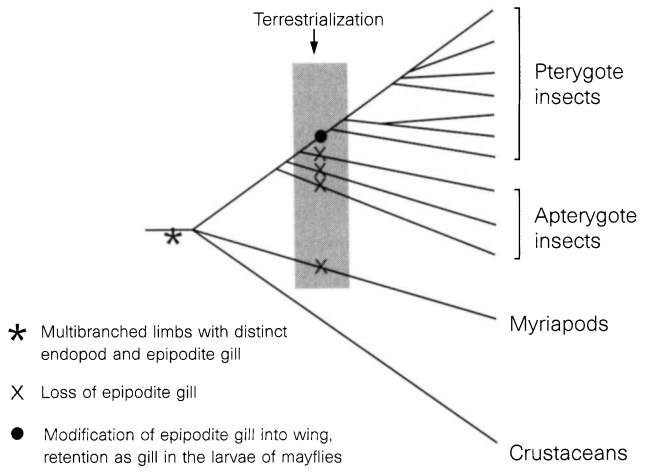

Figure 4 The phylogenetic distribution of respiratory epipodites, wings and gills. Respiratory epipodites are inferred to have been present in the last common ancestor of crustaceans, insects, and possibly myriapods. Thus, the ancestors of insects are likely to have been aquatic animals bearing multibranched appendages with distinct locomotory and respiratory parts (legs and epipodite gills). During the transition from aquatic to terrestrial life epipodite gills must have gradually lost their utility as respiratory and osmoregulatory organs. As a result, gills appear to have been lost independently in myriapods and most of the early lineages of insects (apterygotes). In the lineage that gave rise to the winged insects (pterygotes), we propose that these structures were retained in modified form, perhaps initially as gills in aquatic larvae (as in the abdominal segments of present-day mayfly larvae) and subsequently as wings. The relationships between crustaceans, myriapods and insects remain controversial[9,10] and are therefore presented as an unresolved trichotomy.

have given rise to the insect wings[1–5]. We therefore support the idea that insect wings have evolved from gill-like appendages, and propose that they may be homologous to specific epipodites of crustacean limbs.

Homology of divergent structures can never be proven with certainty. Arguments based on gene expression must take into account that individual genes can acquire different roles in different developmental contexts. For example, both *pdm* and *apterous* show distinctive patterns of expression in wings and legs in *Drosophila*[13,21]. In the case of *pdm*, we have shown that similar distinctive patterns of expression exist in the epipodites and legs (respectively) of crustacean limbs, when these structures exist. Thus we suggest that distinct structural progenitors of legs and epipodites/wings were present in the last common ancestor of crustaceans and insects (Fig. 4). We take this as evidence for a direct evolutionary relationship between epipodites and wings. An alternative interpretation might be that wings do not derive from epipodites but have nevertheless independently coopted a number of gene functions that were already used in epipodites. Although formally possible, we consider this less likely.

The proposed evolution of wings from epipodites presumably involved several genetic and developmental changes, required for repatterning of these structures. At the genetic level, these changes must have utilized and modified gene functions that were already operating in respiratory epipodites. One example may be the use of *apterous* expression and its restriction to the dorsal surface of the wing to initiate a DV patterning system not used in the ancestral limb or in present-day insect legs[16,17]. A second modification must have resulted in the physical separation of the epipodite from the leg, perhaps by fusion of the limb base with the body wall[3,5]. □

Methods

Af-pdm and *Af-ap* were cloned by polymerase chain reaction (PCR) using degenerate primers. Primers were 5′-GGAATTC GA(A/G) CA(A/G) TT(T/C) GCI AA(A/G) AC-3′ and 5′-GCTCTAGA GG(A/G)TT IAT IC(T/G) (T/C)TT (T/C)TC (T/C)TT-3′ for *Af-pdm*, and 5′-GGAATTC GCI GTI GAI AA(A/G) C(A/G)I TGG CA-3′ and 5′-GCTCTAGA TT IGC IC(T/G) IGC (A/G)TT(T/C)TG (A/G)AA-3′ for *Af-ap* (where I is inosine). PCRs were carried out on first-strand cDNA prepared from early larval stages of *Artemia franciscana*; 7 and 4 independent clones were sequenced for *Af-pdm* and *Af-ap* respectively. *Af-pdm* and *Af-ap* were subcloned into expression vectors pATH-1 and pET-23a and expressed in bacteria. Antibodies against Af-PDM and Af-AP were raised in mice by repeated injection of 70-μg doses of bacterially expressed protein in Freund's adjuvant. Immunochemical staining was done using 1:500 dilutions of mouse serum. *Artemia* larvae were sonicated before staining to allow efficient penetration of reagents. The DLL staining in Fig. 3b was done with the antibody described in ref. 24. Detailed protocols are available upon request.

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Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors

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The classical view of fast chemical synaptic transmission is that released neurotransmitter acts locally on postsynaptic receptors and is cleared from the synaptic cleft within a few milliseconds by

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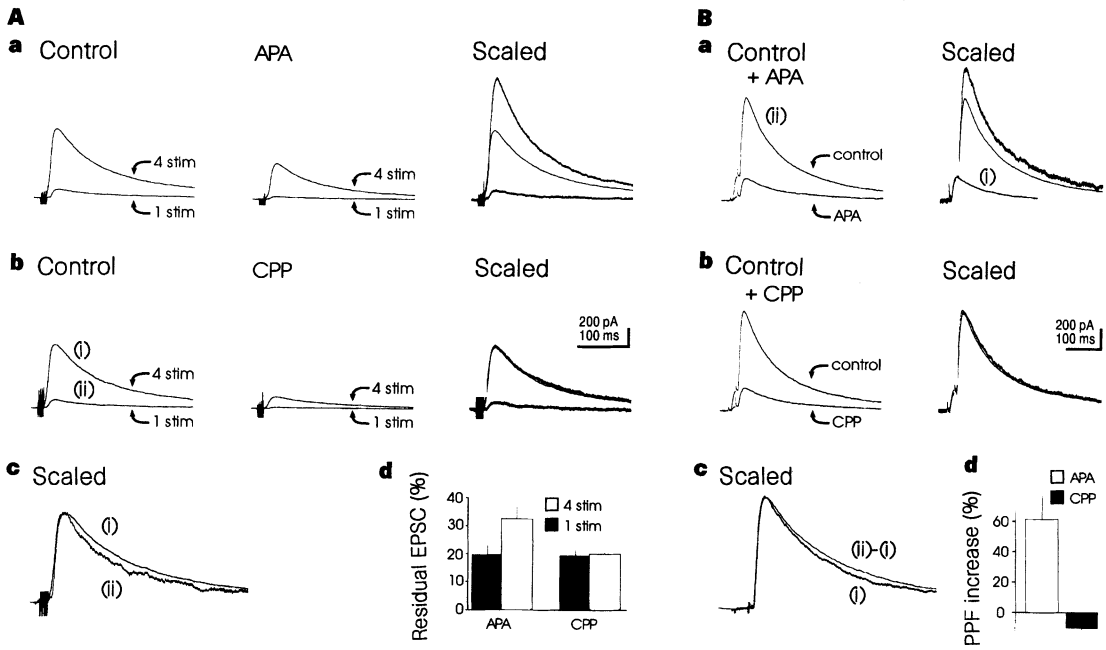

Figure 1 Reduced antagonism of mossy fibre NMDAR EPSCs by the competitive low-affinity NMDAR antagonist APA during brief high-frequency stimulation. **A, a**, A mossy fibre input was stimulated at 0.05 Hz by alternating a single stimulus with four stimuli at 3-ms intervals (superimposed sweeps). The amplitude of the EPSC evoked with 4 stimuli averaged 530 ± 93% of the amplitude of the EPSC evoked with a single stimulus ($n = 6$). Application of APA (1 mM) reduced the EPSC evoked by a single stimulus to a greater extent than the EPSC evoked by four stimuli. **b**, Same neuron as in **a**. The high-affinity NMDAR antagonist CPP (1 μM) reduced both EPSCs to the same extent. **c**, The decay of the EPSC evoked by a single stimulus was faster than the decay of the EPSC evoked by four stimuli. The peak of the EPSC evoked by a single stimulus (see **b**, control) was scaled to the peak of the EPSC evoked by four stimuli. The decay of the EPSC increased from 62 ± 3 ms to 79 ± 5 ms ($n = 6$). **d**, Summary graph of the amplitude of the EPSC recorded in the presence of a non-saturating concentration of the competitive antagonist APA (1 mM; $n = 6$) and CPP (1 μM; $n = 3$) (residual EPSC). **B, a**, A mossy fibre input was stimulated at 0.05 Hz by alternating a single stimulus (i) with two stimuli separated by 20 ms (ii). The paired-pulse facilitation (PPF) in control conditions averaged 262 ± 79% ($n = 6$) (see Methods for calculations). APA (1 mM) reduced the first EPSC to a greater extent than the second, increasing PPF, as shown by scaling of the amplitudes of the first EPSCs in the absence and presence of APA. **b**, Same neuron; CPP (700 nM) reduced both first and second EPSCs to a similar extent. **c**, Decay of the second EPSC (trace (ii) in **a**) after subtraction of the single EPSC (trace (i) in **a**) was slower than the decay of the single EPSC. The decay of the EPSC increased from 60 ± 4 ms to 68 ± 3 ms ($n = 6$). **d**, The change in the ratio (PPF) between the amplitude of the second EPSC (estimated after subtraction of the single EPSC) and the amplitude of the single EPSC is plotted for APA (1 mM, $n = 6$) and CPP (0.7–1 μM; $n = 4$).

diffusion and by specific reuptake mechanisms. This rapid clearance restricts the spread of neurotransmitter and, combined with the low affinities of many ionotropic receptors, ensures that synaptic transmission occurs in a point-to-point fashion[1]. We now show, however, that when transmitter release is enhanced at hippocampal mossy fibre synapses, the concentration of glutamate increases and its clearance is delayed; this allows it to spread away from the synapse and to activate presynaptic inhibitory metabotropic glutamate receptors (mGluRs). At normal levels of glutamate release during low-frequency activity, these presynaptic receptors are not activated. When glutamate concentration is increased by higher-frequency activity or by blocking glutamate uptake, however, these receptors become activated, leading to a rapid inhibition of transmitter release. This effect may be related to the long-term depression of mossy fibre synaptic responses that has recently been shown after prolonged activation of presynaptic mGluRs (refs 2, 3). The use-dependent activation of presynaptic mGluRs that we describe here thus represents a negative feedback mechanism for controlling the strength of synaptic transmission.

Previous studies of cochlear synapses in the chick brain stem[4], cerebellar synapses[5] and cultured neurons[6] indicate that when release is enhanced, the concentration of glutamate can increase and spread from the synapse. If a similar process were to occur at cortical synapses[7], it could have important consequences on the input specificity of these synapses, as well as on their use-dependent modification. We tested for possible use-dependent increases in glutamate concentration at mossy fibre synapses, which contact the proximal dendrites of CA3 pyramidal cells, by using the low-affinity, rapidly dissociating, competitive *N*-methyl-D-aspartate receptor (NMDAR) antagonist amino pimelic acid (APA)[8,9] and comparing its ability to block NMDAR-mediated excitatory postsynaptic currents (NMDAR EPSCs) evoked by single versus repetitive stimuli, which greatly facilitate release of glutamate[10]. With such an antagonist, the degree of reduction of NMDAR EPSCs will decrease as the concentration and/or time course of glutamate in the synaptic cleft increase because the higher concentration of glutamate can rapidly displace the low-affinity antagonist from the receptors[6].

In these experiments, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors were blocked with NBQX (6-nitro-7-sulphamoylbenzo[*f*]quinoxaline-2,3-dione) (10 μM), the cells were held at +30 to +40 mV to record the NMDAR EPSCs, and the ability of APA to antagonize the synaptic response to one versus four stimuli (with an interstimulus interval of 3 ms) was compared (Fig. 1A, a). When the size of the single-stimulus response in APA is scaled up and superimposed on the control single stimulus response (scaled), it is clear that the response to four stimuli is considerably larger than the control response, indicating that APA was less effective at blocking the facilitated response. On the other hand, if in the same cell the high-affinity antagonist CCP (D-carboxy-piperazin-propyl-phosphonic acid) is applied, the degree of antagonism is identical for both responses, as shown by the superimposition of the scaled responses (Fig. 1A, b and d). Similar results were obtained when only two rather than four stimuli were delivered (Fig. 1B). We

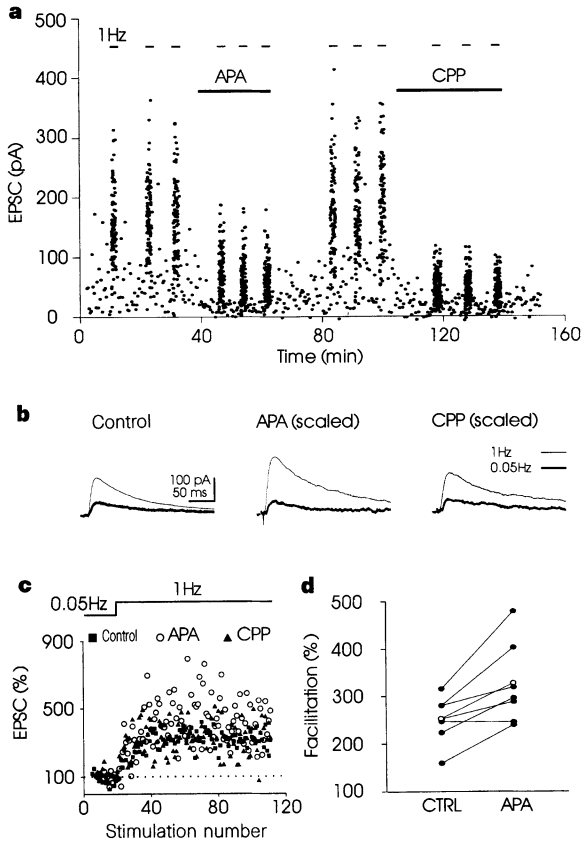

Figure 2 Decreased antagonism of facilitated NMDAR EPSCs by APA. **a**, Mossy fibres were stimulated alternately at 0.05 and 1 Hz. Application of APA (500 μM) inhibited the EPSC evoked at 0.05 Hz to a greater extent than the EPSC evoked at 1 Hz. CPP (250 nM) inhibited EPSCs evoked at 0.05 Hz and at 1 Hz to a similar extent. **b**, Data from the experiment in **a**. The responses at 0.05 Hz in APA or CPP have been scaled to the control responses at 0.05 Hz. **c**, Time course of facilitation of the experiment in **a** after normalization of EPSC amplitudes evoked at 0.05 Hz under control conditions or in the presence of either APA or CPP Note the enhanced facilitation in the presence of APA. **d**, Scatter plot of 7 experiments. Open circles represent average values.

also found that decay of the NMDAR EPSC is slowed when transmitter release is increased by repetitive stimulation (Fig. 1A, c and B, c), consistent with the delayed clearance of glutamate from the synaptic region. Four stimuli prolonged decay by 29 ± 9% ($n = 6$) ($P < 0.02$) and two stimuli prolonged it by 14 ± 3% ($n = 6$) ($P < 0.01$).

Although our results with APA are consistent with an increase in glutamate concentration during repetitive stimuli, a limitation of these experiments is that under control conditions the increase in the amplitude of the EPSC due to two or more stimuli might be limited by receptor occupancy, if glutamate release occurs at the same site during repeated stimulation. In the presence of APA, a fraction of receptors will be protected during the first stimulus, and because of the fast re-equilibration of APA, be available to subsequent stimuli, leading to the observed results. To circumvent this problem, we have used frequency facilitation to enhance transmitter release. As mossy fibres show markedly enhanced release at frequencies at which the NMDAR EPSC completely recovers before the next stimulus, the limitation described above is avoided. As shown in Fig. 2a, mossy fibres were stimulated at 0.05 Hz and then given bouts of 1-Hz stimulation in control conditions and following brief applications of APA or CPP. When the responses are normalized to those recorded during low-frequency stimulation (0.05 Hz) (Fig. 2b, c), it is evident that the 1-Hz responses in CPP scale to the control responses, but that the 1-Hz responses are less inhibited by APA. This decreased effectiveness of APA on 1-Hz responses was seen in six of seven experiments (Fig. 2d). APA significantly increased facilitation from 251 ± 19% to 324 ± 33% ($P < 0.02$) ($n = 6$), whereas CPP had no significant effect ($P > 0.4$) ($n = 4$).

Having established that the concentration of glutamate rises in the synaptic region during repetitive stimulation, we next examined whether this might result in the activation of presynaptic metabotropic glutamate receptors (mGluRs). mGluRs are present on a large number of presynaptic terminals in the mammalian central nervous system (CNS)[11–16], including mossy fibre terminals[2,3,17–21], and when activated by exogenously applied agonists cause a depression of transmitter release. However, the conditions necessary for synaptically released glutamate to gain access to these presynaptic receptors are unknown. In Fig. 3A, a, mossy fibres were stimulated at a frequency of 0.05 Hz and bouts of 1-Hz stimulation were applied. Application of the broad-spectrum mGluR antagonist MCPG ((+)α-methyl-4-carboxyphenylglycine), which is known to antagonize presynaptic mGluRs on mossy fibre synapses[19], had no effect on the responses evoked at low frequency. However, the responses evoked at 1 Hz were enhanced, suggesting that during the 1-Hz stimulation glutamate was able to activate the presynaptic inhibitory receptors. A summary of the effect of MCPG in five similar experiments is shown in Fig. 3A, b and c. A similar enhancement was observed in seven of seven experiments with MCCG ((2*S*,3*S*,4*S*)-methyl-2-(carboxycyclopropyl)-glycine) (500 μM), which selectively antagonizes the presynaptic action of group-2 mGluR agonists at mossy fibre synapses[19].

It could be argued that mGluRs are activated at low-frequency stimulation but that the duration of the presynaptic inhibition is shorter than the interstimulus interval. Alternatively, the mGluRs might require a certain level of repetitive synaptic activation to inhibit transmission. This latter possibility can be ruled out because the inhibition induced by bath-applied mGluR agonists (not shown) was independent of the stimulus frequency. To test the former possibility, we examined the effects of MCPG when stimuli were applied at shorter interstimulus intervals. We chose an interval of 200 ms as this is the optimal interval for presynaptic inhibition mediated by $GABA_B$ receptors[22]. MCPG had no consistent effect on the second response to paired-pulse stimulation (Fig. 3B, a), suggesting that the amount of glutamate released following a single stimulus is unable to activate the mGluRs. If, however, in this same experiment, an additional stimulus was applied immediately before the first, which greatly increased transmitter release, the test response was now clearly enhanced by MCPG (Fig. 3B, a; right traces). A summary graph of five similar experiments (Fig. 3B, b and c) confirms that single stimuli at low-release probability fail to activate mGluRs, but that when release probability is enhanced by paired pulse, stimulation mGluRs are activated.

The need for increased glutamate release for mGluR activation could mean that mGluRs are located within the synaptic cleft and require an increase in glutamate concentration, or that mGluRs are not present in the cleft and require the spread of glutamate from the release site for their activation. Given that the affinity of glutamate for group-2 mGluRs, ~10 μM (ref. 12), is higher than for AMPA receptors, typically greater than 100 μM (ref. 23), the first situation is unlikely. If mGluR activation depends on spread of glutamate from the release site, the activation of these receptors should be enhanced by blocking the uptake of glutamate. Indeed, when the glutamate-uptake blocker L-*trans*-pyrrolidine-2,4-dicarboxylic acid (*trans*-PDC)[24] is applied, the size of the field EPSPs evoked at 1-Hz stimulation, but not at 0.017-Hz stimulation, is reduced (Fig. 4a). This depression is entirely reversed by MCPG, and, in fact, is replaced by an enhancement over the control responses evoked at 1-Hz stimulation (Fig. 4b). Results were similar in four other experiments (Fig. 4c), strongly supporting a model in which glutamate spread from the release site is required for mGluR activation.

In summary, we have found that the concentration of glutamate

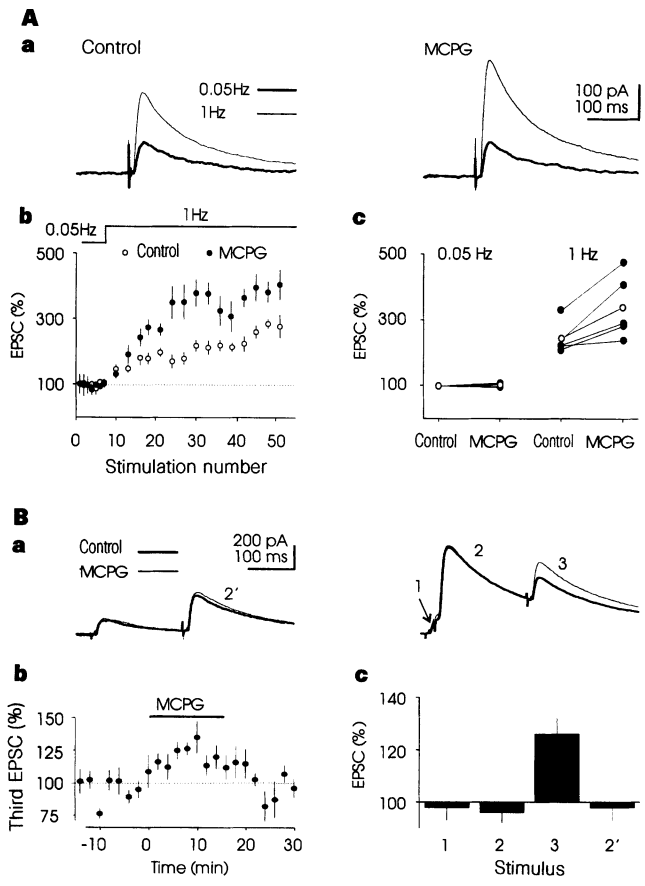

Figure 3 Frequency-dependent activation of presynaptic metabotropic glutamate receptors. **A**, **a**, Mossy fibres were stimulated alternately at 0.05 and 1 Hz (superimposed sweeps). Perfusion of the metabotropic glutamate receptor antagonist MCPG (1.5 mM) had no effect on baseline transmission (0.05 Hz) but increased the mean amplitude of the facilitated response evoked at 1 Hz. **b**, Summary of the time course of facilitation for 5 experiments normalized over the 0.05-Hz stimulation period. **c**, Scatter plot of the 5 experiments. MCPG significantly increased the facilitated response from 247 ± 22% to 343 ± 50% ($P < 0.03$). **B, a**, Mossy fibres were stimulated at 0.033 Hz with paired stimuli separated by 200 ms, and for every other stimulus cycle, a third stimulus preceded the first by 20 ms (response labelled 1 in the traces on the right). MCPG (1.5 mM) had no effect when paired stimuli were given (response labelled 2′ in left-hand traces), but increased the amplitude of the EPSC when preceded by a double stimulus (response labelled 3 in right-hand traces). **b**, Summary of the time course of the action of MCPG on the third EPSC. **c**, Summary of the mean amplitude changes for all EPSCs in the presence of MCPG (0.5–1.5 mM; $n = 8$ for 1, 2 and 3; $n = 3$ for 2′). MCPG increased 3 by 26 ± 6%. In **A**, **b**, the bin size of each data point during 1-Hz stimulation is 3 stimuli. In **A**, **c**, open circles represent average values.

in the mossy fibre synaptic cleft is critically dependent on the frequency of synaptic activation. The mossy fibre synapses are ideally suited for frequency-dependent changes in glutamate concentration because they show dramatic frequency facilitation[10] and a single bouton forms multiple release sites[25,26], an anatomical arrangement favouring spillover onto adjacent synapses (see also refs 4, 5). A consequence of the spread of glutamate from the release site during certain patterns of afferent activity is that mGluRs, which normally are silent during low-frequency mossy fibre transmission, become activated and exert a negative feedback on transmitter release. This proposal is supported by anatomical evidence indicating that group-2 mGluRs, which we have shown contribute to presynaptic inhibition, are localized at the preterminal zone of mossy fibre synapses away from the release sites[2,27]. The use-dependent engagement of presynaptic mGluRs provides a local means for controlling the level of release at which mossy fibre synapses can operate. Furthermore, our findings emphasize that receptor localization relative to transmitter release sites is important and influences the ways in which receptors are engaged during synaptic activity. □

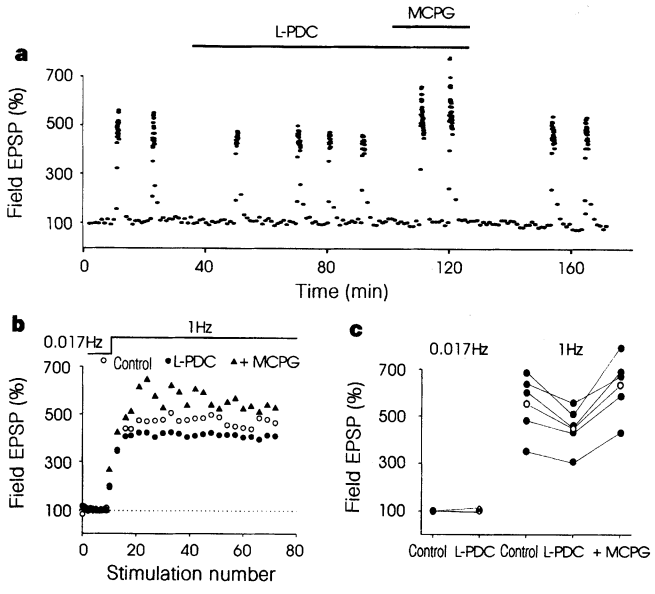

Figure 4 Activation of presynaptic metabotropic glutamate receptor by inhibiting glutamate uptake. **a**, Field recordings of mossy fibre EPSPs evoked alternatively at stimulation frequencies of 0.017 and 1 Hz. The glutamate-uptake blocker L-PDC (30 μM) had no effect on the amplitude of field EPSPs evoked at 0.017 Hz but reduced the average amplitude of the field EPSPs evoked at 1 Hz. This effect was reversed by perfusion of MCPG. **b**, Data from experiment in **a**: time course of facilitation under control conditions or in the presence of L-PDC and MCPG. Note that in the presence of MCPG, facilitation overshoots control values. **c**, Scatter plot of 5 experiments. Average values are 554 ± 61, 448 ± 43 and 634 ± 60% for the facilitated responses in control, L-PDC, and L-PDC with MCPG, respectively. The differences between control and L-PDC, L-PDC and L-PDC with MCPG, and control and L-PDC with MCPG are significant ($P < 0.02$; $P < 0.01$; $P < 0.005$). In **b**, the bin size of each data point during 1-Hz stimulation is 3 stimuli. In **c**, open circles represent average values.

Methods

Standard procedures were used to prepare hippocampal slices (400–500 μm) from 5–15-day-old guinea-pigs. After dissection, slices were maintained at room temperature for at least 1 h in a submerged chamber containing artificial cerebrospinal fluid (ACSF) equilibrated with 95% O_2 and 5% CO_2, and then transferred to a superfusing chamber. The ACSF contained (in mM) 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 11 glucose. For electrophysiological recording, the concentration of $CaCl_2$ and $MgSO_4$ was raised to 4 mM. Whole-cell recordings were made at room temperature in the presence of picrotoxin (100 μM) and NBQX (10 μM). Whole-cell recording electrodes were filled with a solution containing (in mM) 122.5 caesium gluconate, 10 CsCl, 10 HEPES, 10 BAPTA, 8 NaCl, 2 Mg-ATP, 0.3 Na-GTP. The resistance of the patch pipette ranged between 2 and 4 MOhm. Access resistances ranged between 4 and 10 MOhm and were not allowed to vary by more than 15% during the course of the experiment. No series resistance compensation was used. In 5 cells we tested the effect of APA (0.5 mM) on AMPAR EPSCs. Picrotoxin was added to isolate the AMPAR currents and a low concentration of CNQX (0.5 μM) was used to prevent epileptiform activity. APA (0.5 mM) had little effect on AMPAR EPSCs (90 ± 5% of control; $n = 5$) or on paired-pulse facilitation (PPF). The PPF was 2.7 ± 0.4 before APA and 2.9 ± 0.5 during APA. Field recordings were made with a glass electrode placed in the stratum lucidum in the absence of picrotoxin and NBQX. Bipolar steel electrodes were placed in the granule cell layer to stimulate mossy fibres. Only EPSCs depressed by more than 60% or field EPSCs depressed by more than 80% during application of the mGluR agonist L-AP4 (10 μM), which selectively blocks mossy fibre responses in the guinea-pig[17–19], were considered as mossy fibre responses[28]. This was tested in all experiments. Data were acquired and analysed as described previously[28]. Average values are expressed as mean ± s.e.m. Significance was determined by a two-tailed paired *t*-test, unless stated otherwise. PPF was defined as P_2-$P_1/P_1 \times 100$, where P_1 and P_2 are the peak amplitudes of the first (i) and second (ii) EPSC, respectively. P_2 was estimated after subtraction of (i) from (ii) and P_1 was set as the peak amplitude of (i). To determine changes in the decay of the NMDAR EPSC, the first 100 ms of the EPSC was fitted with a single exponential. Drugs used were: caesium hydroxide, D-gluconic acid (Aldrich), picrotoxin, BAPTA, L-AP4 (Sigma), CPP, NBQX, (+)-MCPG (Tocris Cookson), DL-α-amino pimelic acid (ICN Biomedicals), L-*trans*-pyrrolidine-2,4-dicarboxylic acid (Research Biochemicals International).

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Decreased prefrontal dopamine D1 receptors in schizophrenia revealed by PET

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Schizophrenia is believed to involve altered activation of dopamine receptors, and support for this hypothesis comes from the antipsychotic effect of antagonists of the dopamine D2 receptor (D2R)[1]. D2R is expressed most highly in the striatum, but most of the recent positron emission tomography (PET) studies have failed to show any change in D2R densities in the striatum of schizophrenics[2–5], raising the possibility that other receptors may also be involved. In particular, the dopamine D1 receptor (D1R), which is highly expressed in the prefrontal cortex[6], has been implicated in the control of working memory[7,8], and working memory dysfunction is a prominent feature of schizophrenia[9]. We have therefore used PET to examine the distribution of D1R and D2R in brains of drug-naive or drug-free schizophrenic patients. Although no differences were observed in the striatum relative to control subjects, binding of radioligand to D1R was reduced in the prefrontal cortex of schizophrenics. This reduction was related to the severity of the negative symptoms (for instance, emotional withdrawal) and to poor performance in the Wisconsin Card Sorting Test[10]. We propose that dysfunction of D1R signalling in the prefrontal cortex may contribute to the negative symptoms and cognitive deficits seen in schizophrenia.

We examined D1R and D2R in 17 male schizophrenics and 18 healthy male volunteers. Ten of the patients were completely naive for neuroleptics and seven were drug-free for a minimum of two weeks before PET examination. Two PET runs were done in each subject on the same day using [^{11}C]SCH23390 and [^{11}C]N-methyl-

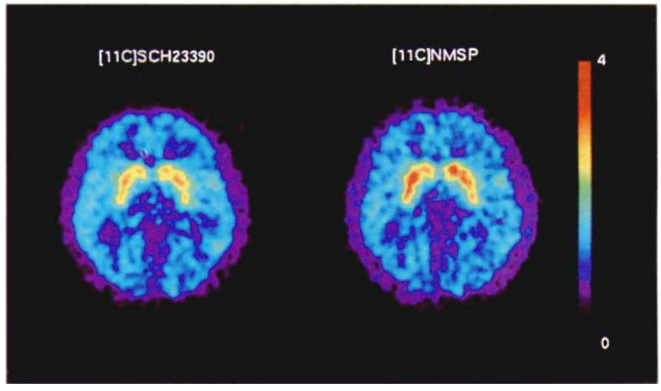

Figure 1 PET visualization of D1R (left image) and D2R (right image) in a control subject. The radioactivity distribution was obtained at the level of striatum, 14 to 40 min after injection of [^{11}C]SCH23390 and 34–60 min after [^{11}C]NMSP injection. Images were normalized with respect to cerebellar radioactivity. The *in vivo* labelling of the striatum and cortex by [^{11}C]SCH23390 (left) represents D1R, and [^{11}C]NMSP (right) binds predominantly to D2R in the striatum but to 5-HR_2 in the cortex.