point mutation (G684R, P687L)[15] that renders it incapable of blocking endocytosis, the co-agonist stimulation caused a sustained decline in NMDAR currents (Fig. 1c) that was not different from that in control recordings.

To determine directly whether conditioning of NMDARs leads to loss of the receptors from the cell surface as a consequence of clathrin-dependent receptor endocytosis, we used a cell enzyme-linked immunosorbent assay (ELISA) (Fig. 1d) with hippocampal neurons in primary culture. Using control extracellular bathing solution we found that there was little internalization of NMDARs during the 15-min experimental period (Fig. 1d). On the other hand, exposing the cells to the conditioning stimulus for 5 min caused 27 ± 3% ($n = 6$) of the pre-labelled NMDARs to be internalized over the subsequent 10-min period (Fig. 1d). To test whether the internalization of NMDARs required clathrin assembly we used extracellular hypertonic sucrose solution, known to block clathrin-mediated endocytosis[16], which was found to prevent the internalization (Fig. 1d, top). NMDAR internalization was produced when the concentration of NMDA in the co-agonist conditioning stimulus was reduced to 50 μM (Fig. 1d, bottom). In contrast, neither the application of NMDA nor glycine alone caused internalization of NMDARs. Furthermore, applying the competitive NMDAR antagonist AP5 (D-2-amino-5-phosphonovaleric acid)[17]—which binds to the glutamate-binding site but does not

activate the receptors—together with glycine did not cause NMDAR internalization. These results indicate that co-agonist activation of NMDARs provokes their robust internalization by clathrin-dependent endocytosis.

When we applied AP5 together with the conditioning NMDA and glycine stimulus during electrophysiological recordings, we found that the test response immediately after the conditioning stimulus had the same amplitude as did the control responses before conditioning (Fig. 2a). Thus, AP5 eliminated the immediate sharp decrease in NMDAR currents produced by the conditioning stimulus, in accord with NMDAR antagonism by AP5 and the lack of effect of AP5 plus glycine on NMDAR internalization. Notably, however, the amplitude of subsequent test responses was not maintained and the responses progressively declined, reaching a level 58 ± 8% of the pre-conditioning control ($n = 4$ cells; Fig. 2a). This decrease in NMDAR amplitude was not different from that produced with the conditioning stimulus lacking AP5. Conditioning with AP5 alone did not cause any change in subsequent test responses. Thus, the conditioning stimulus provoked a sharp decline that was dependent on NMDAR activation during this stimulus, as indicated by the AP5 blockade, and produced a progressive decline that was not prevented by AP5.

Because AP5 is competitive with glutamate at its binding site on the NMDAR, we hypothesized that induction of the progressive decline in NMDAR responses may not require binding to this site but rather may depend on activating the glycine-binding site. This was tested by conditioning with glycine, without NMDA, which caused NMDAR test responses to decline progressively to a stable level at 63.5 ± 2% ($n = 8$ cells) of the control level (Fig. 2b), which was not different from that reached after the NMDA (1 mM) plus glycine (100 μM) conditioning stimulus, with or without AP5. We found that D-serine (30 μM), another endogenous agonist of the glycine site of the NMDAR[18], also produced a progressive decline in NMDAR test responses to a level not different from that reached after conditioning with glycine (Fig. 2b). Moreover, the effect of

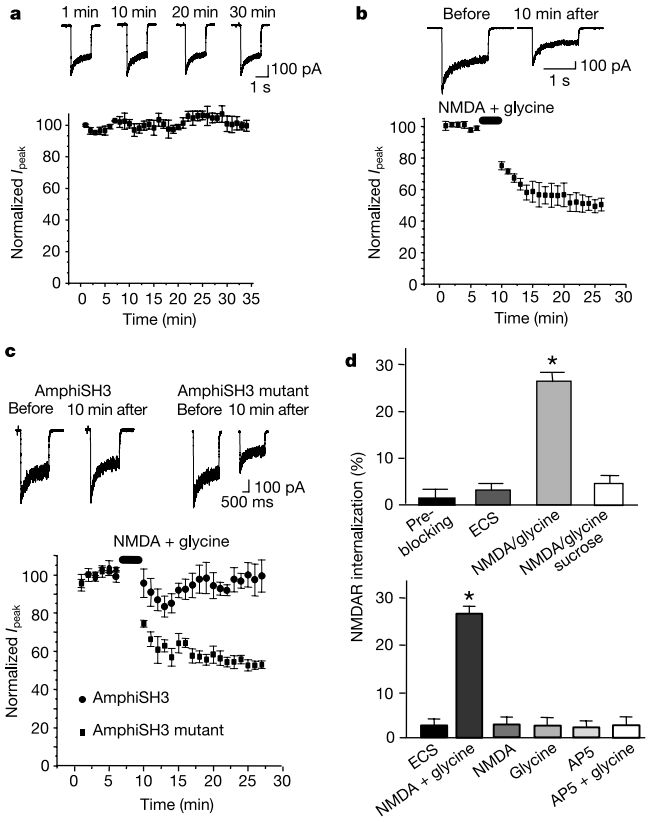

Figure 1 Co-agonist stimulation induces dynamin-dependent NMDA receptor internalization. **a**, Plot of the normalized NMDAR peak currents (mean ± s.e.m.) recorded in acutely isolated rat hippocampal CA1 neurons ($n = 8$ cells). Peak currents were normalized to the average of the first six responses evoked by test applications of NMDA (50 μM) and glycine (1 μM) in this and subsequent figures. Traces (top) show responses to the test applications recorded from one cell at the times indicated. **b**, Normalized NMDAR peak currents ($n = 6$ cells) are plotted before and after conditioning with NMDA (1 mM) plus glycine (100 μM) applied during the time indicated. Shown above are traces of NMDA-evoked whole-cell currents before and 10 min after conditioning stimulation. **c**, Normalized peak NMDA-evoked currents from recordings with intracellular administration of amphiphysin SH3 domain (amphiSH3): wild type (circles; $n = 8$ cells) or G684R, P687L mutant (squares; $n = 5$ cells) protein. **d**, The top panel shows NMDAR internalization (mean ± s.e.m.; percentage of total) as quantified by cell ELISA assay using hippocampal neurons in primary culture. Cultures ($n = 12$ in three separate experiments) were treated with control ECS or ECS containing NMDA (1 mM) plus glycine (100 μM) with or without hypertonic sucrose (0.45 M). For pre-blocking conditions cultures were not treated with ECS. The bottom panel shows quantification of NMDAR internalization for cultures treated with control ECS or ECS supplemented with NMDA (50 μM) plus glycine (100 μM), NMDA (50 μM), glycine (100 μM), AP5 (50 μM), or glycine (100 μM) plus AP5 (50 μM) ($n = 18$ in three separate experiments).

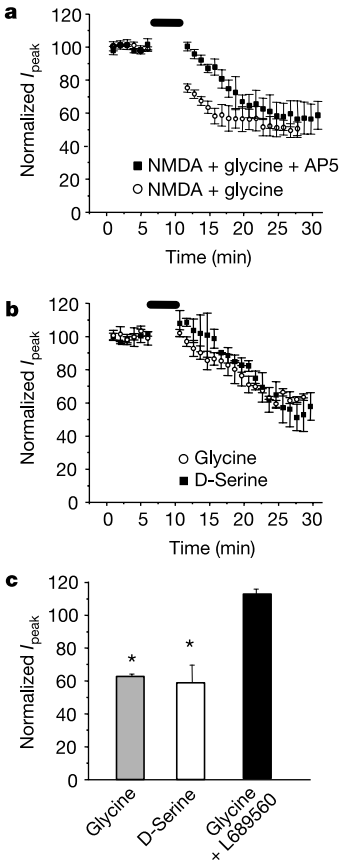

Figure 2 Glycine site stimulation initiates a progressive decline in NMDA receptor-mediated currents. **a**, Normalized peak NMDA-evoked test currents are plotted. The conditioning stimulus of NMDA (1 mM) plus glycine (100 μM) without (open circles; $n = 5$ cells) or with (filled squares; $n = 4$ cells) AP5 (500 μM) was applied during the period indicated by the black bar. **b**, Normalized peak NMDA currents during recordings in which the conditioning stimulus was ECS supplemented with glycine (100 μM, open circles; $n = 8$ cells) or D-serine (30 μM, filled squares; $n = 5$ cells). **c**, Histogram showing averaged, normalized peak NMDA currents 20 min after conditioning with glycine (100 μM; $n = 8$ cells), D-serine (30 μM; $n = 5$ cells), or glycine (100 μM) plus L689560 (1 μM; $n = 5$ cells).

conditioning with glycine was eliminated by co-administering the glycine site antagonist L689560 (refs 19, 20) (Fig. 2c) but not by AP5 (Supplementary Information 2). From these results we conclude that the progressive decline in NMDAR currents was initiated by stimulating the glycine site, independent of the glutamate site, of the NMDAR during the conditioning stimulation.

To determine whether glycine stimulation initiated a slowly developing process in the cells that was independent of NMDAR activity or whether subsequent NMDAR activation was required to produce the decline in the responses, we performed a separate series of experiments in which the start of the test applications was delayed 5 min after the end of the conditioning glycine stimulus. We found that the first NMDAR response after the 5-min delay was not different in amplitude from the test responses immediately in the control period preceding the conditioning stimulation (Fig. 3a; average 97 ± 2% control, $n = 5$ cells). However, the NMDAR responses declined with a time course that paralleled that of the decline of responses when the test applications were started without any delay period (Fig. 3a). Thus, rather than causing the depression of the NMDAR responses, glycine primed the NMDARs for depression, which was subsequently produced by stimulating both co-agonist sites. The priming of depression of NMDAR currents was observed with native receptors in neurons as well as with recombinant NMDARs expressed heterologously (Supplementary Information 3).

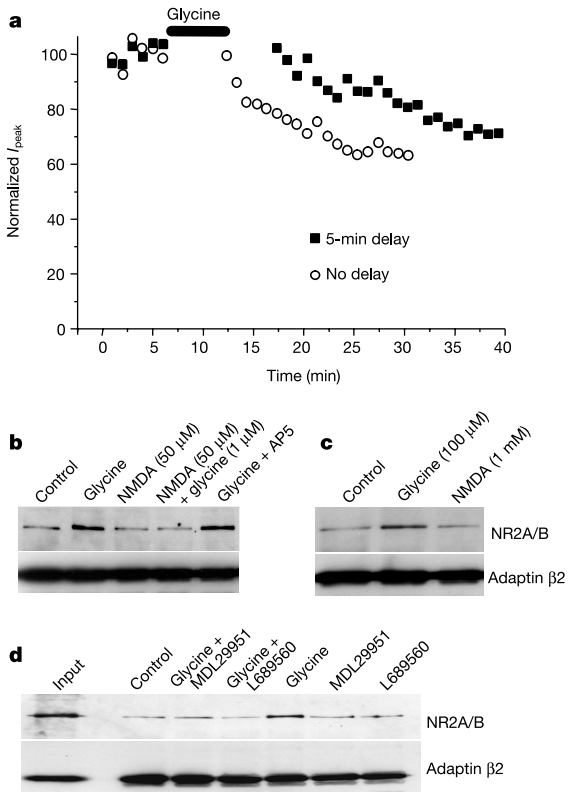

Figure 3 Glycine binding primes the decline of NMDA responses and recruits adaptin β2 to the NMDA receptor complex. **a**, Peak NMDA-evoked test currents plotted for two cells conditioned with glycine (100 μM) during the period indicated. For one cell, test applications were re-started immediately after the glycine conditioning (open circles). For the other cell, there was a delay of 5 min before re-starting the test applications (filled squares). **b**, Co-immunoprecipitation of NMDARs and adaptin β2 from acute hippocampal slices treated for 5 min with control ECS, glycine (100 μM), NMDA (50 μM), NMDA (50 μM) plus glycine (1 μM), or glycine (100 μM) plus AP5 (100 μM). Proteins were immunoprecipitated with anti-adaptin β2, resolved by SDS–PAGE, and analysed by immunoblotting with anti-NMDAR2A/B (top panel). The blot was then re-probed with anti-adaptin β2 (bottom panel). Similar results were observed in each of three separate experiments. **c**, Co-immunoprecipitation of NMDARs and adaptin β2 from slices treated for 5 min with control ECS, glycine (100 μM) or NMDA (1 mM). Shown is the representative of three similar experiments. **d**, Co-immunoprecipitation of NMDARs and adaptin β2 from slices treated for 5 min with control ECS, glycine (100 μM) with or without L689560 (1 μM) or MDL29951 (1 μM), or with L689560 or MDL29951 alone. In the lane marked input, 50 μg of protein without immunoprecipitation was loaded. Similar results were obtained in each of three experiments.

These findings raise the possibility that extracellular binding of glycine to NMDARs causes a transmembrane signalling process by means of the receptors that results in intracellular biochemical alterations. We explored this possibility by examining the association between the NMDARs and the AP2 adaptor protein complex: a principal component of the intracellular endocytic machinery[21]. Using proteins prepared under weakly denaturing conditions from acute hippocampal slices—conditions that preserve many protein–protein interactions—we immunoprecipitated the AP2 complex with an antibody against a common subunit of the complex, the adaptin β2 subunit, and probed for the level of co-immunoprecipitating NMDARs. Under control conditions we found a low level of co-precipitation of NMDARs together with adaptin β2 (Fig. 3b–d); this binding was not detected previously when binding between AMPA receptor subunit GluR2 and adaptin β2 was evident[6], indicating that the level of basal association between AP2 and NMDARs is much less than that between AP2 and AMPA receptors. However, in slices treated with glycine (100 μM) or glycine (100 μM) plus AP5 (100 μM), the level of NMDARs precipitating together with adaptin β2 increased greatly, indicating that glycine enhanced the association of AP2 and NMDARs (Fig. 3b–d). In contrast, treating slices with NMDA (50 μM) or NMDA (50 μM) plus glycine (1 μM) produced no change in the adaptin β2–NMDAR association (Fig. 3b). Moreover, treating slices with 1 mM NMDA also produced no change in the adaptin β2–NMDAR association (Fig. 3c). The increase in precipitation of NMDARs together with adaptin β2 produced by glycine was prevented by L689560 or another glycine site antagonist MDL29951 (refs 20, 22) (Fig. 3d), which themselves had no effect on the association of AP2 and NMDARs. Thus, stimulation of the extracellular glycine site results in an intracellular process, or processes, that leads to recruitment of the endocytic machinery to NMDARs; however, this recruitment, in and of itself, does not cause NMDAR internalization.

To determine whether priming NMDARs with glycine readies the receptors for subsequent internalization on activation, we compared the effect of receptor activation by NMDA (50 μM) plus glycine (1 μM), identical to the co-agonist concentrations used to evoke test NMDAR whole-cell currents, with and without pre-treatment with glycine (100 μM, 5 min). Without glycine pre-treatment, we found that activating the receptors with NMDA (50 μM) plus glycine (1 μM) did not cause them to be internalized (Fig. 4a). However, in neurons that were pre-treated with glycine, subsequent receptor activation did provoke robust NMDAR internalization. The internalization was prevented when receptor activation was blocked by including AP5 with the NMDA and glycine treatment (Fig. 4a), and internalization was produced when NMDA was replaced by the endogenous transmitter glutamate (Fig. 4a). Thus, the internalization process itself was dependent on NMDAR activation. As glycine on its own does not provoke NMDAR internalization (Figs 1d and 4a) our findings indicate that glycine treatment primes, but does not induce, NMDAR endocytosis, and that the receptor internalization process itself is dependent on co-agonist activation of NMDARs.

We next sought to determine whether priming was dependent on stimulating the glycine site of NMDARs. Applying the glycine site antagonist L689560 together with glycine during pre-treatment prevented the co-agonist treatment from producing NMDAR internalization (Fig. 4b). Similarly, L689560 pre-treatment on its

own did not permit subsequent application of co-agonists to stimulate internalization. Thus, glycine site activation is required to prime NMDAR internalization but glycine site occupation with a non-activating ligand is not sufficient to prime the receptors. The effector concentration for half-maximum response (EC_{50}) for the priming effect of glycine was 39 ± 2 μM (Fig. 4c).

To examine whether NMDARs were primed for internalization through the selective activation of the glutamate-binding site, we pre-treated neurons for 5 min with NMDA (100 μM or 1 mM). The pre-treatment was carried out with co-application of L689560 to eliminate any activation of the glycine site. After subsequent treatment with NMDA (50 μM) and glycine (1 μM), there was no difference in NMDAR internalization compared to pre-treatment with extracellular solution (ECS; Fig. 4b). Thus, activating the glutamate site, in contrast to the glycine site, is not sufficient to prime NMDARs for internalization.

To determine whether the internalization of NMDARs after glycine priming was mediated through clathrin-dependent endocytosis, we made use of a dynamin inhibitory peptide, QVPSRPNRAP, which competitively binds with the amphiphysin SH3 domain[15] and prevents endocytosis when administered intracellularly[10]. Myristoylated QVPSRPNRAP (Myr-4–QVPSRPNRAP) is a membrane-permeant form of the peptide that prevents endocytosis[23]. We found that applying Myr-4–QVPSRPNRAP in bath solution inhibited glycine-primed internalization of NMDARs (Fig. 4d). As a negative control we applied the non-myristoylated QVPSRPNRAP, which is not membrane permeant, to bath solution and found that it did not affect the internalization of NMDARs (Fig. 4d). These findings indicate that stimulation of the glycine site alone does not cause the process of NMDAR internalization but rather primes the receptors for clathrin-dependent internalization, which is then produced as a consequence of co-agonist stimulation of the receptor.

In neurons of the central nervous system (CNS), NMDARs are localized at glutamatergic synapses and also at extrasynaptic sites. To investigate directly whether synaptic NMDA receptors are primed by glycine and subsequently internalized, we studied miniature excitatory postsynaptic currents (mEPSCs) during whole-cell recording from hippocampal neurons in primary culture. These mEPSCs show NMDAR- and AMPAR-mediated components[24]. We found that the NMDAR component of the mEPSCs decreased to 61 ± 4% of the initial level ($n = 10$ cells, Fig. 4e, g) after bath application of glycine (100 μM, 5 min) followed by NMDA (50 μM) plus glycine (1 μM, 5 min). In contrast, during control recordings without glycine and NMDA plus glycine treatment, the NMDAR component of mEPSCs was stable (Fig. 4g). Intracellular appli-

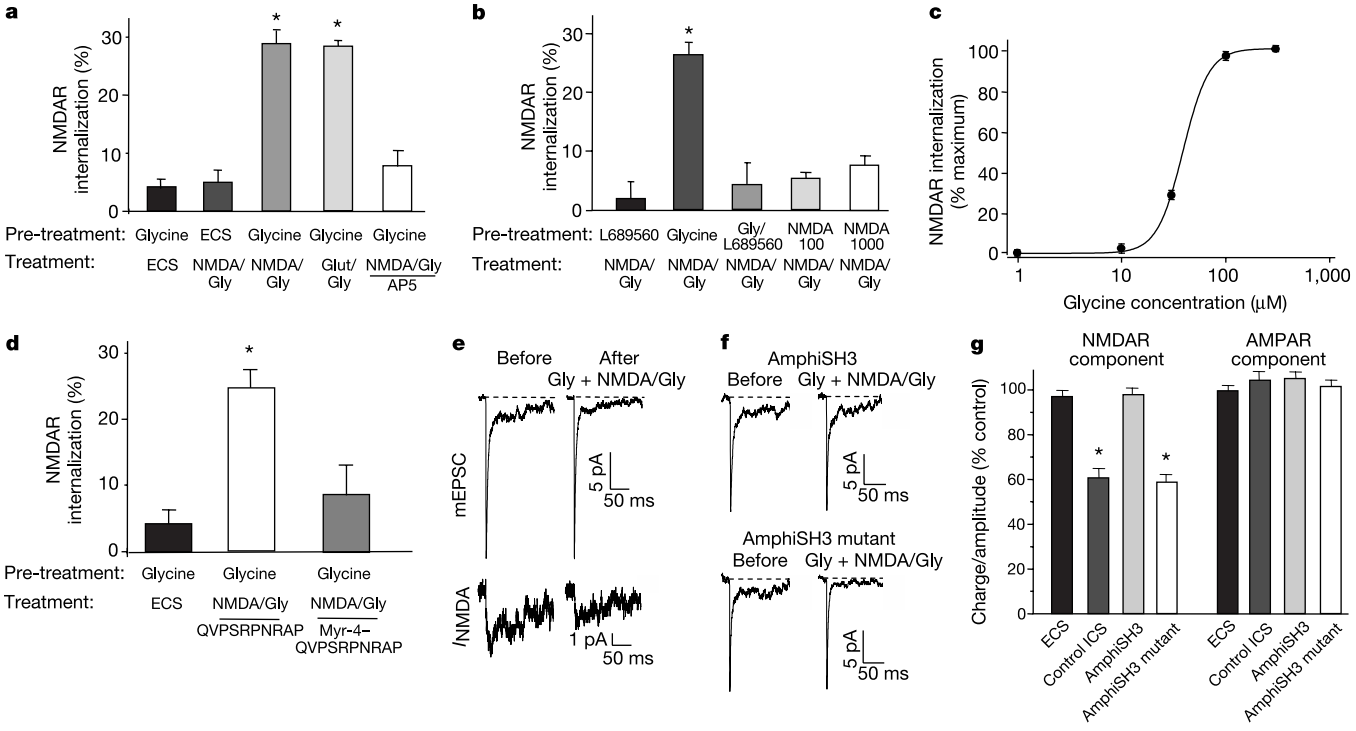

Figure 4 Glycine primes dynamin-dependent internalization of NMDA receptors and leads to preferential suppression of NMDAR- but not AMPAR-mediated synaptic currents. **a**, Cultures were pre-treated with ECS or glycine (100 μM). Next, cultures were treated with ECS or with NMDA (50 μM) plus glycine (1 μM) with or without AP5 (50 μM), or with glutamate (50 μM) plus glycine (1 μM) ($n = 18$). NMDAR internalization was quantified by cell ELISA from hippocampal cultures subject to pre-treatment/treatment models in **a**–**d**. **b**, Pre-treatment (5 min) was as indicated (NMDA 100 μM or 1,000 μM). Treatment: NMDA (50 μM) plus glycine (1 μM, 5 min; $n = 18$). **c**, Concentration-dependence of the glycine priming effect. Each data point is the mean ± s.e.m. of NMDAR internalization subtracted from basal NMDAR internalization at each concentration of glycine ($n = 6$ for each point), normalized to the maximum internalization. The solid curve is the best fit of the data to the logistic equation $E = E_{max}/(1 + (EC_{50}/[glycine])^n)$; where EC_{50} was 39 μM and n was 3.5. **d**, Histogram showing experiments ($n = 6$) in which a membrane-permeant form of the dynamin inhibitory peptide (Myr-4–QVPSRPNRAP; 50 μM) or the membrane non-permeant form (QVPSRPNRAP; 50 μM) were included throughout. **e**, Representative averaged mEPSCs (top traces) or NMDAR components (I_{NMDA}) (bottom traces) obtained before and 10 min after pre-treatment with glycine (100 μM) and treatment with NMDA (50 μM) plus glycine (1 μM, 5 min). **f**, Traces are representative average mEPSCs obtained before and 10 min after pre-treatment with glycine and treatment with NMDA plus glycine. Amphiphysin SH3 domain wild-type (top) or mutant (bottom) fusion protein was included in the recording pipette throughout the experiment. **g**, Mean values in NMDAR components and AMPAR components of averaged mEPSCs after treatment with NMDA plus glycine, expressed as percentage of control. Recordings were done with ECS pre-treatment followed by NMDA plus glycine (ECS; $n = 5$ cells) as indicated; in all other experiments, cells were pre-treated with glycine (100 μM). The intracellular solution was control (Control ICS; $n = 10$ cells), or included amphiphysin SH3 domain fusion protein (AmphiSH3; $n = 12$ cells) or mutant SH3 domain protein (AmpshiSH3 mutant; $n = 8$ cells). Asterisk, $P < 0.005$, t-test, versus ECS.

cation of the wild-type amphiphysin SH3 domain fusion protein during whole-cell recordings prevented the decrease of the NMDAR component of the mEPSCs caused by the glycine followed by NMDA plus glycine treatment ($n = 12$ cells, Fig. 4f, g). However, when the mutant amphiphysin SH3 domain was administered into the cells, the NMDAR component of the mEPSCs declined to $59 \pm 3\%$ of the initial level ($n = 8$ cells, Fig. 4f, g). In contrast to the NMDAR component, the AMPAR component of the mEPSCs was unaffected by treating with glycine followed by NMDA plus glycine (Fig. 4e–g). Together, these results indicate that glycine stimulation differentially primes synaptic NMDARs, but not synaptic AMPA receptors, for internalization.

Our results show that NMDARs undergo regulated internalization on co-agonist stimulation, with a separable priming process initiated by glycine binding. A principal consequence of NMDAR internalization is that the receptors are removed from the cell surface, and are thereby prevented from further activation by extracellular ligands. Thus, neuronal processes that are dependent on NMDAR activation will be suppressed by the regulated internalization of the receptors. Internalized NMDARs might be recycled to the cell surface, be targeted for degradation or be engaged by intracellular biochemical cascades.

The priming that we have discovered is a potential mechanism to explain the basal stability of NMDARs on the cell surface. Under resting conditions in most regions of the CNS, NMDARs may be protected from regulated internalization because the basal extracellular concentration of glycine[20] is just below the 'set point' of the internalization priming mechanism. The priming mechanism may become engaged to permit NMDAR internalization when extracellular glycine concentration is greater than the set point: this may occur in the cerebellum or spinal cord where basal concentrations of glycine greater than 20 μM have been reported; with high levels of neuronal firing activity either physiologically or pathologically, for example during seizures; or in pathological conditions such as CNS ischaemia or glycine encephalopathy, where extracellular glycine concentrations in the tens to hundreds of micromolars are observed[20]. Also, because D-serine can produce the priming effect, it is important to consider not only the glycine concentration but also that of glycine and D-serine combined. Various mechanisms, such as allosteric regulation by polyamines, Mg^{2+} and Ca^{2+}, may cause marked changes in potency at the glycine site[20]. Thus, the priming mechanism might also become engaged if the set point for priming is lowered such that, even without increasing the basal level, the extracellular concentration of glycine and serine might be sufficient to produce the priming effect. We estimate that glycine is about 20–100 times more potent as a co-agonist mediating NMDAR gating as compared with the priming effect discovered here. We suggest two potential explanations for the differing potencies of glycine for these two effects. One is that there might be two different affinities of binding at the glycine site[25–27] (possibly one coupled to gating and the other coupled to priming), and the second is that the "coupling gain"[28] for the coupling between glycine binding and channel gating may be different from that for the coupling between glycine binding and priming (see Supplementary Information 4).

Glycine, without co-agonist activation of the glutamate site, does not evoke NMDAR currents, and thus our findings demonstrate that the priming effect of glycine and the recruitment of AP2 to the receptor complex do not require current flow through NMDARs. A core concept about NMDARs is that their primary signalling mechanisms, depolarization and influx of Ca^{2+} are consequences of transmembrane current flow through the receptor, although it has been suggested that NMDARs may signal independently of ionic flux[12]. From the results of our study, we propose a new concept for signalling by NMDARs: transmembrane signal transduction by selectively stimulating the glycine, but not the glutamate, binding site on the receptors. Hence, glycine functions as more than simply a co-agonist for NMDARs, and the glycine and glutamate binding sites of the receptor can have divergent functions. As NMDARs are implicated in a diversity of physiological and pathological processes throughout the central nervous system[1], glycine priming of NMDAR internalization may have a general role in regulating nervous system functioning in normal and disease states.

Methods

CA1 neuron isolation and whole-cell patch clamp recording

CA1 neurons were isolated from hippocampus slices taken from postnatal Wistar rats (17–25 days) using described procedures[29]. Only the neurons that retained their pyramidal shape were used for recordings. Whole-cell recordings were made at room temperature (20–22 °C). After formation of a whole-cell configuration, the recorded neurons were voltage-clamped (−60 mV) and lifted into the stream of solution supplied by a computer-controlled, multi-barrelled fast perfusion system. The extracellular solution was composed of 140 mM NaCl, 1.3 mM $CaCl_2$, 5 mM KCl, 25 mM HEPES, 33 mM glucose and 500 nM tetrodotoxin, with pH 7.35 and osmolarity 330 mosM. The intracellular solution contained 140 mM CsF, 10 mM BAPTA, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES and 4 mM K_2ATP, with pH 7.3 and osmolarity 300 mosM. NMDAR currents were recorded using an Axopatch 1-D amplifier, data were digitized with DigiData1200A, filtered (2 kHz), and acquired by the pClamp8.1 program. Recordings in which the series resistance varied by more than 10% were rejected.

Cell ELISA assay

Assays were carried out essentially as described[6]. Briefly, hippocampal neurons were cultured in 12-well plates (approximately 1.5×10^5 cells per well). After removing the medium, neurons were placed into extracellular solution (ECS) and cooled to 4 °C to inhibit receptor transport. Cells were then incubated with anti-NMDAR1 monoclonal antibody against the extracellular amino terminus of mouse NMDAR1 (Chemicon; 2.5 μg ml^{-1}) for 30 min at 4 °C for the purpose of pre-labelling NMDA receptors on the cell surface. For experiments in Fig. 1, cells were treated at 37 °C for 5 min with ECS or with NMDA and/or glycine and/or AP5 in ECS at concentrations indicated in the legend; after this treatment period the cells were washed with control ECS for 10 min before fixation. ECS was replaced where indicated with ECS containing hypertonic sucrose (0.45 M). For experiments in Fig. 4, cells were pre-treated at 37 °C for 5 min with ECS or with glycine, NMDA and/or L689560 in ECS, at concentrations indicated in the legend. Cells were then treated for 5 min with control ECS or with 50 μM NMDA and 1 μM glycine followed by incubating for 5 min with control ECS before fixation. AP5 or the dynamin peptides were added to the treatment solution as indicated. NMDA in the treatment solution was replaced, as indicated, by glutamate (50 μM). Cells were fixed for 10 min with 4% paraformaldehyde in PBS, and then half of the samples were permeabilized with 0.1% Triton X-100 in PBS for 5 min. The cells were then rinsed with PBS and incubated for 1 h at room temperature with secondary mouse antibody conjugated to horseradish peroxidase (HRP) (1:1,000, Amersham). After washing with D-PBS, HRP substrate OPD was added to produce a colour reaction that was stopped with 0.2 volume of 3N HCl. The optical density of 1 ml of supernatant was read on a spectrophotometer at 492 nm. The rates of cell surface expression of NMDA receptors were presented as the ratio of colorimetric readings under non-permeabilized conditions versus those under permeabilized conditions. Analysis was done using at least six separate dishes in each group.

Immunoprecipitation of AP2 with NMDARs

Hippocampal slices were prepared from postnatal rats (Wistar 20–24 days) as described[29]. Six slices (400 μm) were treated with ECS containing various drugs such as glycine agonists or antagonists for 5 min and then pooled. All the ECS contain strychnine (1 μM), bicuculline (20 μM) and tetrodotoxin (1 μM). The tissue was then homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, protease inhibitors pepstatin A (20 μg ml^{-1}), leupeptin (20 μg ml^{-1}) and aprotinin (20 μg ml^{-1}), and 1 mM phenylmethylsulphonyl fluoride. Insoluble material was removed by centrifugation at 14,000*g* for 10 min at 4 °C. The protein content of soluble material was determined by Bio-Rad Dc protein assay. Soluble proteins (approximately 500 μg) were incubated overnight with 2.5 μg of anti-AP2 adaptin β2 (BD Biosciences). Immune complexes were isolated by addition of 20 μl of protein G–Sepharose beads, followed by incubation for 1–2 h at 4 °C. Immunoprecipitates were then washed four times with lysis buffer, resuspended in laemmli sample buffer, and boiled for 5 min. The samples were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) using a 7% gel, and transferred to a nitrocellulose membrane. Membranes were immunoblotted with a polyclonal antibody to NMDAR2A/2B (1:500, Chemicon). The blot was developed using the enhanced chemiluminescence western blot detection system (Amersham). The membrane was then stripped and reprobed with anti-AP2 (1:3,000, BD Biosciences).

Miniature EPSC recording

Hippocampal neuron primary cultures were used for electrophysiological recordings 14–18 days after plating as described[29] (see also Supplementary Methods).

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Antibody neutralization and escape by HIV-1

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Neutralizing antibodies (Nab) are a principal component of an effective human immune response to many pathogens, yet their role in HIV-1 infection is unclear[1–6]. To gain a better understanding of this role, we examined plasma from patients with acute HIV infection. Here we report the detection of autologous Nab as early as 52 days after detection of HIV-specific antibodies. The viral inhibitory activity of Nab resulted in complete replacement of neutralization-sensitive virus by successive populations of resistant virus. Escape virus contained mutations in the *env* gene that were unexpectedly sparse, did not map generally to known neutralization epitopes, and involved primarily changes in N-linked glycosylation. This pattern of escape, and the exceptional density of HIV-1 envelope glycosylation generally[7,8], led us to postulate an evolving 'glycan shield' mechanism of neutralization escape whereby selected changes in glycan packing prevent Nab binding but not receptor binding. Direct support for this model was obtained by mutational substitution showing that Nab-selected alterations in glycosylation conferred escape from both autologous antibody and epitope-specific monoclonal antibodies. The evolving glycan shield thus represents a new mechanism contributing to HIV-1 persistence in the face of an evolving antibody repertoire.

HIV-1 virions in plasma have an exceedingly short lifespan (<6 hours), as do productively infected lymphocytes (<1.2 days)[9,10]. As a result, the composition of plasma virus provides a sensitive indicator of biologically relevant selection pressures acting on virus and virus-producing cells[11–13]. We amplified full-length HIV-1 gp160 envelope genes by polymerase chain reaction (PCR) from serial plasma specimens of patients with acute and early infection, expressed the genes *in trans* with *env*-deficient HIV-1, and tested progeny virus for neutralization susceptibility in a sensitive, single-round viral infectivity assay using JC53BL-13 cells[13]. Figure 1a depicts inhibition of virus entry by sequential autologous plasma specimens from a patient (WEAU), beginning 16 days after onset of symptoms of the acute retroviral syndrome, and four days before detection of HIV-1 antibodies by enzyme-linked immunosorbent assay. No Nab activity was observed in the initial day 16 patient plasma specimen compared with normal donor plasma against an early day 16 virus envelope (16-2). However, increasing titres of Nab were detected in plasma specimens between days 72 and 212, reaching half-maximal and 90% maximal inhibitory concentrations (IC_{50} and IC_{90}) of 0.0007 ± 0.0002 and 0.0032 ± 0.0023, respectively. Between days 391 and 772, Nab titres to this early virus declined. Nab activity in patient plasma could be eliminated by pre-treatment of plasma with staphylococcal protein G (SPG), reconstituted by IgG eluted from SPG, and virus pseudotyped with the vesicular stomatitis virus G protein (instead of HIV-1 gp160) was not neutralized by patient plasma (data not shown).