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Mobility of NMDA autoreceptors but not postsynaptic receptors at glutamate synapses in the rat entorhinal cortex

Jian Yang[1], Sophie E. L. Chamberlain[1], Gavin L. Woodhall[2] and Roland S. G. Jones[1]

[1] *Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK*
[2] *The Molecular Biosciences Research Group, School of Life and Health Sciences, Aston University, Birmingham B4 7ET, UK*

NMDA receptors (NMDAr) are known to undergo recycling and lateral diffusion in postsynaptic spines and dendrites. However, NMDAr are also present as autoreceptors on glutamate terminals, where they act to facilitate glutamate release, but it is not known whether these receptors are also mobile. We have used functional pharmacological approaches to examine whether NMDA receptors at excitatory synapses in the rat entorhinal cortex are mobile at either postsynaptic sites or in presynaptic terminals. When NMDAr-mediated evoked EPSCs (eEPSCs) were blocked by MK-801, they showed no evidence of recovery when the irreversible blocker was removed, suggesting that postsynaptic NMDAr were relatively stably anchored at these synapses. However, using frequency-dependent facilitation of AMPA receptor (AMPAr)-mediated eEPSCs as a reporter of presynaptic NMDAr activity, we found that when facilitation was blocked with MK-801 there was a rapid (∼30–40 min) anomalous recovery upon removal of the antagonist. This was not observed when global NMDAr blockade was induced by combined perfusion with MK-801 and NMDA. Anomalous recovery was accompanied by an increase in frequency of spontaneous EPSCs, and a variable increase in frequency-facilitation. Following recovery from blockade of presynaptic NMDAr with a competitive antagonist, frequency-dependent facilitation of AMPAr-mediated eEPSCs was also transiently enhanced. Finally, an increase in frequency of miniature EPSCs induced by NMDA was succeeded by a persistent decrease. Our data provide the first evidence for mobility of NMDAr in the presynaptic terminals, and may point to a role of this process in activity-dependent control of glutamate release.

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Corresponding author R. S. G. Jones: Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath BA2 7AY, UK. Email: r.s.g.jones@bath.ac.uk

Synaptic transmission is a dynamic and plastic process, modified by short, intermediate and long-term regulatory mechanisms. Synaptic strength can be modulated by presynaptic receptors, which provide activity-dependent control of transmitter release. Presynaptic ionotropic receptors have received increasing attention recently (see Engelman & Macdermott, 2004 for review). AMPA receptors (AMPAr; Patel & Croucher, 1997; De Paola *et al.* 2003), NMDA receptors (NMDAr; Berretta & Jones, 1996; Li & Han, 2007; Woodhall *et al.* 2001) and kainate receptors (Agrawal & Evans, 1986; Malva *et al.* 1995; Chittajallu *et al.* 1996; Negrete-diaz *et al.* 2006) can all act as autoreceptors on glutamatergic terminals, and as heteroreceptors on GABA terminals (Bureau & Mulle, 1998; Kullmann & Semyanov, 2002; Duguid & Smart, 2004).

At postsynaptic sites, trafficking of membrane receptors is a determinant of moment-to-moment and long-term modulation of synaptic efficacy (see Carroll & Zukin, 2002; Bredt & Nicoll, 2003; Perez-Otano & Ehlers, 2005; Triller & Choquet, 2005). Interest in receptor mobility has been driven by the demonstration that AMPAr are rapidly inserted or deleted at glutamate synapses during long-term potentiation (LTP) and depression (LTD) (see Ehlers, 2000; Malinow & Malenka, 2002; Song & Huganir, 2002; Bredt & Nicoll, 2003; Collingridge *et al.* 2004). NMDAr were originally thought to be stably anchored at the postsynaptic density (PSD; Ehlers, 2000; Scannevin & Huganir, 2000; Bredt & Nicoll, 2003) but it is now apparent that they also undergo regulated trafficking and that this may be intimately involved in long-term synaptic plasticity (Carroll & Zukin, 2002; Nong *et al.* 2004; van Zundert *et al.* 2004; Perez-Otano & Ehlers, 2005)

The focus regarding glutamate receptor mobility has been on recycling of receptors between the synaptic membrane and intracellular pools. However, diffusion

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of receptors within cell membranes may also regulate synaptic efficacy. Glycine (Meier *et al.* 2001; Dahan *et al.* 2003), $GABA_A$ (Jacob *et al.* 2005; Thomas *et al.* 2005), AMPAr (Borgdorff & Choquet, 2002; Groc *et al.* 2004) and NMDAr (Groc *et al.* 2004) have all been shown to undergo lateral diffusion in the postsynaptic membrane primarily using direct imaging and optical tracking. Lateral diffusion of postsynaptic NMDAr has been demonstrated using a pharmacological approach (Tovar & Westbrook, 2002; Zhao *et al.* 2008), in which irreversible block of NMDAr responses by MK-801 at synapses on hippocampal neurones was followed by an anomalous recovery during washout of the blocker. This suggested that receptors that were use-dependently blocked following activation by glutamate at the PSD could be replaced by non-blocked receptors from a distal source. A number of experimental approaches indicated that this replacement occurred by lateral diffusion of receptors from extrasynaptic sites in the postsynaptic membrane rather than by insertion of new receptors from cytosolic stores (Tovar & Westbrook, 2002; Zhao *et al.* 2008).

In the entorhinal cortex (EC) presynaptic NMDAr facilitate spontaneous release at glutamate synapses (Berretta & Jones, 1996; Woodhall *et al.* 2001; Yang *et al.* 2006). These receptors also mediate short-term, frequency-dependent facilitation of excitatory transmission (Berretta & Jones, 1996; Woodhall *et al.* 2001; Sjöström *et al.* 2003; Brasier & Feldman, 2008; Chamberlain *et al.* 2008), and may play a role in LTP; Humeau *et al.* 2003; Samson & Pare, 2005), LTD; (Sjöström *et al.* 2003; Bender *et al.* 2006; Corlew *et al.* 2007), and activity-dependent signalling via astrocytes (Jourdain *et al.* 2007). The role of presynaptic NMDAr in synaptic plasticity could be modulated by mobility of receptors in the terminal membrane. In the present study we used physiological approaches to examine whether pre- or postsynaptic NMDAr undergo lateral diffusion at glutamate synapses in the EC. Some of these results have been published in abstract form (Yang *et al.* 2008).

Methods

Ethical information

Experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, European Communities Council Directive 1986 (86/609/EEC) and the University of Bath ethical review document.

Slice preparation

Slices containing EC and hippocampus were prepared from male Wistar rats (3–5 weeks), which were anaesthetized with an I.M. injection of ketamine ($120\ mg\ kg^{-1}$) plus xylazine ($8\ mg\ kg^{-1}$) and decapitated. The brain was rapidly removed and immersed in oxygenated artificial cerebrospinal fluid (aCSF) chilled to 4°C. Slices (350–400 μm) were cut using a Vibroslice, and stored in aCSF bubbled with 95% O_2–5% CO_2, at room temperature. Following recovery for at least 1 h, individual slices were transferred to a recording chamber mounted on the stage of a Zeiss Axioskop FS or an Olympus BX50WI microscope. The chamber was perfused ($2.5\ ml\ min^{-1}$) with oxygenated aCSF (pH 7.4) at 31–33°C. The aCSF contained (in mM): NaCl (126), KCl (3), NaH_2PO_4 (1.25), $NaHCO_3$ (24), $MgSO_4$ (2), $CaCl_2$ (2), and D-glucose (10). Neurones were visualized using differential interference contrast optics and an infrared video camera.

Electrophysiological recordings

Patch pipettes were pulled from borosilicate glass on a Flaming/Brown microelectrode puller. For recording spontaneous (sEPSCs), miniature (mEPSCs) or evoked (eEPSCs) EPSCs, pipettes were filled with a caesium gluconate-based solution containing (in mM): D-gluconate (100), Hepes (40), QX-314 (1), EGTA (0.6), NaCl (4), $MgCl_2$ (5), TEA-Cl (1), ATP-Na (4), GTP-Na (0.3), MK-801 (2). Solutions were adjusted to $290\ mosmol\ l^{-1}$, and to pH 7.25–7.3 with CsOH. Whole-cell voltage clamp recordings (holding potential –60 mV unless otherwise stated) were made from neurones in layer V of the medial division of the EC, using an Axopatch 200B amplifier. Series resistance compensation was not employed, but access resistance (10–30 MΩ) was monitored at regular intervals throughout each recording and cells were discarded from the analysis if it changed by more than ±10%. Liquid junction potentials were estimated using the calculator of pCLAMP 8 software, and compensated for in the holding potentials.

eEPSCs were elicited by electrical stimulation (bipolar pulses, 10–50 V, 0.02 ms duration) via a bipolar tungsten electrode placed on the surface of the slice in layer V of the lateral EC. The stimulation intensity was adjusted to give a submaximal (approx. 60% maximum amplitude) response. Unless otherwise stated, MK-801 was included in the patch pipettes allowing us to record AMPA-mediated responses in isolation and to monitor activity at presynaptic NMDAr uncontaminated by post-synaptic receptor effects, an approach that we (Berretta & Jones, 1996; Woodhall *et al.* 2001; Yang *et al.* 2006, 2007), and others (Sjöström *et al.* 2003; Samson & Pare, 2005; Bender *et al.* 2006; Jourdain *et al.* 2007; Li & Han, 2007; Brasier & Feldman, 2008), have used successfully to block postsynaptic NMDAr in recorded neurones.

Determination of receptor mobility

Postsynaptic. We used an approach described by Tovar & Westbrook (2002) and used by others (Harris & Pettit, 2007; Zhao *et al.* 2008) to monitor postsynaptic NMDAr mobility. Repeated stimulation delivered in the presence of the channel blocker, MK-801 results in progressive block of NMDAr accessed by glutamate released during synaptic activation. As this block is essentially irreversible, recovery of the synaptic response after washout of MK-801 provides a reporter of receptor mobility. A modification of this approach was used here to monitor the mobility of postsynaptic NMDAr in the EC in two series of experiments. Both were conducted with an AMPAr antagonist (NBQX), and a $GABA_A$r antagonist (bicuculline) in the bath. In the first study, MK-801 was *not* included in the patch pipette solution. When whole-cell access was achieved, neurones were held at +40 mV and isolated, long duration NMDAr-mediated EPSCs were evoked at low frequency (0.05 Hz). These vary in their deactivation kinetics from neurone to neurone (see Fig. 1*A*) but can be abolished by NMDAr antagonists (Chamberlain *et al.* 2008). When eEPSC amplitudes were stable, MK-801 (10 μM) was bath applied for a period of 5 min. Four minutes after MK-801 perfusion commenced, stimulation frequency was increased to 5 Hz for 40–50 s, resulting in a rapid decline in eEPSC amplitude to around 0–10% of the amplitude recorded at low frequency. Stimulation frequency was then restored to 0.05 Hz, and MK-801 was washed out of the bath. Responses to low frequency stimulation were then monitored for the remaining lifetime of the recording (e.g. Fig. 2). After 35 min, all stimulation was halted for a period of 15 min, before being resumed at low frequency until the end of the recording.

In a second series of experiments, the same protocol was followed, but MK-801 was omitted from the external perfusion and included in the patch pipette. Again, when whole-cell access was achieved, NMDAr-mediated eEPSCs were recorded at low frequency (0.05 Hz). After 6 min, stimulation frequency was increased to 5 Hz for 40–50 s and this resulted in a rapid decrease of the eEPSCs due to blockade of the postsynaptic NMDAr by intracellular MK-801 dialysed into the cell via the patch pipette. When the eEPSCs had reached a stable low level, stimulation was returned to 0.05 Hz, and recovery of the responses monitored for the lifetime of the patch recordings (Fig. 2).

Presynaptic. Monitoring presynaptic receptor mobility is more difficult. In addition to the electrophysiological approach (Tovar & Westbrook, 2002; Harris & Pettit, 2007), optical tracking of quantum dot or fluorescent antibody-labelled receptors has been used to study postsynaptic receptor trafficking (e.g. Groc *et al.* 2004; Washbourne *et al.* 2004). These approaches are complicated at presynaptic sites because the size of terminals precludes direct electrophysiological access, or sufficient visual resolution. Direct imaging of the movement of receptors in spines and dendrites has been largely conducted in cultured neurones and applying this to terminals in native tissue would be technically more difficult. It requires specific labelling of presynaptic receptors without contamination by those at postsynaptic sites, and a very high degree of visual spatial resolution to visualize and track receptor movement in very small terminals.

To overcome these limitations we have developed a variation of the functional pharmacological approach (Tovar & Westbrook, 2002; Harris & Pettit, 2007) to test for the mobility of presynaptic NMDAr in EC slices. All experiments were conducted with MK-801 in the patch pipette. When whole-cell access was gained, neurones were voltage clamped at 0 mV, and synaptic stimulation delivered at 5 Hz for 30–40 s to allow blockade of postsynaptic NMDAr by MK-801 dialysed into the cell via the patch pipette solution. Membrane potential was then clamped at –60 mV and single shock stimulation delivered at low frequency (0.05 Hz) to evoke isolated AMPAr-mediated EPSCs. At intervals of 2 min, the single shock was replaced with stimulation at 3 Hz for 5 s. Such stimulation results in a frequency-dependent facilitation of the AMPAr-mediated EPSC, which we have shown previously to arise primarily from activation of presynaptic NMDAr (Woodhall *et al.* 2001; Chamberlain *et al.* 2008). We used the degree of frequency-dependent facilitation of AMPAr-mediated eEPSCs as a quantitative measure of presynaptic NMDAr activation. After a control recording of 3–4 episodes of 3 Hz stimulation, with single shock stimulation at 0.05 Hz restored between each episode, MK-801 (10 μM) was bath applied for 5 min and then washed out for the remaining lifetime of the recording (see Figs 3, 4, 5 and 7). The stimulation protocol continued uninterrupted during the period of MK-801 application and during the remainder of the recording. The protocol thus included at least 3–5 periods of stimulation at 3 Hz in the presence of MK-801 (including those occurring when the blocker was being washed from the bath, see Fig. 1*A*), and this was sufficient to cause use-dependent block of frequency-facilitation. In a second group of neurones the stimulation protocol was completely halted for a period of 15 min starting approximately 6 min after the beginning of the MK-801 wash. The rationale for this was to determine if the stimulation, and subsequent evoked release of glutamate, could elicit a use-dependent unbinding of the blocker from the receptors and hence be responsible for recovery. In a third group, the same experimental protocol was employed except that MK-801 perfusion was accompanied by bath perfusion with NMDA (25 μM), and both agents were subsequently washed out. The latter

studies aimed to determine the effects of global blockade of NMDAr on the terminals.

Control studies

MK-801 washout. The majority of the studies involved monitoring response parameters (pre- or postsynaptic) after removal of MK-801 from the bath, and relied on its ability to irreversibly block NMDAr. It was important to attempt to monitor the rate of removal of the drug from the bath after termination of perfusion. The period of application (5 min in all experiments) was timed from the moment the drug solution reached the bath, to the moment it was replaced by drug-free ACSF. The recording chamber had a volume of approximately 500 μl, so with a perfusion rate of 2.5 ml min^{-1} the bath solution should be completely exchanged 4–5 times per minute during washout. Since NMDAr blockade is use dependent, we monitored the washout of MK-801 using the following protocol. Isolated NMDAr-mediated eEPSCs were recorded as above. Control responses were evoked every 15 s for 5–7 min, and then stimulation was halted prior to perfusion with MK-801 for 5 min. Repetitive stimulation (2 Hz for 50 s) was then delivered 4 min into the MK-801 perfusion ($n = 8$), or 2, 7, 12, or 17 min ($n = 3$ in each case) after the start of washout, followed by a return to low frequency stimulation. Responses were averaged over the subsequent 2 min for comparison.

Stability of NMDAr-mediated effects. Experiments on postsynaptic NMDAr mobility rely on the stability of the evoked responses over time. To control for this, in seven neurones we employed the protocol used to monitor postsynaptic mobility (see above) but without the addition of MK-801 to the bath perfusion or the patch pipette. Likewise, meaningful experiments on presynaptic receptor mobility are dependent on frequency-dependent facilitation of AMPAr-mediated EPSCs remaining stable, without rundown over time. To monitor this, in eight neurones we performed experiments designed to monitor presynaptic mobility as described above, but again, without addition of MK-801 to the bath perfusion.

Data analysis

Data were recorded to computer hard disk using Axoscope software. Minianalysis (Synaptosoft, USA) was used for analysis of EPSCs off-line. In experiments with postsynaptic NMDAr, the mean peak amplitudes of at least five NMDAr-mediated eEPSCs evoked at low frequency were determined every 2 min in the control period before the high frequency stimulation, and subsequently throughout the study. In the studies of presynaptic NMDAr, the average peak amplitude of the 5–6 responses before each episode of 3 Hz stimulation was determined. During the period of 3 Hz stimulation the amplitude of the 5–6 largest events was determined and normalized to the average amplitude of the preceding low frequency events prior to it. Inter-event interval (IEI), amplitude, rise (10–90%) and decay times of AMPAr-mediated sEPSCs and mEPSCs were also determined in some studies of presynaptic NMDAr. Events were detected automatically using a threshold-crossing algorithm. Threshold varied from neurone to neurone but was always maintained at a constant level in any given recording. At least 200 events were sampled during a continuous recording period for each neurone under each condition. Cumulative probability distributions of IEI were compared using the Kolmogorov–Smirnoff (K–S) test. The statistical significance of changes in amplitudes and frequencies was determined with a paired *t* test or one-way ANOVA. All error values indicated in the text and figures refer to S.E.M.

Materials

Salts used in preparation of aCSF were 'Analar' grade and purchased from Merck/BDH (UK). All drugs were applied by bath perfusion. NMDA, MK-801 ((+)-dizocilpine maleate), NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), D-2-AP5 (D-2-amino-5-phosphonovalerate), Ro 25-6981 ((α*R*,β*S*)-α-(4-hydroxy-phenyl)-β-methyl-4-(phenylmethyl)-1-piperidinepropanol hydrochloride) and bicuculline methiodide were obtained from Tocris (UK). TTX came from Alomone Laboratories (Israel).

Results

Control studies

Studies of both pre- and postsynaptic receptor mobility (see below) rely on monitoring recovery after blockade of NMDAr with MK-801. Experiments to determine the rate of washout of the blocker from the slices after a brief period (5 min) of bath perfusion are illustrated in Fig. 1*A*. During perfusion with MK-801 ($n = 4$), isolated NMDAr-mediated eEPSCs were blocked when repetitive stimulation was applied at 2 Hz. A similar stimulation protocol applied 2 min after the start of washout ($n = 3$) was equally effective, showing that sufficient MK-801 was still available in the bath/slices to exert a use-dependent block of the receptors. However, when stimulation was delayed by 7 min after the start of washing ($n = 3$) responses were only reduced by around 50–60%. After 12 min, repetitive stimulation was much less effective ($n = 3$) and with a further 5 min delay (17 min, $n = 3$) blockade was virtually absent. The data

indicate that MK-801 washes relatively rapidly (around 15 min) from the bath/slices, following brief applications at the perfusion rate and bath volume employed in all experiments described below.

We also determined the stability of postsynaptic NMDA-mediated responses in seven neurones (Fig. 1*B*). During repetitive stimulation (MK-801 absent), the slow eEPSCs decreased in amplitude by around 10–30%. Control amplitudes recovered within 3–4 min. Subsequently there was a weak but consistent increase over the next 15–25 min, although this did not reach significance at any point.

Figure 1*C* shows the stability of the frequency-dependent facilitation of AMPAr-mediated eEPSCs used to monitor presynaptic NMDAr-mediated activity ($n = 8$). MK-801 was present in the patch solution in

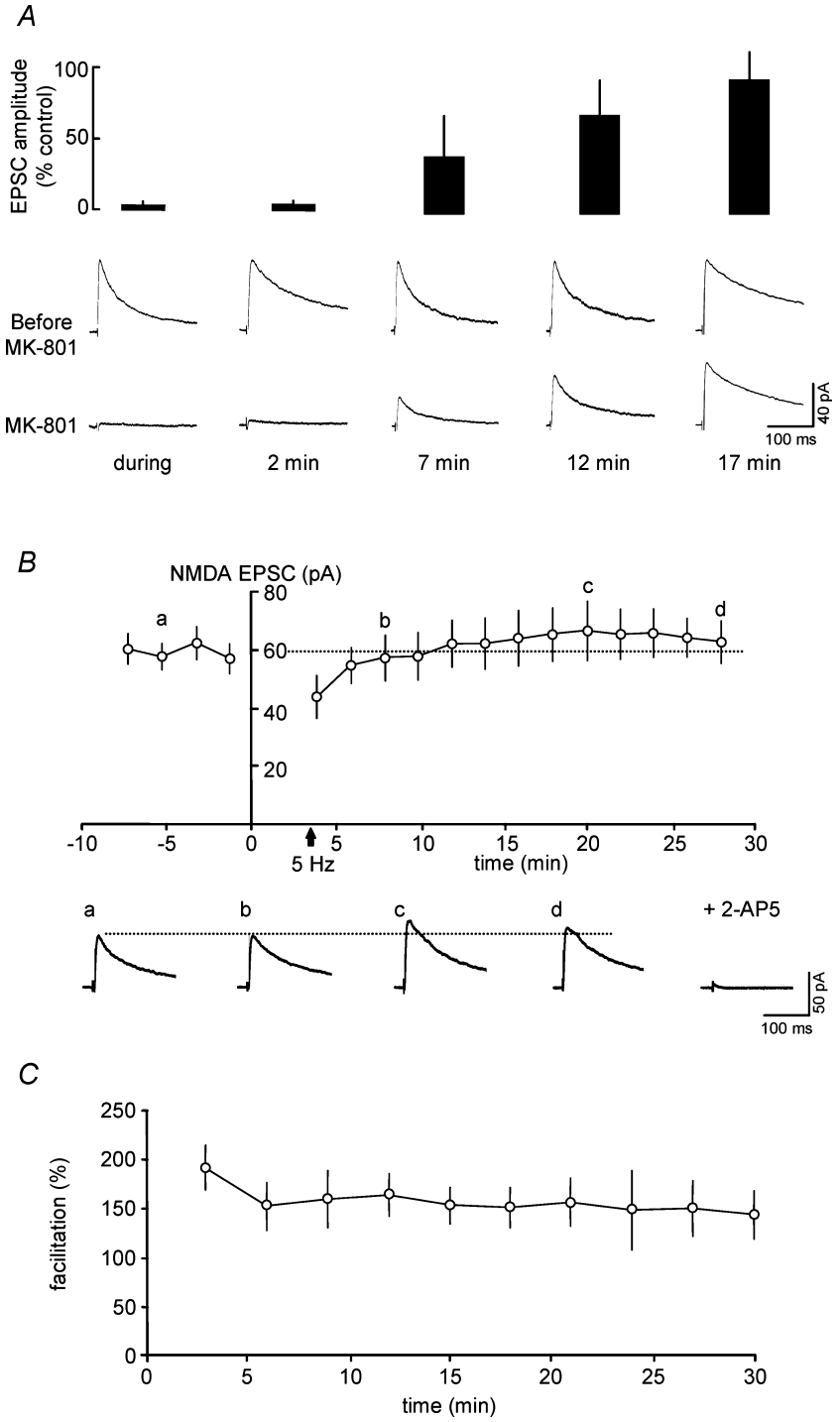

Figure 1. Washout of MK-801 and stability of NMDAr-mediated responses
A, isolated NMDA EPSCs were recorded in the presence of NBQX and bicuculline at a holding potential of +40 mV. Responses were evoked by repetitive stimulation (2 Hz, 40–50 s) before the application of MK-801 (5 min), during its application and at various time points after. The bars show the average reduction in eEPSC amplitude at each time point. The responses below are averages of 6–7 responses recorded in selected individual neurones at each corresponding time point, before or after application of MK-801. The time course of recovery of events at the 7 and 12 min time points is shown in Fig. 2*C*. *B*, stability of postsynaptic NMDAr EPSCs in the absence of MK-801. Each point is the average of 6–8 responses. During repetitive stimulation at 5 Hz for 40–50 s, eEPSC amplitude decreased overall, but recovered to control levels within 3 min. Thereafter, responses were stable. The slight increase noted around 15–25 min was consistent, but not significant. *C*, stability of frequency-dependent facilitation of AMPAr eEPSCs. Frequency-facilitation was quantified as described in the text (low frequency stimulation at 0.05 Hz interleaved with episodes of stimulation at 3 Hz for 5 s) and monitored at 3 min intervals. The graph shows pooled data from 8 neurones. There was an initial decline in facilitation after the first episode but thereafter it remained stable. Stimulation artefacts in the records shown here and in all subsequent figures have been partly blanked for clarity.

four of these studies but absent in the remainder. As there was no detectable difference between groups they were pooled. There was a small but consistent decline in facilitation between the first and second episodes of repetitive stimulation, but thereafter it remained stable throughout the 30 min of recording.

Mobility of postsynaptic NMDAr

The pharmacological approach (see Tovar & Westbrook, 2002; Harris & Pettit, 2007; Zhao *et al.* 2008) used utilizes the properties of blockade by MK-801 to assess mobility of postsynaptic NMDAr. In our first set of experiments we recorded isolated NMDAr-mediated eEPSCs. In these experiments, MK-801 was *not* included in the patch pipette. As noted above, these responses were stable when evoked at low frequency, but rapidly reduced or abolished when the stimulation frequency was increased to 5 Hz in the presence of bath-applied MK-801.

The results from one experiment are shown in Fig. 2*Aa*. The responses were recorded in the presence of bicuculline and NBQX. Each trace is the average of 5–6 responses. Stimulation at 5 Hz in the presence of MK-801 dramatically reduced the NMDAr EPSC. Responses recorded 2 min and 35 min after the start of washout of MK-801 show that they remain blocked and there is no evidence for recovery of the post-synaptic NMDAr-mediated eEPSCs. The time course of experiments in nine neurones is shown in Fig. 2*Ab* and clearly shows the lack of recovery of responses at any point during washout (up to 70 min in some neurones). The use-dependent nature of MK-801 antagonism means that only receptors accessed by glutamate at the activated synapses will be blocked. However, the failure of the eEPSCs to recover during washout suggests that these are not replaced by non-blocked receptors moving into the synapses from distal extrasynaptic sites or by receptors from cytoplasmic storage sites. Note also that all stimulation was halted for a period of 15 min during washout (dotted line), but that responses remained at close to zero when it was restarted. Although control experiments (Fig. 1*A*) clearly show that there is little MK-801 remaining in the bath/slices after 15 min, we wanted to check the possibility that failure to recover was due to residual MK-801, or to rebinding of MK-801 recently dissociated from the receptors. The fact that recovery was absent whether or not stimulation was delivered indicates that this was not the case. Finally, although our control experiments to monitor stability of the NMDAr test responses (Fig. 1*B*) only extended to 30 min compared to the 70 min tested here, it was clear that the lack of recovery could not be attributed to an extensive or permanent rundown of the postsynaptic responses. Thus, the experiments suggest that mobility of NMDAr at these synapses in the EC is limited, or that if it does occur, it may be much slower compared to CA1 (see Tovar & Westbrook, 2002; Zhao *et al.* 2008). However, it should also be noted that Harris & Pettit (2007) have conducted similar experiments and found no evidence for receptor mobility in CA1.

We performed a second set of experiments ($n = 9$) with a slightly different approach and these are illustrated in Fig. 2*B*. In these neurones MK-801 was included in the patch pipette instead of being bath applied. Internal dialysis with MK-801 via the patch pipette begins following whole-cell access, but NMDAr EPSCs could still be evoked over the control period of recording with the stimulation at low frequency (0.05 Hz; Fig. 2*B*), although there was a slow, progressive decline in amplitude. This was rapidly accelerated when high frequency (5 Hz) stimulation was applied, as a result of the use-dependent block of the postsynaptic receptors. On average, the reduction in amplitude of the slow eEPSC by internal MK-801 ($84 \pm 7\%$) was slightly less than that seen with bath-applied MK-801 ($94 \pm 4\%$), suggesting that some postsynaptic NMDAr may have remained unblocked. However, as was the case with bath-applied MK-801, a return to low frequency stimulation was not associated with recovery of the eEPSCs, whether stimulation was applied or not (Fig. 2*B*). In these experiments we confirmed that the small residual eEPSC was NMDAr-mediated by application of 2-AP5. These data support the suggestion that the mobility of postsynaptic NMDAr is low. They also show that this lack of mobility is a characteristic of the synapses on the layer V neurones themselves, since only the NMDAr in the recorded neurones will be blocked and any NMDAr-mediated poly-synaptic events will remain unaltered.

One possible explanation for the lack of mobility could be that the repetitive stimulation is sufficient to cause spillover of glutamate resulting in blockade of both synaptic and extrasynaptic receptors. Harris & Pettit (2008) showed recently that significant recruitment of extrasynaptic NMDAr at synapses in CA1 required stimulation at 25 Hz or above, although they utilized much shorter trains of stimuli. Of course, the situation may be different at layer V synapses, but by limiting the stimulation frequency to 5 Hz in our experiments we hoped to avoid blockade of extrasynaptic receptors and so give ourselves the best chance of detecting receptor mobility. It should be noted that, in the experiments where MK-801 was present internally, there was a small, NMDAr-mediated EPSC remaining after the period of 5 Hz stimulation, suggesting that some of the postsynaptic receptors were not blocked. However, there was still no evidence of recovery during the subsequent 60 min. In addition, in the experiments where we monitored MK-801 washout (Fig. 1*A*) we used a lower stimulation frequency (2 Hz). When stimulation was delayed until

the blocker was already being removed from the bath the residual NMDAr eEPSC clearly indicated that a substantial proportion of the postsynaptic NMDAr (whether synaptic or extrasynaptic) were unblocked, but still there was no recovery (Fig. 2*C*). Finally, Scimemi *et al.* (2004) showed that glutamate spillover could result in MK-801-induced blockade of NMDAr at synapses some distance apart, even when stimulation was delivered at only 0.25 Hz. We have found that if 5 Hz stimulation is replaced with low frequency (0.05 Hz) stimulation at layer V synapses, substantial blockade of postsynaptic NMDAr does not occur within the 5 min perfusion of MK-801 employed

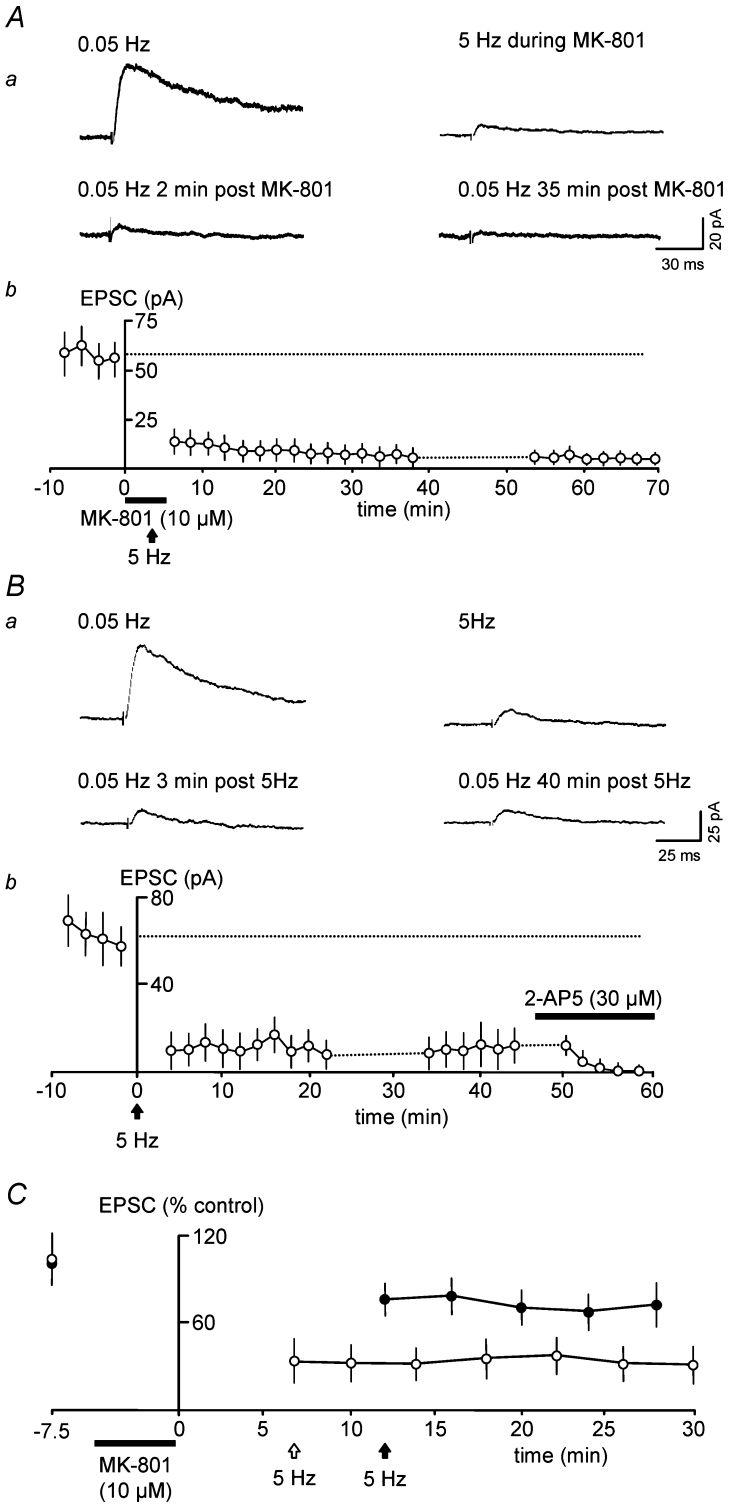

Figure 2. Lack of mobility of postsynaptic NMDAr
Aa, synaptic responses evoked at +40 mV in the presence of NBQX (10 μM) and bicuculline (20 μM), but *without* MK-801 in the patch pipette. The NMDAr-mediated EPSC (0.05 Hz, 5 events averaged) was dramatically reduced by stimulation at 5 Hz for 40–50 s (last 5 events of train averaged) in the presence of bath-applied MK-801. After MK-801 was washed out and stimulation restored to 0.05 Hz there was no recovery of the responses (5 events averaged in each case) in the short or long term. *Ab,* time course of experiments pooled from 9 neurones. Each point is the average of mean amplitudes of EPSCs at each time interval in the 9 neurones. At some of the later time points (60 min plus) not all neurones are included as some recordings were lost at this stage. During the period indicated by the dotted line, low frequency (0.05 Hz) stimulation was halted. *Ba,* similar protocol to *A*, except that MK-801 was included in the patch pipette and omitted from the bath perfusion. The results were essentially the same. *Bb*, pooled data from 9 neurones. In these experiments there was usually a small residual EPSC, which was blocked by 2-AP5. *C,* time course data for the neurones at the 7 min (○, $n = 3$) and 12 min (●, $n = 3$) time points illustrated in Fig. 1*A*. No recovery of eEPSC amplitude was seen during the washout of MK-801.

in our studies, and requires perfusion periods of around 20 min, with the attendant problems of a much extended period of washout. However, we have done this in two neurones and found that even with the very low frequency there is no suggestion of any recovery over a subsequent 35 min of washout of MK-801 (not shown). Thus, we feel confident that the lack of recovery of the NMDAr response is due to a lack of mobility rather than due to a global blockade of all postsynaptic receptors.

Mobility of presynaptic receptors

Unless otherwise indicated, MK-801 was included in the patch pipette to block postsynaptic NMDAr in these experiments. Under these conditions eEPSCs are mediated primarily by postsynaptic AMPAr (Berretta & Jones, 1996; Woodhall *et al.* 2001). As noted above, we used frequency-dependent facilitation of AMPAr-mediated eEPSCs (Woodhall *et al.* 2001; Chamberlain *et al.* 2008) as a reporter of the function of presynaptic NMDAr in order to investigate their mobility. To quantify frequency-facilitation, responses evoked at low frequency (0.05 Hz) were interspersed with periods of high frequency stimulation, and response amplitudes during the latter normalized to the average amplitude of the preceding low frequency events. When frequency-dependent facilitation was stable, it was then blocked by bath perfusion of MK-801 for 5 min, and the blocker then washed out. Alternate periods of low and high frequency stimulation were continued uninterrupted throughout the application of MK-801 and washout period.

In the first set of experiments we examined changes in facilitation, recorded uninterrupted, after a brief application of MK-801 in 10 neurones. Averaged eEPSCs from one neurone are illustrated in Fig. 3*A*. The frequency-dependent facilitation of eEPSCs during 3 Hz stimulation is readily apparent. During application of MK-801 this was abolished but low frequency responses were unaffected. However, in contrast to the postsynaptic NMDAr-mediated responses, it is clear that after MK-801 was washed out, frequency-facilitation mediated by presynaptic NMDAr progressively recovered towards control levels over a period of 30–40 min. The time course of this recovery in 10 neurones is shown in Fig. 3*B*. Initially, the average change in response amplitude during 3 Hz stimulation became slightly negative because a weak frequency-dependent depression of eEPSCs was often seen when facilitation was blocked. However, this was quickly replaced by facilitation again, which progressively increased after MK-801 was washed out. Facilitation was more variable than prior to application of the blocker, but had returned to control levels by 40–45 min. Note that these experiments, and those below employed the same recording chamber, perfusion rate and period of application of MK-801 employed in the experiments on postsynaptic NMDAr EPSCs. Although we have not conducted experiments using frequency-facilitation to monitor MK-801 washout we would expect that the blocker would have a similar time course of availability at both the presynaptic receptors and postsynaptic receptors and that washout of MK-801 would be complete after about 15 min. Frequency-facilitation was monitored in the absence of any drugs (Fig. 1*C*) and was stable over at least 30 min, so state-dependent alterations in presynaptic receptor sensitivity were unlikely to influence recovery.

Thus, anomalous recovery of frequency-dependent facilitation provides a strong indication that NMDAr in the presynaptic membrane may be relatively mobile, particularly compared to their postsynaptic counterparts. Presynaptic NMDAr, close to the release sites and activated by glutamate, will be use-dependently blocked by MK-801 during repetitive stimulation. They should remain blocked during and after washout of MK-801, as its binding in the channel is essentially irreversible. The anomalous recovery of facilitation suggests that the blocked presynaptic receptors may be replaced at the release sites by others that were originally outside the range of the released glutamate, did not undergo a use-dependent block, and were able to move into the vicinity of the release sites.

An alternative possibility is that the presynaptic NMDAr are stably anchored at release sites, and that recovery of facilitation occurred as a result of use-dependent unblocking of these receptors by glutamate released as a result of stimulation in the washout period. If this were the case we would expect that halting stimulation would negate recovery during this period, and that recovery would only occur when the stimulation was recommenced. We examined this possibility in seven neurones. Results from one neurone are illustrated in Fig. 4*A*. Facilitation was again abolished by bath application of MK-801. Shortly following the start of the washout period, all stimulation (both high and low frequency) was halted for 15 min. However, when stimulation was recommenced, it was clear that recovery had occurred despite the hiatus. Summary data for the seven neurones is shown in Fig. 4*B*. Allowing for the period of no stimulation, the time course of recovery was remarkably similar to that seen in neurones where the stimulus protocol was unchecked throughout (Fig. 3). In these experiments we applied NMDAr antagonists after recovery to confirm that the facilitation seen during anomalous recovery was due to activation of presynaptic NMDAr. In four neurones we used 2-AP5 (30 μM) and this rapidly abolished facilitation. We have recently shown that frequency-dependent facilitation in layer V is mediated by NMDAr containing the NR2B subunit (Chamberlain *et al.* 2008). In three further studies we used the NR2B-selective antagonist, Ro 25-6981 (500 nM), and this also abolished facilitation (see Fig. 7*A*), showing that the presynaptic receptors that replace those

following MK-801 blockade are likely to be the same as those initially present at release sites. Because 2-AP5 and Ro 25-6981 had the same effect these studies have been pooled in Fig. 4*B*.

The use-dependent nature of MK-801 blockade means that only receptors accessed by glutamate in the vicinity of the release sites will be blocked. Recovery would then result from non-blocked receptors moving from distal inaccessible sites on or in the terminals. It follows then that if we are able to globally block these receptors then recovery should not occur. We investigated this in a third set of experiments. We combined bath application of MK-801 with concurrent bath application of NMDA ($25\ \mu$M) to globally block surface-expressed NMDAr. Results from one neurone, together with pooled data from the seven neurones tested, are shown in Fig. 5. The concurrent application of MK-801 and NMDA rapidly abolished frequency-dependent facilitation. However, prolonged washout of both agents was not accompanied by anomalous recovery. Thus, these studies provide strong evidence that the recovery of facilitation shown in the previous experiments was due to the presence of non-blocked receptors in the distal terminal membrane, able to move into the proximity of the glutamate release

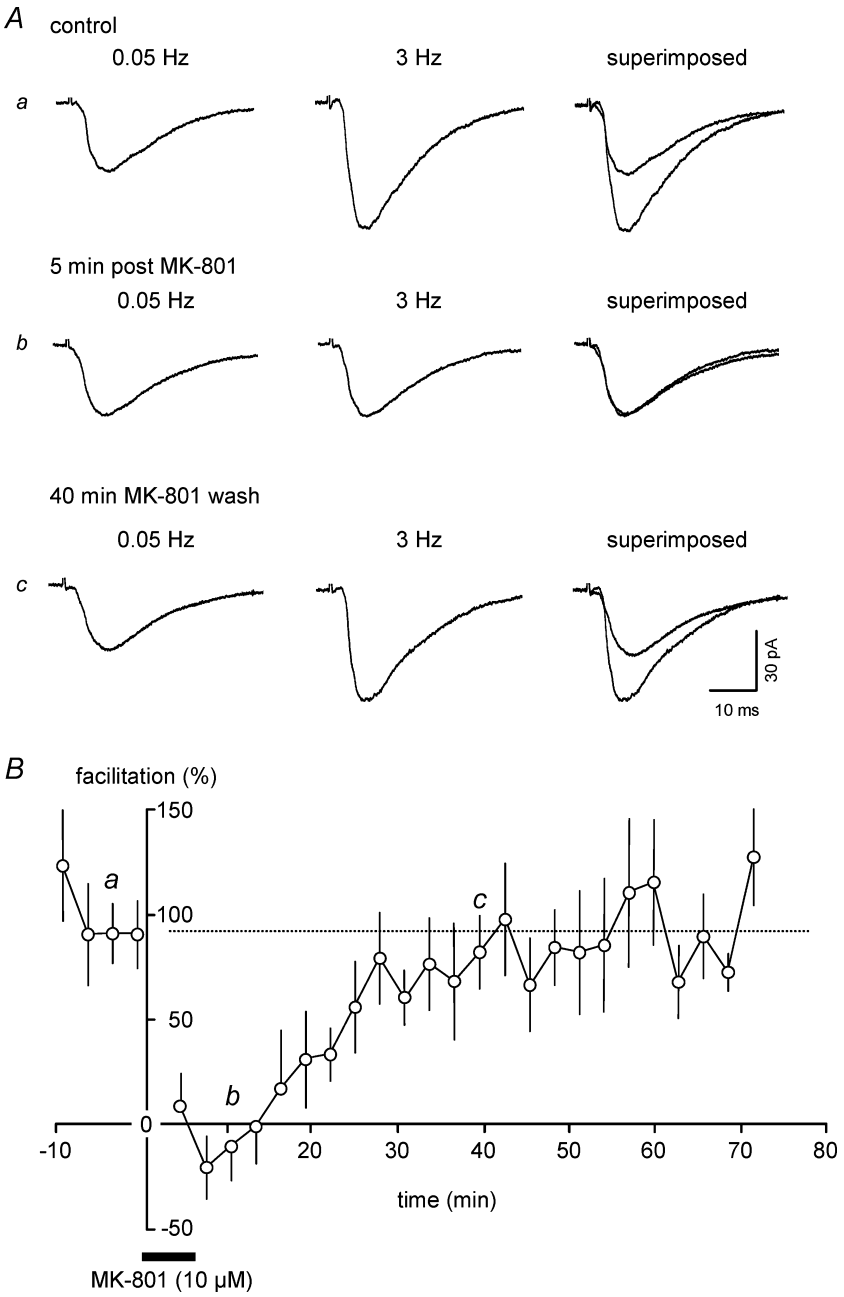

Figure 3. Mobility of presynaptic NMDAr
The responses are AMPAr-mediated eEPSCs (with MK-801 in patch pipette, average of 5 responses) evoked with interleaved low (0.05 Hz) and high (3 Hz, 5 s every 2 min) frequency stimulation. *A*, there was a clear frequency-dependent facilitation of the AMPAr eEPSCs during 3 Hz stimulation (*a*). During, and shortly following, bath application of MK-801 (*b*), the amplitude of low frequency responses was unaffected, but the frequency-facilitation was largely abolished. However, responses recorded after 40 min (*c*) show a full recovery of facilitation. *B,* time course of studies pooled from 10 neurones. Blockade of frequency-facilitation by MK-801 revealed a brief, weak overall frequency depression of eEPSCs followed by a progressive anomalous recovery.

sites. Receptors located in cytoplasmic storage sites would not be accessible to either agonist or blocker so they would remain unblocked during perfusion with the two drugs. Thus, the fact that no recovery was seen after the combined treatment strongly suggests that the recovery seen with MK-801 alone reflects lateral diffusion of NMDAr already inserted in the terminal membrane.

We cannot completely rule out the possibility that anomalous recovery of facilitation reflects MK-801 unbinding from a proportion of presynaptic NMDAr. However, the experiments above suggest this is very unlikely. Tovar & Westbrook (2002) investigated this possibility by transiently applying the competitive antagonist 2-AP5 during recovery from MK-801 to prevent use-dependent dissociation of the channel blocker. This manipulation did not alter the rate of recovery. We considered a similar approach in our studies of presynaptic NMDAr, but ruled it out because of technical difficulties associated with our experimental situation. Tovar & Westbrook (2002) used a highly reduced culture preparation and a fast perfusion system for drug application capable of solution exchanges in the vicinity of the recorded cell in 30–40 ms, allowing perfusion of the competitive antagonist to be turned on and off almost instantaneously. That is not possible in native tissue with an extracellular perfusion system. Recovery of responses from even a short period of perfusion with

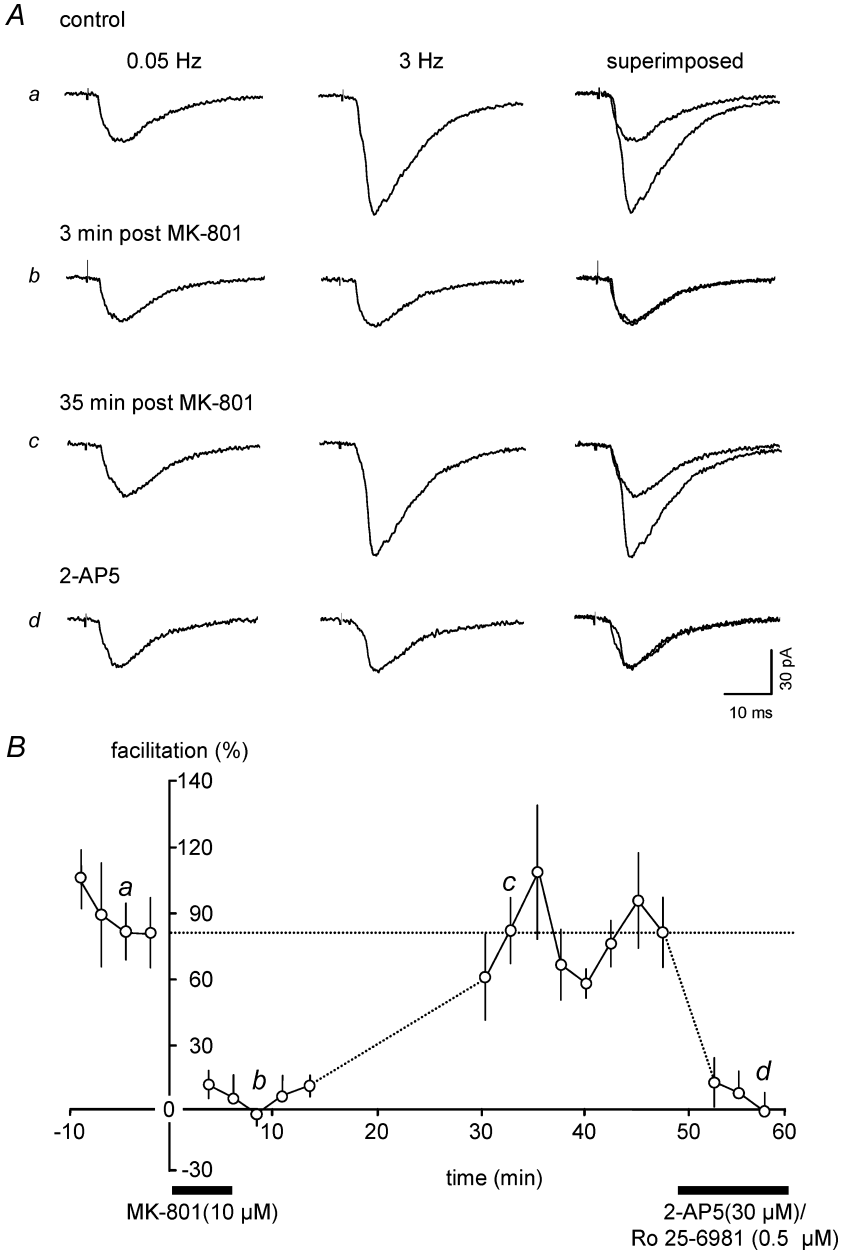

Figure 4. Anomalous recovery is not dependent on continuous stimulation
The protocol was the same as in Fig. 2, where low frequency-stimulation (0.05 Hz) was interleaved with 3 Hz stimulation (3 Hz, 5 s, every 2 min). *A,* frequency-facilitation of AMPAr-mediated eEPSCs (*a*) is again blocked in the presence of MK-801. Full recovery was seen after 35 min (*c*) despite the fact that no stimulation was delivered between 15 and 30 min. *B*, time course of the studies pooled from 7 neurones. In these neurones, after anomalous recovery, we applied either 2-AP5 ($n = 4$) or Ro 25-6981 ($n = 3$), either of which blocked the recovered frequency-dependent facilitation.

2-AP5 is gradual (see Fig. 7*B*), and in our experimental set-up, recovery from competitive blockade would simply be superimposed on the recovery due to (presumed) receptor mobility, and would not confirm or deny the possibility that unbinding of MK-801 was occurring. However, we believe that the lack of anomalous recovery following global blockade of facilitation with combined application of MK-801 and NMDA, and the failure of halting stimulation to alter the rate of recovery, strongly suggest that use-dependent unbinding of the blocker is unlikely to be responsible for recovery. This conclusion is also indirectly supported by the lack of recovery of postsynaptic NMDAr function after blockade with MK-801.

Activity-dependent changes in presynaptic receptor function

We have shown previously that presynaptic NMDAr in the EC are tonically activated by ambient glutamate, since antagonists induce a decrease in spontaneous glutamate release (Berretta & Jones, 1996; Woodhall *et al.* 2001; Yang *et al.* 2006, 2007). Therefore, we predicted that changes in frequency-facilitation of eEPSCs might be paralleled by changes in spontaneous glutamate release. Thus, we determined changes in glutamate-mediated sEPSCs in the neurones illustrated in Figs 3 and 5.

In the neurones where MK-801 alone was bath applied, blockade of frequency-dependent facilitation (Fig. 3) was

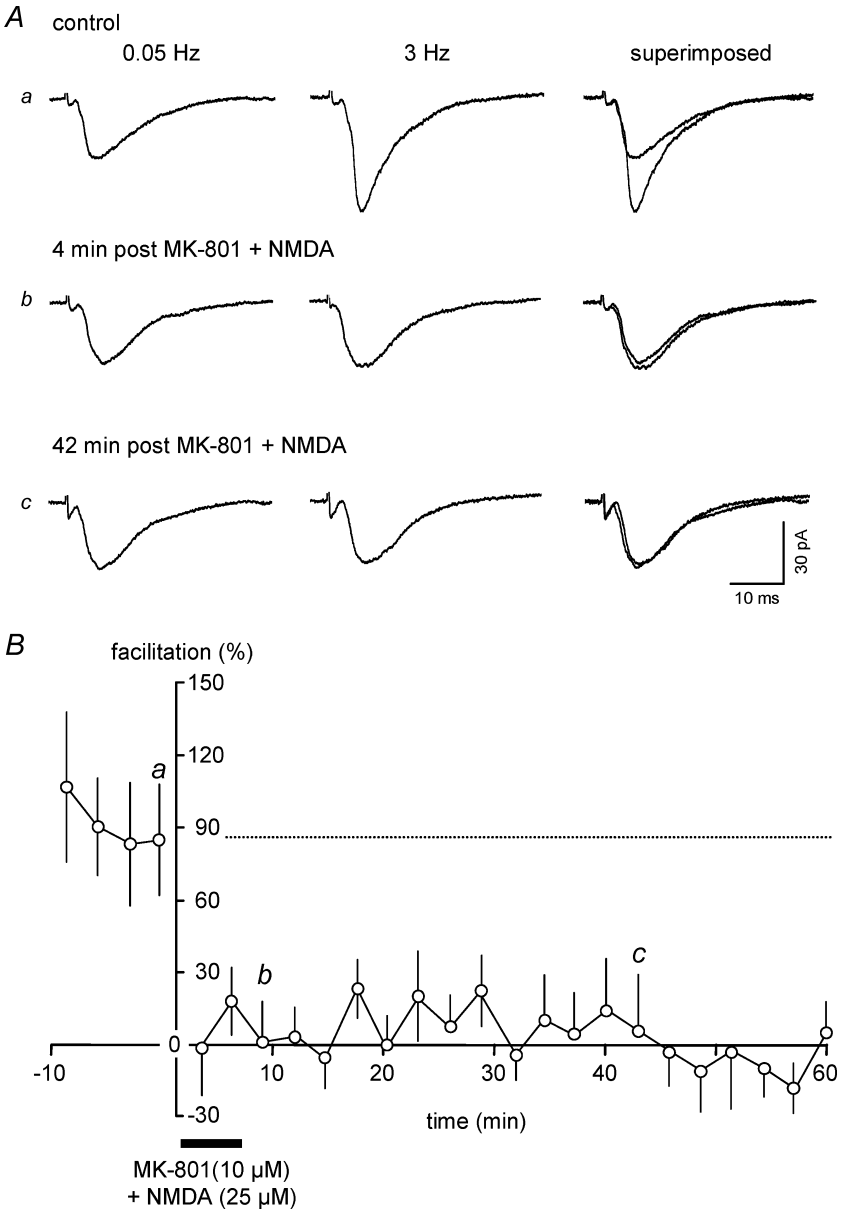

Figure 5. Anomalous recovery does not occur after global blockade of NMDAr
The protocol was the same as in Fig. 2, where low frequency stimulation (0.05 Hz) was interleaved with 3 Hz stimulation (5 s, every 2 min). *A*, the records in one neurone show that frequency-facilitation is abolished by combined application of MK-801 together with NMDA, again with no detectable effect on low frequency responses (*b*). However, now, despite prolonged washing of both agents, there was no evidence for anomalous recovery of facilitation (*c*). *B*, time course of experiments pooled from 7 neurones.

accompanied by a substantial decrease in frequency of sEPSCs (Fig. 6*Aa*). Mean IEI increased from a control value of 306 ± 80 ms to 615 ± 136 ms at the start of the washout period. When facilitation of eEPSCs had returned to control levels 40 min later, mean IEI decreased to 264 ± 75 ms, These changes reflect a decrease in frequency of around 50% by MK-801 and an increase of about 15% associated with anomalous recovery. K–S analysis of cumulative probability distributions of IEI showed that both the increase after MK-801 ($P < 0.001$) and decrease

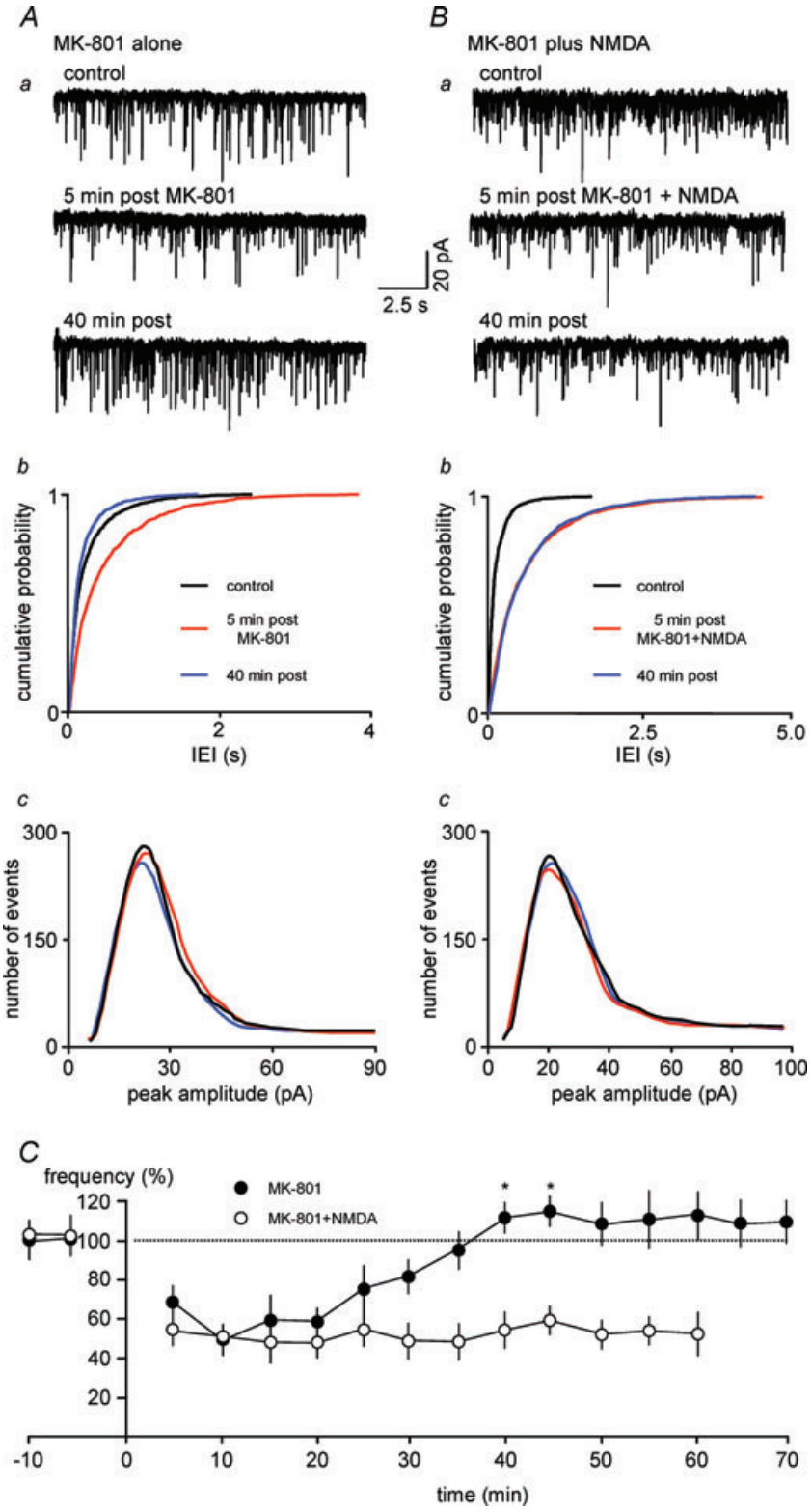

Figure 6. Anomalous recovery is paralleled by changes in sEPSCs
A, sEPSCs were recorded in the 10 neurones included in Fig. 3. The recordings from one neurone shown in *Aa* were obtained before application of MK-801, 5 min after washing the drug from the bath, and 40 min after washout, when recovery of frequency-dependent facilitation was complete. Cumulative probability analysis of pooled data of IEI from all 10 neurones is shown in *Ab* and the distribution of peak amplitudes of events at the same time points is shown in *Ac*. Blockade of frequency-dependent facilitation of eEPSCs by MK-801 (Fig. 3) was accompanied by a decrease in frequency of sEPSCs (rightward shift in IEI cumulative probability distribution) with little overall change in amplitude. Recovery of frequency-facilitation of eEPSCs (see Fig. 3) was accompanied by an increased sEPSC frequency (leftward shift in IEI) with again little overall change in amplitude although there was a slight shift towards larger amplitudes during recovery. *B,* analysis of sEPSCs in the 7 neurones illustrated in Fig. 5. Combined application of NMDA and MK-801 shifted the cumulative probability distribution of sEPSC IEI to the right, reflecting a decrease in frequency of events. However, after 40 min of washout of both agents, there was no recovery of sEPSC frequency. Again there was little change in amplitude distributions. *C*, time course of changes in sEPSCs in the two groups. For this analysis, mean IEI of all events in 5 min blocks was determined and converted to frequency for each neurone. Changes in frequency were determined as a percentage of the mean control frequency at each time point, and averaged across all neurones in each group.

after washout ($P < 0.01$) were significant compared to control (Fig. 6*Ab*). There was no significant change in mean amplitudes (control: 15.5 ± 3.1 pA; MK-801: 14.0 ± 3.1 pA; wash: 14.0 ± 2.9 pA), and little overall change in frequency distribution of peak amplitudes (Fig. 6*Ac*). Mean rise times (1.5 ± 0.1 *versus* 1.6 ± 0.1 *versus* 1.6 ± 0.3 pA) and decay times (control: 3.5 ± 0.5 *versus* 3.7 ± 0.3 *versus* 3.7 ± 0.2 ms) were not significantly altered at any stage, suggesting that the changes in sEPSCs occurred as a consequence of the blockade and recovery of presynaptic NMDA receptors.

The effects on sEPSCs differed when MK-801 was bath applied in conjunction with NMDA (same 7 neurones as illustrated in Fig. 5). In this case, sEPSC frequency was reduced but failed to recover during washout. In the control situation, IEI was 251 ± 41 ms. Five minutes following the washout of the agonist and the blocker, IEI was increased to 552 ± 76 ms, again reflecting a decrease in frequency of around 50%. After 40 min of washing, IEI remained elevated at 598 ± 91 ms (Fig. 6*Aa*,*b*). Thus, the failure of frequency-dependent facilitation of eEPSCs to recover following global blockade of NMDAr was paralleled by a reduction in tonic facilitation of spontaneous release, which likewise failed to recover. Again, mean amplitudes were unaffected throughout (control: 12.8 ± 1.8 pA; MK-801: 12.5 ± 1.5 pA; wash: 13.3 ± 1.7 pA) and there was little change in amplitude distributions (Fig. 6*Bc*). Mean rise (1.6 ± 0.2 *versus* 1.9 ± 0.2 *versus* 1.7 ± 0.1 pA), and decay times (control: 4.2 ± 0.5 *versus* 5.3 ± 0.6 *versus* 5.1 ± 0.5 ms) were again unaffected.

Figure 6*C* shows the time course of changes in sEPSC frequency in the two sets of experiments. It is clear that recovery was not seen after global blockade of presynaptic NMDAr, but did occur when only NMDAr accessible to synaptically released glutamate were blocked. The increase above control levels was small, but consistent and persistent during the period of recording, although it only reached significance at two time points. This increase in spontaneous glutamate release during anomalous recovery of frequency-dependent facilitation (Fig. 6*Aa*,*b*) could be indicative of an activity-dependent increase in trafficking of NMDAr when those receptors close to the release sites are compromised. Facilitation of eEPSCs did become much more variable after recovery from MK-801. Although there was no significant overall increase in frequency-dependent facilitation of eEPSCs there was a relatively clear increase in at least five neurones, three in experiments when stimulation was uninterrupted (Fig. 3) and two when it was halted during recovery (Fig. 4). An example of the latter is shown in Fig. 7*A*. In this neurone, frequency-facilitation was around 50% in control, and this progressed to around 80%, 40 min after washout of MK-801. In this neurone facilitation was abolished by Ro 25-6891, demonstrating that it was likely to be mediated exclusively by NR2B-containing receptors.

To look further at this enhancement of presynaptic NMDAr activity after receptor blockade, we determined the effect of a short period of competitive NMDAr blockade in a group of 6 neurones using a similar protocol to that used to monitor recovery from MK-801 blockade. In these experiments MK-801 was included in the patch pipette but 2-AP5 was substituted for bath application of MK-801 to abolish frequency-dependent facilitation ($n = 5$). The results of these studies are summarized in Fig. 7*B*. As expected, 2-AP5 abolished frequency-facilitation and this recovered relatively rapidly over 15–20 min when the drug was washed out. However, there was also an increase in frequency-facilitation beyond control levels following recovery, which then declined back towards control over a further 20–30 min. The increase in facilitation was significant ($P < 0.05$) at several points. As noted, frequency-facilitation is mediated primarily by NR2B receptors (Woodhall *et al.* 2001; Chamberlain *et al.* 2008), and in preliminary experiments ($n = 2$, not shown) we have shown that brief blockade with Ro 25-6891 is also followed by a weak increase above control during recovery. Thus, the data do suggest that an overall reduction of activation of presynaptic NMDAr, presumably NR2B, could initiate an accelerated diffusion of receptors towards the release sites.

Finally, we have determined whether the opposite may be true by applying NMDA alone in the absence of external MK-801. Application of NMDA (25 μM) often resulted in the generation of large recurrent bursts of oscillatory activity, which appeared to be network driven, making meaningful analysis of sEPSCs and eEPSCs problematic. In addition, eEPSCs *per se* were increased in amplitude as might be expected, but more importantly, the agonist resulted in a change in profile of activity at 3 Hz from facilitating to depressing, or a variable mix of facilitation and depression. To avoid these complications, in the current experiments we examined mEPSCs recorded in the presence of TTX (1 μM). A brief application of NMDA ($n = 4$) resulted in a substantial decrease in IEI of mEPSCs from 213 ± 44 ms to 125 ± 27 ms, reflecting an increase in frequency from around 4.5 Hz to 8 Hz. However, after washout of the agonist, mEPSC frequency then declined to below baseline levels where it remained for at least 45 min. IEI at 25 min was 320 ± 66 ms and at 45 min it was 330 ± 66 ms reflecting a frequency of approximately 3.0 Hz at both time points.

The results of these studies are summarized in Fig. 8. Figure 8*B* shows analysis of IEI and peak amplitudes of sEPSCs in the neurone illustrated in Fig. 8*A*. Both the leftward shift in cumulative probability distribution elicited by NMDA, and the subsequent rightward shift during recovery, were highly significant (K–S test, $P < 0.001$). The frequency distribution of event

amplitudes was not greatly altered by NMDA, or during recovery from it. Mean control amplitude in this neurone was 23.3 ± 0.5 pA, 21.9 ± 0.4 pA during NMDA, 22.3 ± 0.4 after 25 min recovery and 23.5 ± 0.5 after 45 min. Pooled data from the four neurones tested are shown in Fig. 8*C*, and these largely reflect those seen in the neurone in Fig. 8*A*. However, overall there was a small shift towards slightly larger amplitude events in the presence of NMDA, reflected by a small, but significant ($P < 0.05$) increase in mean peak amplitude from 16.8 ± 0.4 pA in

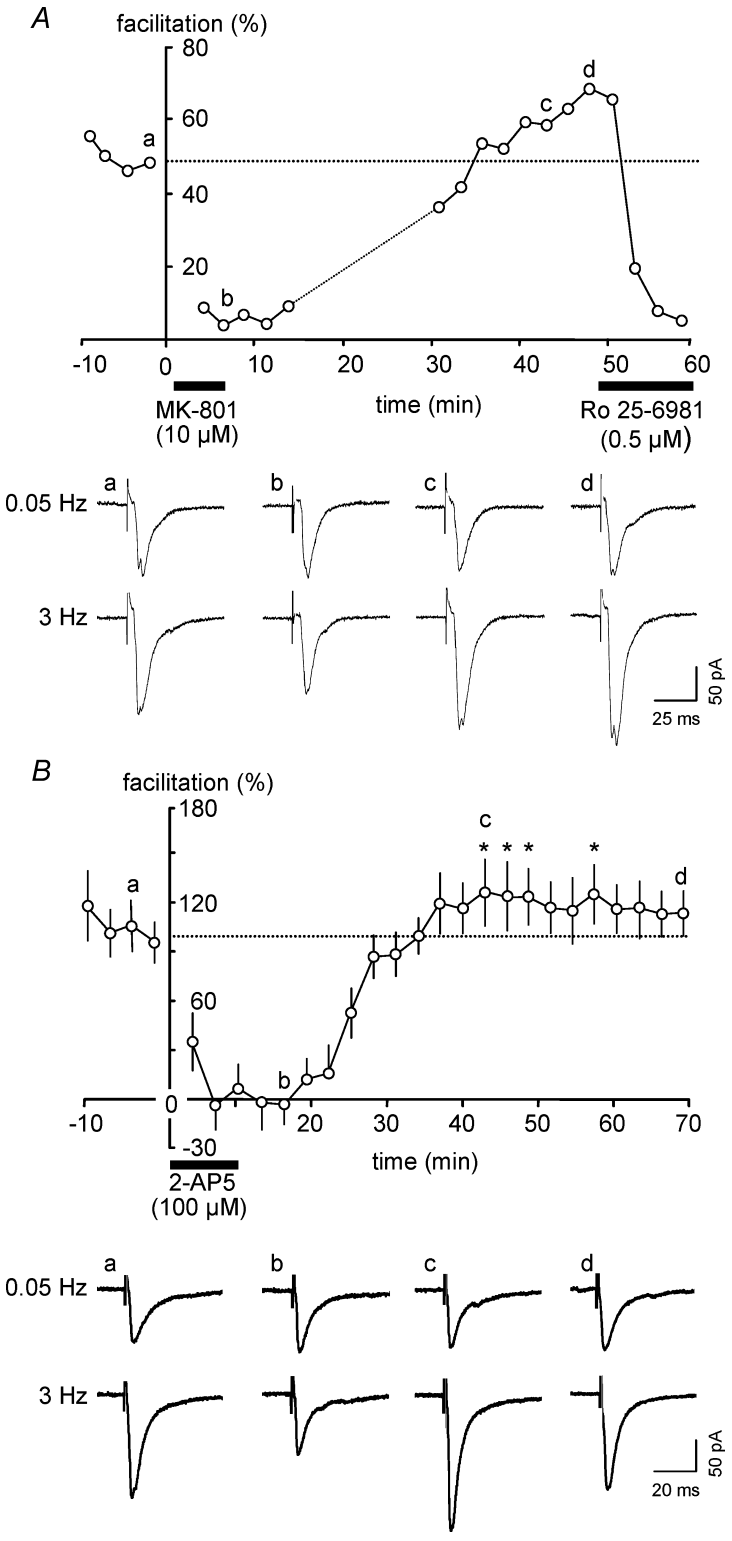

Figure 7. Blockade of NMDAr may promote presynaptic NMDAr mobility
A, results from one neurone included in the analysis of Fig. 4. Stimulation at 3 Hz was delivered for 5 s every 2 min. Again, each point represents the mean facilitation occurring during these periods relative to the mean amplitude of responses at 0.05 Hz in the intervening periods. Following anomalous recovery, frequency-dependent facilitation increased beyond control levels. The NR2B-selective antagonist Ro 25-6981 abolished the enhanced facilitation. *B*, in 5 neurones 2-AP5 was used to a block frequency-dependent facilitation instead of MK-801. Recovery was succeeded by a period where facilitation exceeded that seen in control conditions followed by a gradual decline towards baseline levels by 60–70 min.

control to 18.5 ± 0.5 pA when NMDA was added. Peak amplitudes declined again during recovery to 15.6 ± 0.3 at 25 min and 15.9 ± 0.5 pA at 45 min, slightly, but not significantly, below baseline. The time course of the changes in sEPSC frequency are shown in the graph in Fig. 8*D*, which clearly illustrates the rise induced by NMDA followed by the persistent decrease. Changes in frequency were significant ($P < 0.01$) at all time points.

Discussion

Glutamate release at cortical synapses is facilitated by presynaptic NMDAr (Berretta & Jones, 1996; Woodhall *et al.* 2001; Sjöström *et al.* 2003; Jourdain *et al.* 2007; Li & Han, 2007; Brasier & Feldman, 2008; Li *et al.* 2008). We have now demonstrated that presynaptic NMDAr are likely to be dynamically mobile, and able to exchange between locations close to release sites and more distal sites in the

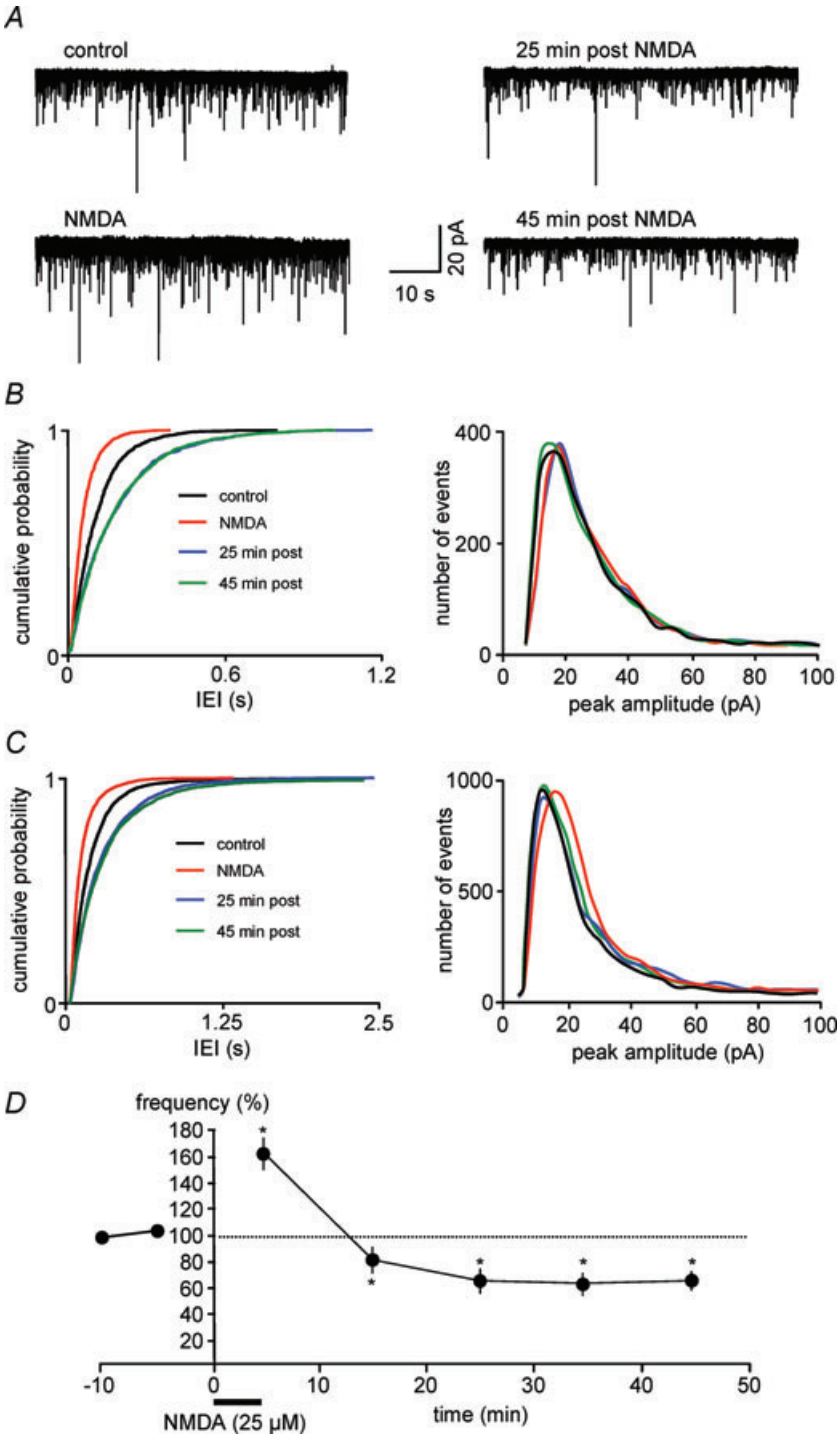

Figure 8. Activation of NMDAr may decrease presynaptic receptor mobility
A, recordings from a layer V neurone showing the effects of a brief (5 min) application of a low concentration (25 μM) of NMDA on mEPSCs recorded in the presence of TTX (1 μM). The agonist caused a substantial increase in mEPSC frequency, but following its washout there was a sustained decrease in frequency over the subsequent recording period (up to 50 min). This is reflected by the analysis of cumulative probability of IEI shown in *B*. Each plot consists of 1500 mEPSCs. The shift to the left in NMDA reflects the change to shorter intervals and a higher frequency. During recovery, the distributions are persistently located to the right of control reflecting the decreased frequency. Frequency histograms of amplitudes of the same events show little change in amplitude distribution at any time point. *C*, IEI and amplitude analysis of data pooled from 4 neurones. Each neurone contributed 500 events to the pooled analysis. The results mirror those in the neurone illustrated in *A* and *B* except that there was a slight shift in amplitude distribution towards larger events in the presence of NMDA. *D*, time course of the effects of NMDA. Mean IEI of all events in 5 min blocks was determined and converted to frequency for each neurone. Changes in frequency were determined as a percentage of the mean control frequency at each time point, and averaged across all neurones.

terminal membrane. In contrast, we found that NMDAr in the postsynaptic membrane are likely to reside in relatively stable synaptic and extrasynaptic pools.

Mobility of postsynaptic NMDAr

There has been increasing interest in the movement of receptors between synaptic and extrasynaptic compartments. Most of this attention has focused on the mobility of postsynaptic receptors as a basis for long-term synaptic plasticity (see Carroll & Zukin, 2002; Collingridge *et al.* 2004; Perez-Otano & Ehlers, 2005; Lau & Zukin, 2007). Trafficking appears to occur in at least two compartments. Receptors may cycle between the synaptic membrane and intracellular sites (see Carroll *et al.* 1999; Collingridge *et al.* 2004; Nong *et al.* 2004; Groc & Choquet, 2006), or by lateral diffusion in the cell membrane without internalization (Choquet & Triller, 2003; Groc *et al.* 2004; Triller & Choquet, 2005; Groc & Choquet, 2006). Both forms of trafficking contribute to functional mobility of NMDAr, and it seems likely that the two probably interact, with receptors moving into and out of the PSD by lateral diffusion, and then recycled via endocytosis at more distal sites in spines (see Washbourne *et al.* 2004; Groc & Choquet, 2006; Lau & Zukin, 2007). NMDAr were originally thought to be stably anchored at the PSD, and that AMPAr receptors were more mobile, However, evidence now suggests that both receptors can be exchanged between the synaptic membrane and internal stores as well as between synaptic and extrasynaptic sites (Collingridge *et al.* 2004; Groc *et al.* 2004; Nong *et al.* 2004; Triller & Choquet, 2005; Groc & Choquet, 2006).

A number of studies have used biochemical, immuno-fluorescence techniques, and direct imaging to monitor postsynaptic receptor trafficking (e.g. Groc *et al.* 2004; Washbourne *et al.* 2004). We have examined functional mobility using the pharmacological approach of monitoring anomalous recovery of NMDAr EPSCs after blockade with MK-801 (Tovar & Westbrook, 2002; Harris & Pettit, 2007; Zhao *et al.* 2008). Tovar & Westbrook (2002) found that NMDAr could move by lateral diffusion between synaptic and extrasynaptic compartments at autaptic synapses in hippocampal cultures, and Zhao *et al.* (2008) reported similar findings in CA1 synapses in hippocampal slices. In contrast, Harris & Pettit (2007) found no exchange of synaptic and extrasynaptic receptors in CA1 pyramidal neurones using the same approach. Clearly our results are in agreement with the latter study, as we found no indication of mobility of postsynaptic NMDAr in the EC, where they appear to be stably anchored or only slowly mobile. Of course, we are studying different synapses to those in previous studies and there is no reason *a priori* to assume that mobility of postsynaptic receptors is a common feature of all glutamate synapses. However, this does not explain the differences between studies in the hippocampus. One possible factor could be a developmental decline in receptor mobility. There is considerable evidence to suggest that subunit composition of postsynaptic NMDAr changes during development, particularly with respect to synaptic *versus* extrasynaptic location and synaptic plasticity (e.g. Wenzel *et al.* 1997; Rumbaugh & Vicini, 1999; Tovar & Westbrook, 1999; Liu *et al.* 2004; Groc *et al.* 2006*b*). Mobility at cultured autaptic synapses (Tovar & Westbrook, 2002) was studied during early development (6 days *in vitro*). Rapid exchange of synaptic NMDAr from NR2B- to NR2A-containing is pronounced at postnatal days (P) 2–9 but declines markedly by P16–21 (Bellone & Nicoll, 2007). Our studies were conducted at P21–35, so it is possible that receptor mobility at EC synapses has already stabilized at this stage. We have recently shown that postsynaptic NMDAr at these synapses may contain a high proportion of triheteromeric NR1/NR2A/NR2B receptors (Chamberlain *et al.* 2008), a situation suggested to represent a stable mature situation (Tovar & Westbrook, 1999). Again, however, this does not explain the contrasting results in hippocampus reported by Harris & Pettit (2007; P14–21) and Zhao *et al.* (2008; P21) in hippocampal slices.

Mobility of presynaptic NMDAr

In contrast to the lack of mobility of NMDAr at postsynaptic sites, using a similar physiological approach we found clear evidence that NMDAr in presynaptic terminals in the EC are highly mobile. The data strongly suggest that this mobility results from lateral diffusion of receptors in the terminal membrane. We cannot entirely dismiss the possibility of trafficking of NMDAr to and from internal stores in the terminals. One scenario is that receptors are translocated from cytoplasmic sites to the terminal membrane at distal locations and subsequently move into the proximity of the release sites and access to synaptically released glutamate by lateral diffusion, analogous to the situation that may occur in postsynaptic spines (Groc & Choquet, 2006; Lau & Zukin, 2007).

Our results provide a first functional demonstration of trafficking of NMDAr in presynaptic terminals. Immuno-labelling studies of NMDAr subunits have shown them located in proximity to the terminal membrane of active zones (Wang & Pickel, 2000; Fujisawa & Aoki, 2003; Adams *et al.* 2004; Kotak *et al.* 2005). However, receptors have also been seen close to the membrane at extra-junctional sites in terminals and axons, as well in the cytoplasm distal to the active zones (e.g. Aoki *et al.* 1994; Conti *et al.* 1999; Wang & Pickel, 2000; Adams *et al.* 2004; Kotak *et al.* 2005; Jourdain *et al.* 2007). This provides physical evidence for the existence of extra-junctional presynaptic NMDAr as a source of receptors to be trafficked to the active zones. Interestingly, immuno-labelling of NR2B subunits has been detected

in association with vesicular organelles in hippocampal terminals (Saldanha *et al.* 2004; Jourdain *et al.* 2007), perhaps representing a storage/delivery mechanism for trafficking of NMDAr to the terminal membrane.

Few previous studies have considered trafficking of presynaptic NMDAr. Electron microscopy combined with immuno-labelling indicated that the number of NR2A and NR2B subunits in terminals in rat somatosensory cortex *in vivo* appeared to increase and decrease, respectively, during NMDAr-blockade (Aoki *et al.* 2003; Fujisawa & Aoki, 2003). This was taken as evidence that presynaptic NR2A and NR2B subunits undergo regulated trafficking in these terminals. Presynaptic NMDAr may originate in the somatic endoplasmic reticulum and be transported along axons to terminals, analogous to the transport of NMDAr to spines along dendrites (see Lau & Zukin, 2007). The anterograde transport of NMDAr along vagal axons was suggested to reflect trafficking of NMDAr into terminals to act as autoreceptors (Cincotta *et al.* 1989). O'Donnell *et al.* 2004) observed NMDAr receptors in the axoplasm of spinal axons and suggested these were being transported along microtubules to primary afferent terminals, where they would also act as autoreceptors (albeit inhibitory; Bardoni *et al.* 2004). Scaffolding proteins such as PSD-95, and SAP-102 (Kornau *et al.* 1995; Niethammer *et al.* 1996; Fujita & Kurachi, 2000) anchor postsynaptic NMDAr at the PSD, and such proteins also occur in cortical terminals (Valtschanoff *et al.* 1999; Aoki *et al.* 2001). Our studies demonstrate a rather dynamic mobility of presynaptic NMDAr, but the presence of scaffolding proteins presynaptically could suggest that receptors that diffuse from distal sites may be anchored near active zones by mechanisms similar to those at the PSD.

We have not yet fully examined the functional role of presynaptic NMDAr mobility. One possibility is a role in activity-dependent plasticity. We found that anomalous recovery from MK-801, or acute blockade with 2-AP5 could be followed by a period of enhanced frequency-dependent facilitation of AMPAr-mediated transmission and of spontaneous glutamate release. These effects could be explained by an increased trafficking of NMDAr into the vicinity of release sites in response to decreased activation of the existing receptors during the period of block. In contrast, when we activated presynaptic receptors with NMDA the frequency of mEPSCs greatly increased (see also Woodhall *et al.* 2001), but this was succeeded by a persistent decrease. Thus, we could postulate that mobility of the presynaptic NMDAr receptors is involved in an intermediate form of self-regulation of synaptic strength at glutamate synapses. We know that the presynaptic NMDAr are likely to be exclusively NR2B-containing (Woodhall *et al.* 2001; Chamberlain *et al.* 2008), so the level of activity at the presynaptic NR2B receptors may control an activity-dependent signal leading to accelerated or decreased mobility, and increased or decreased numbers of the same receptor close to release sites. As noted above, blockade of NMDAr with 2-AP5 *in vivo* appears to result in trafficking of NR2B subunits *out* of synaptic terminals in somatosensory cortex (Fujisawa & Aoki, 2003), but these studies were conducted immediately following the 2-AP5 application, not after recovery so it is difficult to relate these studies to ours. In the context of activity-dependent mobility, it is also of interest that hearing loss induced by cochlear ablation in young (P10) gerbils rapidly results in an increase in the number of presynaptic NR2B subunits at synapses in the superficial layers of auditory cortex (Kotak *et al.* 2005), and this could reflect increased trafficking of receptors induced by reduced afferent input.

Further implications for synaptic plasticity

Considerable attention has been focused recently on the role of trafficking of NMDAr in postsynaptic dendrites as a basis for long-term potentiation (LTP) and long-term depression (LTD) (Carroll & Zukin, 2002; Nong *et al.* 2004; van Zundert *et al.* 2004; Perez-Otano & Ehlers, 2005). Both forms of enduring plasticity have been demonstrated at layer V synapses (Yun *et al.* 2002; Solger *et al.* 2004; Craig & Commins, 2007). However, our results suggest that surface mobility of postsynoptic NMDAr is unlikely to be involved in NMDAr-dependent changes in synaptic strength at these synapses, although we cannot rule out a contribution of NMDAr exchanging between membrane and cytoplasmic stores.

There is increasing evidence that presynaptic NMDAr are involved in LTP (Humeau *et al.* 2003; Samson & Pare, 2005) and LTD (Sjöström *et al.* 2003; Bender *et al.* 2006; Corlew *et al.* 2007) at other glutamate synapses. We do not yet know whether presynaptic NMDAr can act as mediators or modulators of long-term plasticity in the EC. If they do, the ability of NMDAr to alter synaptic transmission by migration in the presynaptic membrane could well play a role in plasticity or metaplasticity and we now aim to investigate this possibility. Finally, we have previously shown that presynaptic NMDAr function is greatly enhanced in the EC in chronically epileptic animals, and that some anticonvulsants may target presynaptic NMDAr (Yang *et al.* 2006, 2007). Whether alterations in presynaptic NMDAr mobility are a factor in the action of anticonvulsant drugs and whether a pathological change in mobility may contribute to chronic epileptogenesis, will also be subjects for future investigation.

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