Kainate receptors are involved in synaptic plasticity

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The ability of synapses to modify their synaptic strength in response to activity is a fundamental property of the nervous system and may be an essential component of learning and memory[1]. There are three classes of ionotropic glutamate receptor, namely NMDA (*N*-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole-4-propionic acid) and kainate receptors[2]; critical roles in synaptic plasticity have been identified for two of these. Thus, at many synapses in the brain, transient activation of NMDA receptors leads to a persistent modification in the strength of synaptic transmission mediated by AMPA receptors[3,4]. Here, to determine whether kainate receptors[5–7] are involved in synaptic plasticity, we have used a new antagonist, LY382884 ((3S, 4aR, 6S, 8aR)-6-((4-carboxyphenyl)methyl-1,2,3,4,4a,5,6,7,8,8a-decahydroisoquinoline-3-carboxylic acid), which antagonizes kainate receptors at concentrations that do not affect AMPA or NMDA receptors. We find that LY382884 is a selective antagonist at neuronal kainate receptors containing the GluR5 subunit. It has no effect on long-term potentiation (LTP) that is dependent on NMDA receptors but prevents the induction of mossy fibre LTP, which is independent of NMDA receptors. Thus, kainate receptors can act as the induction trigger for long-term changes in synaptic transmission.

We have previously described two compounds, LY293558 ((3S, 4aR, 6R, 8aR)-6-[2-(1(2)H-tetrazol-5-yl)ethyl]-decahydroisoquinoline-3-carboxylic acid)[8] and LY294486 ((3SR, 4aRS, 6SR, 8aRS)-6- ((((1H-tetrazol-5-yl) methyl) oxy) methyl) -1,2,3,4,4a,5,6,7,8,8a decahydroisoquinoline-3-carboxylic acid)[9], that inhibit responses mediated by kainate receptors containing the GluR5 but not the GluR6 subunit. However, these kainate receptor antagonists are also potent AMPA receptor antagonists, limiting their use except under circumstances where synaptic transmission mediated by AMPA receptors is blocked. We therefore examined other members of this family of decahydroisoquinoline compounds using ligand-binding assays; one compound, LY382884, was identified as

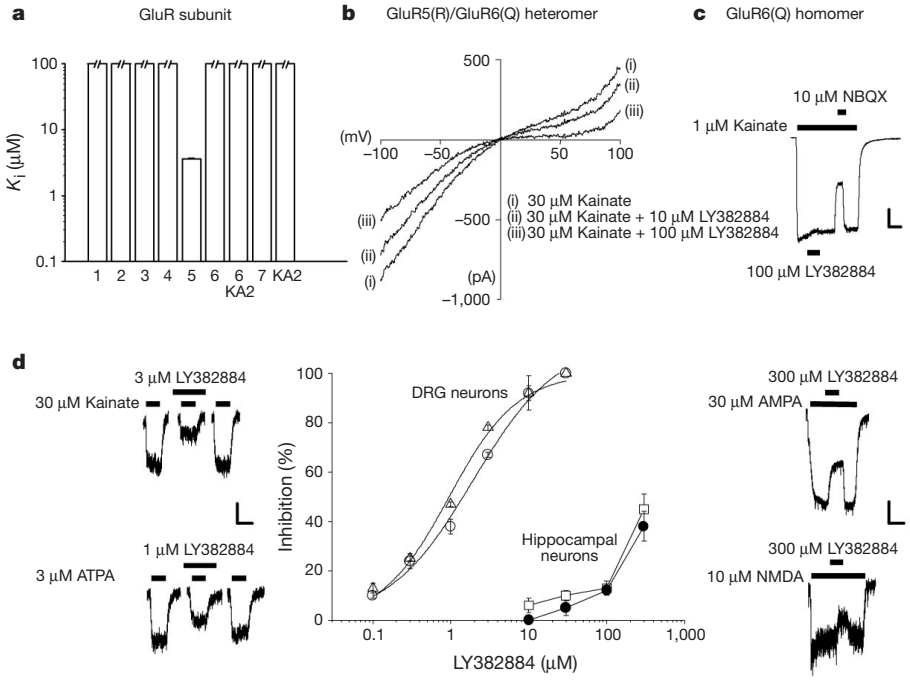

Figure 1 LY382884 is a selective GluR5 kainate receptor antagonist. **a**, Selective displacement of binding to human recombinant GluR5 receptors. K_i values were estimated from 11-point competition assays, from 3–4 separate preparations. Histograms with open tops indicate a binding affinity >100 μM (displacements using 100 μM LY382884: GluR1, 23 ± 5%; GluR2, 26 ± 3%; GluR3, 5 ± 3%; GluR4, 24 ± 2%; GluR6, 1 ± 1%; GluR7, 26 ± 6%; KA2, 8 ± 3%; GluR6 + KA2, 25 ± 16%). **b**, LY382884 antagonizes GluR5/GluR6 heteromeric receptors. A voltage ramp was applied during steady-state responses to kainate (30 μM). Cells with a significant response generated by heteromeric receptors were characterized by less rectification than homomeric GluR6(Q) (the ratio of currents at +50/−50 mV was 0.66 ± 0.07 for heteromeric receptors (n = 8) and 0.06 ± 0.03 for homomeric GluR6(Q) (n = 5)). Kainate currents were inhibited by LY382884 (10 and 100 μM) by 18 ± 3 and 41 ± 3%, respectively (−50 mV), and by 30 ± 6 and 70 ± 6%, respectively (+50 mV). The voltage-dependence of the antagonism was significant (P < 0.01, n = 8). **c**, LY382884 does not affect 1 μM kainate currents in cells expressing homomeric GluR6(Q) (4 ± 3%; n = 3). The effectiveness of NBQX (10 μM) is shown for comparison. Holding voltage (V_h) = −70 mV; scale bar represents 100 pA vertical and 10 s horizontal. (LY382884 (100 μM) also had little effect on 30 μM kainate currents in GluR6(Q) homomers (6 ± 1%; n = 5)). **d**, Functional selectivity of LY382884 at native ionotropic glutamate receptors. The graph shows concentration-dependent antagonism by LY382884 of responses of DRG neurons to 3 μM ATPA (triangles) and 30 μM kainate (open circles) and of hippocampal neurons to 10 μM NMDA (filled circles) and 30 μM AMPA (squares) (V_h = −70 mV). Data were obtained from at least three separate cells at each concentration. Scale bars for representative currents are 50 pA (kainate), 75 pA (ATPA), 200 pA (AMPA) and 50 pA (NMDA) (vertical) and 10s (horizontal).

having considerably greater selectivity for GluR5 than for GluR2 (ref. 10). The ability of LY382884 to displace binding at a wide range of recombinant AMPA and kainate receptors is shown in Fig. 1a. Binding to GluR5 was displaced with an inhibition constant (K_i) of 4.0 ± 0.2 μM ($n = 4$), whereas binding to GluR1–4, GluR6, GluR7, KA2 and a heteromeric assembly of GluR6 and KA2 was displaced with K_i values in excess of 100 μM. The kainate receptor antagonist activity of LY382884 is shown for recombinant and native receptors in Fig. 1b–d. LY382884 antagonized kainate-induced currents in GluR5/GluR6 heteromers (Fig. 1b) but not in GluR6 homomers (Fig. 1c). The heteromer was made by co-expressing GluR6(Q), which alone generated strongly rectifying currents, with GluR5(R), which alone was non-functional; this co-expression yielded a more linear *I–V* relationship than GluR6 alone, in response to voltage ramps between −100 and +100 mV. In cells displaying this more linear rectification, LY382884 dose dependently reduced the current induced by 30 μM kainate and increased rectification, presumably because a greater proportion of GluR6 homomers contributed to the residual current (Fig. 1b). The absence of functional antagonism at recombinant GluR6 kainate receptors is shown in Fig. 1c, which compares LY382884 with the AMPA/ kainate receptor antagonist NBQX (2,3-dihydroxy-6-nitro-7-sulphamoylbenzo(*f*)quinoxaline). LY382884 had no observable antagonistic effects at human GluR6 kainate receptors at concentrations of up to 100 μM. In rat dorsal root ganglion (DRG) neurons, which express GluR5 kainate receptors[8,11,12], LY382884 inhibited currents evoked by kainate (30 μM) and a selective GluR5 kainate receptor ligand, ATPA ((*RS*)-2-amino-3-(3-hydroxy-5-*tert*-butylisoxazol-4-yl)propanoic acid) (3 μM) in a concentration-dependent manner (half maximal concentration (IC_{50}) 0.95 ± 0.16 μM ($n = 6$) and 1.19 ± 0.79 μM ($n = 6$), respectively. We also established the antagonistic activity of LY382884 at hippocampal AMPA and NMDA receptors (Fig. 1d). LY382884 had little or no effect on currents evoked by AMPA (30 μM) or NMDA (10 μM) at a concentration of 10 μM.

To determine whether LY382884 can be used to antagonize neuronal kainate receptors selectively in an intact slice preparation, we performed experiments on the CA1 region of the hippocampus. We recorded excitatory postsynaptic potentials (EPSPs) mediated by AMPA receptors intracellularly and determined their sensitivity by sequentially applying increasing concentrations of the antagonist. LY382884 depressed the synaptic AMPA receptor-mediated response with an IC_{50} of 87 μM ($n = 3$; Fig. 2a). In these experiments, 10 μM was the maximum concentration that could be used before AMPA receptor-mediated synaptic transmission was affected. Neither monosynaptic γ-aminobutyric acid $(GABA)_A$ and $GABA_B$ receptor-mediated synaptic transmission[13] nor passive membrane properties were affected by 10 μM LY382884 ($n = 5$; data not shown). Next, we determined the effectiveness of 10 μM LY382884 as a kainate receptor antagonist by testing its ability to inhibit the depression of AMPA receptor-mediated synaptic transmission induced by ATPA, using field potential recordings in slices obtained from juvenile rats[14]. ATPA depressed field EPSPs (fEPSPs), at a concentration of 1 μM ($n = 5$) or 3 μM ($n = 6$), respectively, by 57 ± 6 and 63 ± 6% under control conditions but by only 6 ± 2 and 18 ± 9% in the presence of LY382884 (data not shown).

Given the high density of kainate receptors in area CA3 (ref. 15), we extended our analysis to this region. LY382884 similarly antagonized the depression of AMPA receptor-mediated EPSPs induced by ATPA. Thus, 1 μM ATPA depressed associational/commissural and mossy-fibre-evoked fEPSPs, respectively, by 34 ± 2 and 42 ± 11% under control conditions but by only 6 ± 4 and 10 ± 5% in the presence of 10 μM LY382884 ($n = 4$; Fig. 2b). Synaptic transmission in area CA3 is also inhibited by activation of metabotropic glutamate (mGlu) receptors, raising the possibility that ATPA and LY382884 are acting through these receptors. However, the mGlu receptor antagonist (S)-α-methyl-4-carboxyphenylglycine (MCPG) had no effect on ATPA-induced depression ($n = 5$), and LY382884 had no effect on depression induced by the mGlu receptor agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid ((1S,3R)-ACPD; $n = 6$; Fig. 2c). Furthermore, 10 μM LY382884 had no effect on depolarization of CA3 neurons induced by 30 μM (RS)-DHPG ((RS)-3,5-dihydroxyphenylglycine) (mean depolarizations before and after addition of LY382884, 10 ± 3 and 12 ± 2 mV, respectively; $n = 8$), precluding an effect of the antagonist on postsynaptic group I mGlu receptors. CA3 neurons possess postsynaptic kainate receptors that can be activated synaptically by brief, high frequency tetanus[16,17]. LY382884 (10 μM) antagonized this kainate receptor-mediated excitatory postsynaptic current (EPSC), recorded under whole-cell voltage-clamp conditions at −70 mV, by 38 ± 4% ($n = 7$; Fig. 2d).

Having established that LY382884 is a selective kainate receptor antagonist, we wished to determine whether kainate receptors are involved in the induction of LTP at mossy fibre synapses, given that

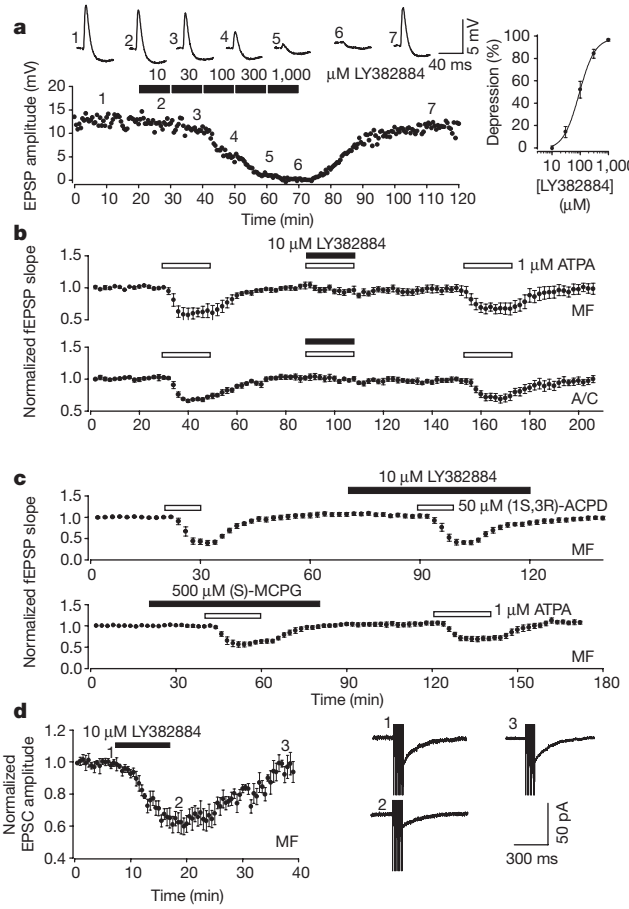

Figure 2 LY382884 as an antagonist in rat hippocampal slices. **a**, Concentration-dependent antagonism of AMPA receptor-mediated EPSPs. The left graph shows a single representative example and the right graph plots peak synaptic depression versus [LY382884] for three neurons (EC_{50} = 87 μM; neurons held at −73 ± 3 mV). **b**, Pooled data ($n = 4$) showing reversible antagonism by LY382884 of ATPA-induced depression of field EPSPs in area CA3, evoked alternatively by stimulation of mossy fibres (MF) and associational/commissural (A/C) inputs. **c**, Pooled data to show that LY382884 does not affect (1S,3R)-ACPD-induced depression ($n = 6$) and that MCPG does not affect ATPA-induced depression ($n = 5$) of mossy-fibre-evoked fEPSPs. **d**, Reversible antagonism by LY382884 of kainate receptor-mediated EPSCs, evoked by high-frequency stimulation (10 shocks at 100 Hz) of mossy fibres, in the presence of a cocktail of antagonists[16] to prevent activation of AMPA, NMDA or GABA receptor-mediated synaptic currents ($n = 7$).

LTP at these synapses is classically independent of NMDA receptors[18]. Mossy fibre LTP was completely prevented, in a reversible manner, by LY382884. (The mean potentiation 60 min after tetanization (100 Hz, 1 s, test intensity) in the presence and after washout of LY382884 was 1 ± 4% and 48 ± 10%, respectively; $n = 7$; Fig. 3a, b.) The ability of LY382884 to block the induction of LTP was pathway specific, as NMDA receptor-dependent LTP in the CA3 region of the hippocampus, evoked by tetanic stimulation of associational/commissural fibres, was fully resistant to the actions of LY382884. The mean potentiation 60 min after tetanization in the presence of LY382884 was 45 ± 10 ($n = 3$), compared with 47 ± 6% in interleaved control experiments ($n = 6$; Fig. 3c). Furthermore, NMDA receptor-dependent LTP at CA1 synapses was also insensitive to LY382884 ($n = 7$; data not shown).

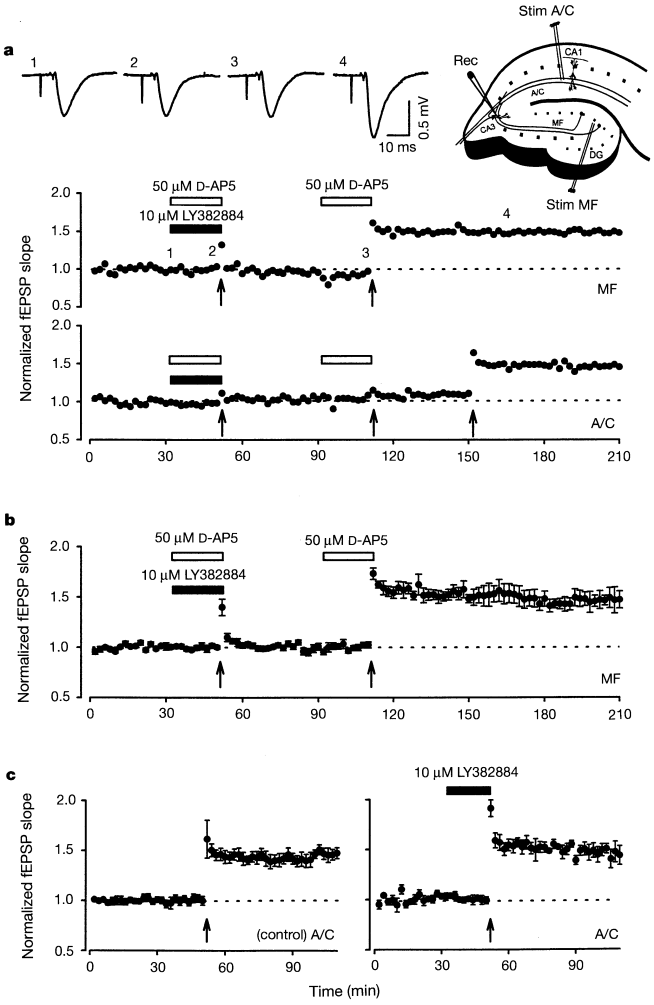

Figure 3 LY382884 specifically blocks the induction of mossy fibre LTP. **a**, A single example to show reversible block of the induction of mossy fibre (MF) LTP by LY382884. Note that the tetani were delivered in the presence of the NMDA receptor antagonist D-AP5, except for the third tetanus delivered to the associational/commissural (A/C) fibres. The traces in this and subsequent figures are averages of four successive records obtained at the times indicated on the graphs by the corresponding numbers. Inset, positions of the stimulating and recording electrodes. **b**, Pooled data for seven experiments showing reversible block of mossy-fibre-induced LTP. In this and subsequent graphs, data points are the average of four successive measurements and the time of each tetanus (100 Hz, 1 s, test intensity) is indicated by an arrow. **c**, The pooled data shows NMDA receptor-dependent LTP in the CA3 region of the hippocampus, evoked by tetanic stimulation of A/C fibres, under control conditions ($n = 6$) and in the presence of LY382884 ($n = 3$).

It was possible that LY382884 blocked the induction of LTP by an action that was independent of its ability to antagonize kainate receptors. Mossy fibre LTP involves the activation of the cAMP–protein kinase A (PKA) pathway and can be induced by the stimulation of adenylyl cyclase by forskolin[19]. Figure 4a shows input-specific, long-lasting (>4 h), PKA-dependent mossy fibre LTP induced by our standard tetanus protocol. LY382884 had no effect on forskolin-induced mossy fibre LTP ($n = 6$) when compared with interleaved controls ($n = 6$; Fig. 4b). Thus, its ability to inhibit LTP is unlikely to be due to an action on signal transduction or expression mechanisms involved in mossy fibre LTP.

The finding that kainate receptors are involved in the induction of mossy fibre LTP is unexpected, as two other antagonists, kynurenate and CNQX, have been reported not to block mossy-fibre-induced LTP[20,21]. We confirmed that kynurenate could cause substantial antagonism of AMPA receptor-mediated synaptic transmission without preventing the induction of LTP, as determined following washout of kynurenate (this result was observed when 3 mM was applied for 20 min, our standard perfusion time to achieve a steady-state concentration; Fig. 5a). We verified that AMPA receptors are not required for induction of mossy fibre LTP using GYKI53655 (30 μM, 20 min), a more selective AMPA receptor antagonist, which

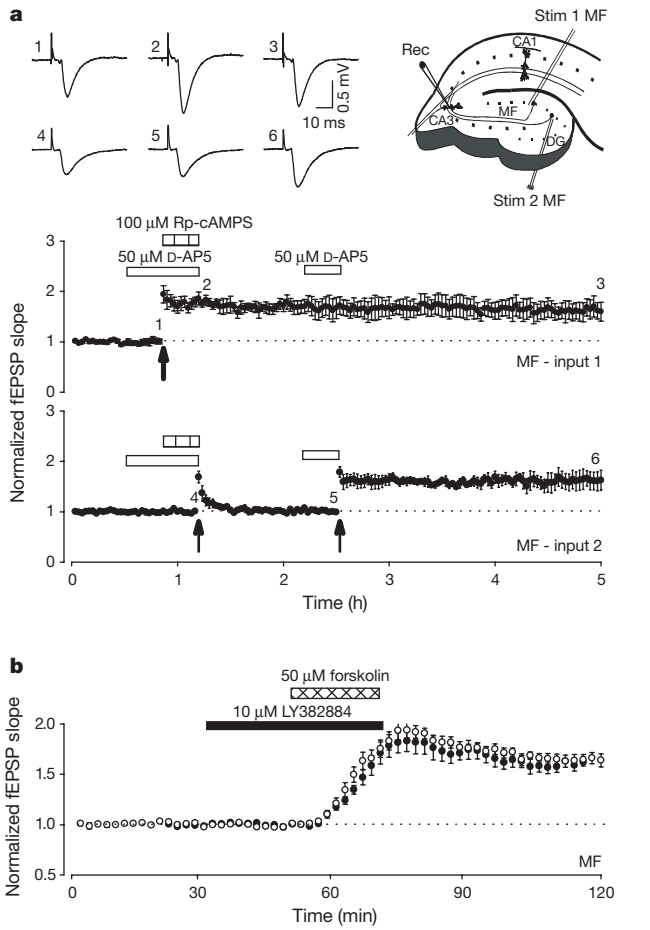

Figure 4 LY382884 does not affect forskolin-induced LTP. **a**, Pooled data ($n = 3$) to show that a single tetanus, delivered in the presence of D-AP5, induced input-specific, PKA-dependent LTP that lasted at least 4 h. Note that Rp-cAMPS (Rp-adenosine 3,5-cyclic monophosphothioate triethylamine) was applied immediately after tetanization of input 1 and washout was commenced immediately after tetanization of input 2. Inset, electrode arrangements. **b**, Pooled data to show that LY382884 does not affect LTP induced by application of forskolin. Data are from six slices treated with LY382884 (open symbols) and six interleaved controls (filled circles). Forskolin produced no potentiation of the associational/commissural input (data not shown).

reversibly blocked mossy fibre synaptic transmission. Three hours after the start of washout of GYKI53655, the non-tetanized input had recovered to baseline values (+5 ± 2%), whereas the tetanized input exhibited stable LTP (of 89 ± 25%; $P < 0.05$; $n = 5$).

LTP was, however, fully blocked by 10 mM kynurenate ($n = 5$; Fig. 5b). We also found that 10 μM CNQX, a concentration that antagonizes kainate-induced currents mediated by GluR5 in CA3 neurons[16], blocked the induction of mossy fibre LTP ($n = 4$; Fig. 5c). In these two sets of experiments, we also examined the effects of LY382884 alone and of D(-)-2-amino-5-phosphonopentanoate (D-AP5) alone on mossy fibre LTP; in all cases, LY382884 blocked LTP whereas LTP was readily obtained in the presence of D-AP5 (the mean potentiation 60 min after tetanization in the presence of LY382884 alone was −2 ± 1%; 60 min after tetanization in the presence of D-AP5 alone it was 36 ± 11%; $n = 9$). CNQX (K_i for displacement of GluR5 binding, 2.9 ± 0.2 μM; $n = 3$) is about as effective as LY382884, whereas kynurenate is very weak as an antagonist at GluR5-expressing cells, with dose-dependent antagonism over the millimolar range. In three separate systems, kynurenate produced significantly ($P < 0.05$) more antagonism at 10 mM than at 3 mM: displacement of 100-μM kainate binding in GluR5-expressing HEK293 cells was 98 ± 1% at 10 mM kynurenate and 60 ± 1% at 3 mM ($n = 3$); inhibition of 100 μM kainate currents in DRG neurones was 89 ± 3 and 76 ± 1%, respectively ($n = 3$); depression of synaptic responses mediated by kainate receptors in CA3 neurons was 84 ± 7 and 42 ± 8%, respectively ($n = 4$). Thus, three structurally unrelated compounds antagonized both events mediated by kainate receptors containing GluR5 and mossy fibre LTP in the same rank order of potency: LY382884 = CNQX ≫ kynurenate.

LY382884 shows selectivity between homomeric GluR5 and GluR2 receptors[10]; it has been used in studies of global ischaemia[10] and nociception[22,23]. We have shown that LY382884 antagonizes responses mediated by kainate receptors at concentrations below those that affect synaptic processes mediated by AMPA or NMDA receptors. Like LY293558 (ref. 8) and LY294486 (ref. 9), LY382884 is highly selective for the GluR5 kainate receptor subunit. The earlier kainate receptor antagonists have been used to identify a role for GluR5 subunits in excitatory synaptic transmission in the hippocampus[24,25] and amygdala[26]. However, these compounds also antagonize AMPA receptors. Given its greater selectivity, LY382884 is a more useful antagonist with which to explore the functions of GluR5 in the brain.

Although our data indicate that the kainate receptor involved in the induction of LTP may have at least one GluR5 subunit, this does not preclude roles for other kainate receptor subunits in synaptic plasticity. Indeed, as the kainate receptor-mediated component of excitatory synaptic transmission at mossy fibre synapses[16,17] is both antagonized by GluR5 antagonists[24] and absent in GluR6 knockout mice[27], both subunits may contribute to native kainate receptors in these neurons. The sensitivity of a GluR5/GluR6 heteromer to LY382884 is consistent with this possibility. Our findings do not indicate whether mossy fibre LTP is induced pre- or postsynaptically[28,29], as GluR5-containing kainate receptors are located at both sites. However, it may be possible to develop antagonists that are selective for pre- or postsynaptic kainate receptors at mossy fibres to address this question. Other receptors, such as mGlu receptors, may activate separate transduction processes that are also required for mossy fibre LTP[20,29,30]. Hitherto, LTP has been viewed as usually being induced by the synaptic activation of NMDA receptors and expressed as an enhancement of synaptic transmission mediated by AMPA, and under certain circumstances NMDA, receptors[3]. At synapses that exhibit LTP independently of NMDA receptors, it has been assumed that ionotropic glutamate receptors are not required for the induction of LTP[28,29]. We have shown that kainate receptors are involved in the induction of LTP at a synapse where NMDA receptors do not have a role. It will be interesting to determine whether this principle extends to other synapses where NMDA receptor-independent LTP has been found and whether, by analogy to the NMDA receptor, the biophysical properties of kainate receptors[5–7] endow properties of functional significance to this form of LTP.

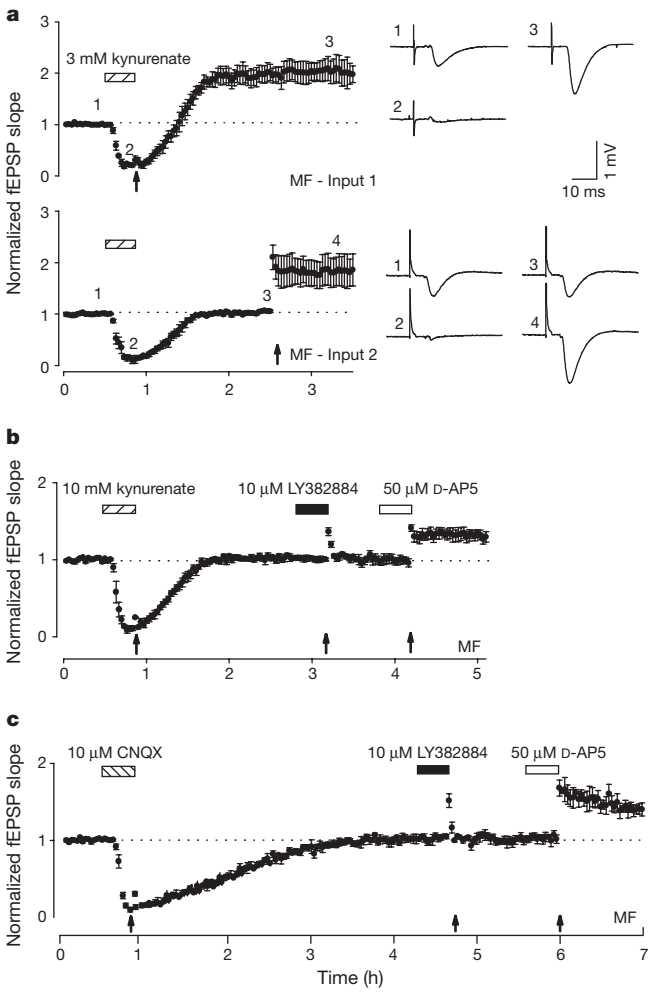

Figure 5 Kynurenate and CNQX block the induction of mossy fibre LTP. **a**, Pooled data ($n = 4$) to show that 3 mM kynurenate blocks AMPA receptor-mediated synaptic transmission but not mossy fibre LTP. A tetanus was delivered to input 1 during blockade of synaptic transmission and to input 2 following recovery from the effects of kynurenate. The traces are from a single experiment. **b**, Pooled data ($n = 5$) to show that 10 mM kynurenate, like 10 μM LY382884, blocks the induction of mossy fibre LTP. **c**, Pooled data ($n = 4$) to show that 10 μM CNQX, like 10 μM LY382884, blocks the induction of mossy fibre LTP.

Methods

Ligand-binding studies

These experiments were performed using cell membranes prepared from frozen HEK293 cells expressing recombinant AMPA or kainate receptor subunits and using [^{3}H]-AMPA and [^{3}H]-kainate, respectively, as described[9].

Construction of the heteromer

A GluR6(Q) stable HEK293 cell line was transfected with $GluR5_{2b}$(R) in the vector pCEP4, using Lipofectamine 2000. After three weeks of selection with 250 μg ml^{-1} hygromycin, the cells reached 30% confluency and were split for electrophysiology.

Electrophysiology using isolated cells

Whole-cell voltage clamp recordings were made using extracellular solutions comprising (in mM) NaCl (138), $CaCl_2$ (5), KCl (5), $MgCl_2$ (1), HEPES (10) and glucose (10); pH 7.4.

For HEK293 cells and hippocampal neurons, intracellular solutions comprised (in mM) CsCl (140), $MgCl_2$ (1), diTris creatine phosphate (14), HEPES (10), BAPTA (15) and creatine phosphokinase (50 units ml^{-1}); pH 7.15. For DRG neurons[8], intracellular solutions comprised (in mM) $CsMeSO_4$ (125), CsCl (15), CsBAPTA (5), HEPES (10), $CaCl_2$ (0.5) $MgCl_2$ (3) and MgATP (2); pH 7.2. Experiments were performed at room temperature (20–22 °C). Drugs were applied by bath perfusion and exchange of solutions under these conditions took about 5 s. Voltage ramps were conducted between −100 and +100mV in 1 s. Experiments using recombinant receptors and DRG neurons were performed after incubation of cells with 250 μg ml^{-1} concanavalin A for 10 min to remove receptor desensitization. Hippocampal pyramidal neurons were cultured from E17 Sprague Dawley rat fetuses. AMPA receptor-mediated currents were obtained from cells (6–12 days *in vitro*) in the presence of tetrodotoxin (1 μM); NMDA receptor-mediated currents were recorded from cells (10–12 days *in vitro*) in the presence of glycine (10 μM) but without added magnesium. Curve fitting to the data points was based upon the equation $y = 100(D^n/(D^n + EC_{50}^n))$ using a slope fixed to a value of 1 and where D is the drug concentration. For antagonists, $EC_{50} = IC_{50}$. IC_{50} values were estimated from data obtained from at least four separate cells.

Electrophysiology in slice

Experiments were performed on transverse rat hippocampal slices (400 μm) maintained in medium comprising (in mM) NaCl (124), KCl (3), $NaHCO_3$ (26), NaH_2PO_4 (1.25), $CaCl_2$ (2), $MgSO_4$ (1) and D-glucose (10) (bubbled with O_2/CO_2 : 95/5%). Extracellular fEPSPs were recorded in areas CA1 and CA3 using glass microelectrodes (2–4 MΩ) containing 4 M NaCl, as described[14]. Intracellular recordings were obtained using sharp glass microelectrodes (40–80 MΩ) filled with $KMeS0_4$ (2M)[13]. Whole-cell patch-clamp recordings were obtained blind using glass microelectrodes (5–7 MΩ; seal resistance ~10 GΩ) filled with a solution comprising (in mM) $CsMeSO_3$ (120), NaCl (1), $MgCl_2$ (1), Mg-ATP (4), BAPTA (10), *N*-(2,6-dimethyl-phenylcarbamoylmethyl)-triethylammonium bromide (QX-314) (5) and HEPES (5), adjusted to pH 7.3, as described[16]. Data are presented as mean ± s.e.m. throughout.

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Activation of the epithelial Na^+ channel (ENaC) requires CFTR Cl^- channel function

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It is increasingly being recognized that cells coordinate the activity of separate ion channels that allow electrolytes into the cell. However, a perplexing problem in channel regulation has arisen in the fatal genetic disease cystic fibrosis, which results from the loss of a specific Cl^- channel (the CFTR channel) in epithelial cell membranes[1]. Although this defect clearly inhibits the absorption of Na^+ in sweat glands[2,3], it is widely accepted that Na^+ absorption is abnormally elevated in defective airways in cystic fibrosis[4,5]. The only frequently cited explanation for this hypertransport is that the activity of an epithelial Na^+ channel

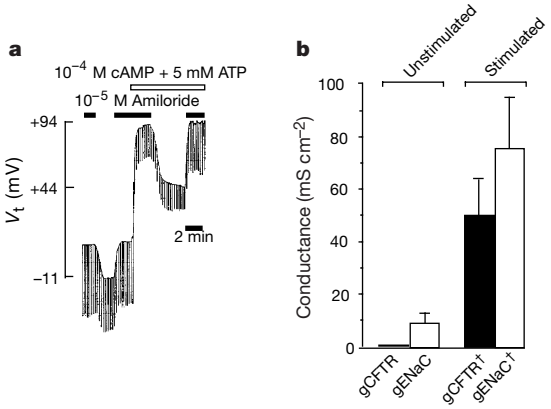

Figure 1 Effect of cAMP and ATP activation of CFTR Cl^- conductance (gCFTR) on amiloride-sensitive epithelial Na^+ conductance (gENaC). **a**, Diffusion potentials and constant current–pulse potentials across the apical membrane. Without stimulation, CFTR is completely deactivated, but a small stimulation-insensitive, amiloride-sensitive Na^+ diffusion potential and gENaC remain. However, stimulation results in a significant increase in both gCFTR and gENaC† (see text). **b**, Mean conductances of gCFTR (filled columns) and gENaC (open columns) measured under baseline conditions (Unstimulated) and after gCFTR when stimulated with 0.1 mM cAMP plus 5 mM ATP (Stimulated) ($P < 0.01$, $n = 15$).