European Journal of Neuroscience, Vol. 15, pp. 1859–1862, 2001

SHORT COMMUNICATION
Rapid regrowth of hippocampal mossy fibres and preceding maturation of NMDA receptor-mediated neurotransmission

Yuji Ikegaya, Ryuta Koyama, Maki K. Yamada, Nobuyoshi Nishiyama and Norio Matsuki
Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113–0033, Japan

Keywords: dentate gyrus, hippocampus, lesion, *N*-methyl-D-aspartate, transection

Abstract

Early in postnatal development, glutamatergic synapses contain primarily NMDA receptors and progressively acquire AMPA receptor function. To determine whether this transformation occurs in a process of regenerative synaptogenesis following axotomy, we investigated the recovery of AMPA and NMDA receptor-mediated neurotransmission after the transection of mossy fibres (MF) in organotypic hippocampal cultures. An NMDA component could already be elicited 1 day after the lesion and reached a saturated level after 3 days. Thereafter, an AMPA component appeared and slowly matured after 10 days. The preceding establishment of NMDA receptor function implies that immature MF synapses are functionally silent at least for the first several days of recovery. The appearance of AMPA receptor-mediated neurotransmission was unchanged in the presence of an NMDA-receptor antagonist or tetrodotoxin, which suggests that the AMPA receptor maturation is virtually independent of neuronal activity. Thus, the conversion of silent to functional synapses is not unique to synaptic plasticity or developmental processes but also occurs in recovery after brain damage, but its mechanism is likely to differ from NMDA receptor-dependent recruitment of AMPA receptors in synaptic plasticity.

Introduction

Although a functioning excitatory synapse in the vertebrate central nervous system contains both α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors in the postsynaptic membrane, recent evidence indicates that, early in postnatal development, neurons contain functionally 'silent' synapses, which exhibit purely NMDA receptor-mediated postsynaptic responses and do not conduct signals at resting membrane potentials (Durand *et al*., 1996; Wu *et al*., 1996). As development progresses, these synapses gradually become functional, probably by recruiting functional AMPA receptors. Such a switching of silent synapses to functional synapses offers a powerful mechanism for mediating synaptic plasticity (Isaac *et al*., 1995; Liao *et al*., 1995). However, it remains uncertain whether this transformation is also used in a process of recovery after brain damage, e.g. synapse reformation and network reorganization following axon degeneration or neuronal cell death.

In organotypic cultures of hippocampal slices, we and colleagues indicated that even after an axotomy, the hippocampal mossy fibre (MF) is able to regrow into the appropriate target, i.e. the proximal segment of the apical dendrites of CA3 pyramidal cells, and elaborate functional synapses with the target cells (Zimmer & Gahwiler, 1987; Dailey *et al*., 1994; Ikegaya *et al*., 1997; Ikegaya *et al*., 1998; Mizuhashi *et al*., 2001). Therefore, the MF regrowth provides an opportunity to observe how synapses acquire physiological function during recovery from the injury. Using this culture system, the present work shows that regenerated MF synapses are kept silent for a few days and subsequently acquire AMPA receptor function.

Correspondence: Dr Yuji Ikegaya, as above.
E-mail: ikegaya@tk.airnet.ne.jp

Received 10 February 2002, revised 6 April 2002, accepted 15 April 2002

Materials and methods

Hippocampal slice cultures were prepared from 6–8-day-old Wistar/ST rats (SLC, Shizuoka, Japan), as previously described (Ikegaya, 1999), according to the Japanese Pharmacological Society guide for the care and use of laboratory animals. After decapitation, brains were aseptically removed and cut into 300-μm-thick slices in oxygenated ice-cold Gey's balanced salt solution. The entorhino-hippocampi were dissected out and cultured using a membrane interface technique. The culture medium, consisting of 50% minimal essential medium (Life Technologies, Grand Island, NY, USA), 25% horse serum (Cell Culture Laboratory, Cleveland, OH, USA) and 25% Hanks' balanced salt solution (Cell Culture Laboratory), was changed every 3.5 days. After 10–12 days *in vitro*, cultures were sectioned by using a razor blade along the axis running between the tips of the inferior and superior layers of stratum granulosum (Ikegaya *et al*., 1997). The major aim of this procedure was to cut the connections established between the dentate gyrus and the CA3 region, i.e. the MF tract (Fig. 1A).

For the quantitative assessment of functional MF connections, field excitatory postsynaptic potentials (fEPSPs) evoked by stimulation of stratum granulosum were extracellularly recorded from stratum

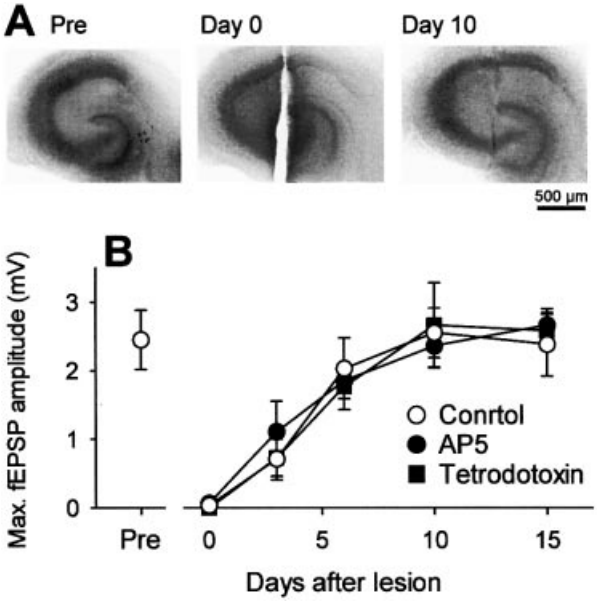

FIG. 1. Activity-independent recovery of hippocampal organotypic cultures after MF transection. (A) Representative images of Nissl-stained slices immediately before (Pre, i.e. intact slices) or immediately after (Day 0) and 10 days after (Day 10) the sectioning. The cleft between two stumps was emphasized in the day 0 slice because the tissues shrank slightly after fixation. (B) Functional recovery of MF synaptic transmission following complete MF lesion in the chronic presence or absence of 25 μM D-AP5 or 0.5 μM tetrodotoxin was assessed by recording MF synaptic responses. Each data point is an average (± SEM of 7–9 slices) maximal fEPSP amplitude at the indicated days after lesion.

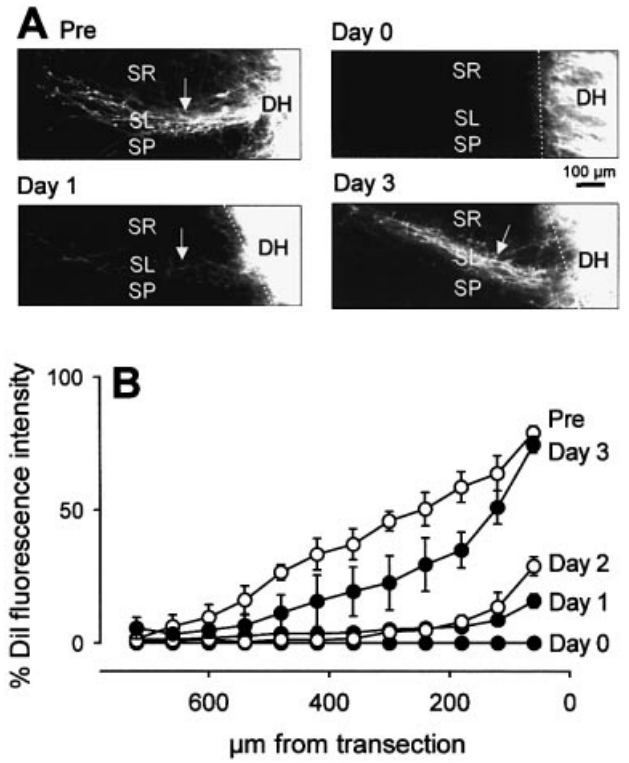

FIG. 2. Rapid re-elongation of transected MFs. (A) The MFs were visualized by iontophoretic DiI injection immediately before (Pre) and 0, 1, and 3 days after lesion. The white broken lines indicate lines of cutting plane. The arrows indicate the MFs, which run through stratum lucidum, a normal MF trajectory. SR, stratum radiatum; SL, stratum lucidum; SP, stratum pyramidale; DH, dentate hilus. (B) Relationship between distances from the transection and DiI fluorescence intensity. Each data point is an average (± SEM of 14–17 slices) intensity relative to the DiI-injected area of the dentate hilus.

pyramidale of the CA3b region (≈ 500 μm from the CA3c/hilus boundary) in warmed (32 °C) artificial cerebrospinal fluid (ACSF) consisting of (in mM) NaCl, 124; $NaHCO_3$, 26; KCl, 5; KH_2PO_4, 1.24; $CaCl_2$, 2.4; $MgSO_4$, 1.3; and glucose, 10. In our experiments, the fEPSPs were recorded as positive responses, i.e. upward traces (see Fig. 3A), because the recording electrode was positioned in pyramidal cell layer. These responses displayed a high degree of paired-pulse facilitation (PPF) (the PPF ratio was 196.4 ± 15.6% at 50 ms paired-pulse interval; mean ± SEM) and were efficiently attenuated by bath application of 0.5–1 μM (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl) glycine (Tocris Cookson, Ballwin, MO, USA), a group II metabotropic glutamate receptor agonist. These are typical of MF-mediated fEPSPs (Claiborne *et al*., 1993; Yokoi *et al*., 1996). The maximal size of fEPSPs was used as an index of the number of functional synaptic contacts of the MFs (Mizuhashi *et al*., 2001). In some experiments, AMPA and NMDA receptor-mediated fEPSPs were pharmacologically isolated using ACSF containing 25 μM D-2-amino-5-phosphonopentanoic acid (D-AP5; Sigma, St. Louis, MO, USA) and Mg^{2+}-free ACSF containing 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Sigma), respectively. At the end of each experiment, we confirmed that the AMPA and NMDA components were completely blocked by 10 μM CNQX and 25 μM D-AP5, respectively (data not shown). The kinetics of NMDA synaptic responses were fast compared with those of AMPA synaptic responses, probably owing to the much higher affinity for glutamate of NMDA receptors than AMPA receptors (Patneau & Mayer, 1990).

To visualize MF pathways, the MFs were iontophoretically labelled with 1,1′-dioctadecyl 3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR, USA) (Koyama *et al*., 2002). After fixation with 4% paraformaldehyde, a glass micropipette filled with 0.5% DiI was inserted into the dentate hilus, and a single positive pulse (100 V, 10 s) was applied through the pipette. After 7 days of incubation, the labelled fibres were observed with the confocal imaging system.

Results

Although the sectioning of cultured hippocampal slices *per se* induced no apparent cell death as assessed by Nissl staining (Fig. 1A) or propidium iodide uptake (data not shown), no synaptic response was evoked in the stratum pyramidale of CA3 by stimulation of the dentate gyrus immediately after the lesion (Fig. 1B), suggesting that our procedure successfully produced a complete transection of the MF tract without impairing cell viability.

As the slices were then maintained in culture, MF synaptic responses emerged after 3 days, thereafter gradually increased in size and eventually reached an apparent steady state at day 10 (Fig. 1B). At this time, the response amplitude was almost the same as the baseline level prior to the lesion ($P > 0.1$, Student's *t*-test).

By using the neuronal tracer DiI, we examined the time course of MF re-elongation. The MFs were not evident in the CA3 region immediately after the transection but, surprisingly, a few fibres could already be detected after 1 day (Fig. 2A). After 3 days, the number of established MFs was almost indistinguishable from control levels (Fig. 2A). The DiI fluorescence intensity was quantitatively analysed

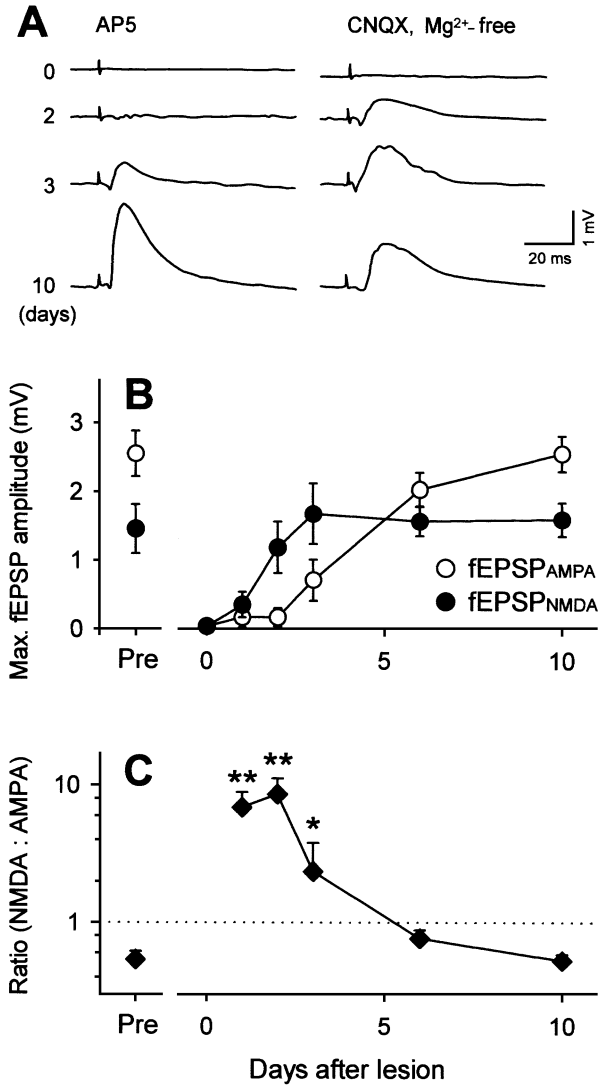

FIG. 3. Time course of recovery of NMDA receptor-mediated neurotransmission. (A) Typical traces of AMPA (left) and NMDA (right) receptor-mediated synaptic responses at 0, 2, 3 and 10 days after lesion. AMPA and NMDA receptor-mediated fEPSPs were pharmacologically isolated by ACSF containing 25 μM D-AP5 and by Mg^{2+}-free ACSF containing 10 μM CNQX, respectively. (B) Summary data for the time course of recovery of the maximal amplitude of AMPA and NMDA receptor-mediated fEPSPs. (C) The ordinate indicates the ratio of the NMDA component to the AMPA component. The data were the same as Fig. 3B. $*P < 0.05$, $**P < 0.01$ vs. Pre; Tukey's test after one-way analysis of variance. Data represent means ± SEM of 5–9 slices.

along stratum lucidum from dentate hilus to CA2 (Fig. 2B). The average recovery of DiI signal in stratum lucidum at day 3 was 83.4 ± 26.8% of the prelesion level (mean ± SEM of 17 slices), which was much higher than the rate of fEPSP recovery (29.0 ± 12.4% in Fig. 1B, $P < 0.05$, Student's t-test). We thus suspected that, at early stages of recovery, MF synapses are silent.

To address this possibility, AMPA and NMDA receptor-mediated neurotransmissions were pharmacologically isolated from the same slices (Fig. 3). Because normal synaptic transmission is mediated primarily by AMPA receptors, the recovery of AMPA receptor-mediated fEPSPs had a very similar time course to that seen in Fig. 1B; it began after 3 days and lasted over the next several days of development until reaching baseline levels at day 10. On the other hand, NMDA receptor-mediated fEPSPs were detectable at day 1, and became comparable to control levels after 3 days (Fig. 3B). As a result, the ratio of the NMDA component to the AMPA component was significantly high during the first 3 days of recovery, as compared to control (Fig. 3C), suggesting that newly formed MF synapses are functionally silent.

The stimulus intensity was adjusted to produce fEPSPs with an amplitude of ≈50% of maximum, and paired-pulse stimulation with a 50-ms interval was delivered in order to evoke PPF. Three days after the axotomy, the PPF ratio of NMDA responses was 206.5 ± 18.8% (mean ± SEM, $n = 5$ slices), which was almost the same as that of AMPA responses (198.9 ± 16.7%, $P > 0.1$, Student's t-test). When the stimulating electrode was placed on the CA3a stratum radiatum to stimulate undamaged associational/commissural fibres at day 3, normal AMPA responses were recorded (103.5 ± 8.4% of intact slices; $n = 4$ slices). Thus, we consider that the early recovery of the NMDA component was not a misinterpretation resulting from a possible alteration of MF presynaptic release probability or a contribution of disynaptic activation of CA3 pyramidal cells via their recurrent inputs.

To determine whether the prior activity of NMDA receptors at immature MF synapses is required for the subsequent acquisition of AMPA receptor function, slices were cultured in the continuous presence of 25 μM D-AP5, an NMDA receptor antagonist. They displayed no difference in fEPSP recovery compared to control slices (Fig. 1B). Likewise, the development of MF responses was normal even in the presence of tetrodotoxin (Fig. 1B). These results suggest that neither NMDA receptor function nor neuronal activity was essential for AMPA receptor maturation.

Discussion

One of the major questions in neuroscience is how synapses acquire physiological properties during maturation. Although many studies have demonstrated that, early in development, glutamatergic synapses are conditionally silent and then progressively acquire physiological function (Durand *et al*., 1996; Wu *et al*., 1996), it remained unclear whether this transformation also occurs in a process of regeneration following brain damage. Using experimental axotomy of hippocampal MFs *in vitro*, we have shown for the first time that newly formed synapses after the injury are silent but progressively become functional. This maturation is unlikely to depend on neuronal activity. Activity-dependent, NMDA receptor-mediated recruitment of AMPA receptors has been suggested particularly in synaptic plasticity (reviewed in Feldman & Knudsen, 1998; Carroll & Malenka, 2000). It is therefore possible that, in a recovery process, the functional switching involves a mechanism different from that of synaptic plasticity. In cultures of hippocampal neurons, AMPA receptors have been shown to accumulate at new synaptic junctions even when spontaneous spiking activity or synaptic transmission is blocked (Craig *et al*., 1994; Mammen *et al*., 1997; Friedman *et al*., 2000). Thus, we consider that the recovery process contains an analogous mechanism to developmental synapse maturation.

Among the afferent fibres onto CA3 pyramidal cells, the MFs have a postnatal development (Amaral & Dent, 1981). Recent electrophysiological studies showed that at early developmental stages (postnatal 2–7 days), the MFs may contain ≈65% silent synapses (Gasparini *et al*., 2000). The authors could not determine, however, whether all MF synapses initially have NMDA receptor function and subsequently gain AMPA receptor function because the period of MF

development is not limited; some immature axons may form synaptic contacts with pyramidal cells from the first postnatal day but others develop even after the second postnatal week (Amaral & Dent, 1981). In the present study, MF synapses, which were forced to simultaneously develop after sectioning, exhibited silent responses during the first days. We therefore believe that naturally occurring MF synaptogenesis in development is also accompanied by a conversion of silent into functional synapses.

Finally, it is noteworthy that MF regrowth itself was considerably more rapid than expected. On the basis of electrophysiological experiments, MF synaptic maturation has long been believed to require at least several days (Muller *et al*., 1994; Ikegaya *et al*., 1997; Ikegaya, 1999). However, the assessment from the isolated NMDA component and DiI labelling revealed that some, if not all, of the MFs can already elongate and make synapses within 24 h after damage. The appearance of AMPA receptor function is therefore the rate-determining step in full recovery; if the functional switching is promoted, the regeneration is also accelerated. Thus, elucidating the mechanisms for the acquisition of AMPA receptor function may be beneficial to medical treatment of brain injuries including trauma, infection and ischemia.

Abbreviations

ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid; D-AP5, D-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DiI, 1,1′-dioctadecyl 3,3,3′3′-tetramethylindocarbocyanine perchlorate; fEPSP, field excitatory postsynaptic potential; MF, mossy fibre; NMDA, *N*-methyl-D-aspartate; PPF, paired-pulse facilitation.

References

Amaral, D.G. & Dent, J.A. (1981) Development of the mossy fibers of the dentate gyrus: I. A light and electron microscopic study of the mossy fibers and their expansions. *J. Comp. Neurol.*, **195**, 51–86.

Carroll, R.C. & Malenka, R.C. (2000) Delivering the goods to synapses. *Nature Neurosci.*, **3**, 1064–1066.

Claiborne, B.J., Xiang, Z. & Brown, T.H. (1993) Hippocampal circuitry complicates analysis of long-term potentiation in mossy fiber synapses. *Hippocampus*, **3**, 115–121.

Craig, A.M., Blackstone, C.D., Huganir, R.L. & Banker, G. (1994) Selective clustering of glutamate and gamma-aminobutyric acid receptors opposite terminals releasing the corresponding neurotransmitters. *Proc. Natl Acad. Sci. USA*, **91**, 12373–12377.

Dailey, M.E., Buchanan, J., Bergles, D.E. & Smith, S.J. (1994) Mossy fiber growth and synaptogenesis in rat hippocampal slices in vitro. *J. Neurosci.*, **14**, 1060–1078.

Durand, G.M., Kovalchuk, Y. & Konnerth, A. (1996) Long-term potentiation and functional synapse induction in developing hippocampus. *Nature*, **381**, 71–75.

Feldman, D.E. & Knudsen, E.I. (1998) Experience-dependent plasticity and the maturation of glutamatergic synapses. *Neuron*, **20**, 1067–1071.

Friedman, H.V., Bresler, T., Garner, C.C. & Ziv, N.E. (2000) Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron*, **27**, 57–69.

Gasparini, S., Saviane, C., Voronin, L.L. & Cherubini, E. (2000) Silent synapses in the developing hippocampus: lack of functional AMPA receptors or low probability of glutamate release? *Proc. Natl Acad. Sci. USA*, **97**, 9741–9746.

Ikegaya, Y. (1999) Abnormal targeting of developing hippocampal mossy fibers after epileptiform activities via L-type Ca^{2+} channel activation in vitro. *J. Neurosci.*, **19**, 802–812.

Ikegaya, Y., Ikeda, Y., Saito, H. & Nishiyama, N. (1998) Suppression of synaptogenesis by epileptiform discharges in hippocampal slice culture. *Biol. Pharm. Bull.*, **21**, 231–234.

Ikegaya, Y., Yoshida, M., Saito, H. & Nishiyama, N. (1997) Epileptic activity prevents synapse formation of hippocampal mossy fibers via L-type calcium channel activation in vitro. *J. Pharmacol. Exp. Ther.*, **280**, 471–476.

Isaac, J.T., Nicoll, R.A. & Malenka, R.C. (1995) Evidence for silent synapses: implications for the expression of LTP. *Neuron*, **15**, 427–434.

Koyama, R., Yamada, M.K., Nishiyama, N., Matsuki, N. & Ikegaya, Y. (2002) Group II metabotropic glutamate receptor activation is required for normal hippocampal mossy fiber development in the rat. *J. Physiol. (Lond.)*, **539**, 157–162.

Liao, D., Hessler, N.A. & Malinow, R. (1995) Activation of postsynaptically silent synapses during pairing-induced LTP in CA1 region of hippocampal slice. *Nature*, **375**, 400–404.

Mammen, A.L., Huganir, R.L. & O'Brien, R.J. (1997) Redistribution and stabilization of cell surface glutamate receptors during synapse formation. *J. Neurosci.*, **17**, 7351–7358.

Mizuhashi, S., Nishiyama, N., Matsuki, N. & Ikegaya, Y. (2001) Cyclic nucleotide-mediated regulation of hippocampal mossy fiber development: a target-specific guidance. *J. Neurosci.*, **21**, 6181–6194.

Muller, D., Stoppini, L., Wang, C. & Kiss, J.Z. (1994) A role for polysialylated neural cell adhesion molecule in lesion-induced sprouting in hippocampal organotypic cultures. *Neuroscience*, **61**, 441–445.

Patneau, D.K. & Mayer, M.L. (1990) Structure–activity relationships for amino-acid transmitter candidates acting at *N*-methyl-D-aspartate and quisqualate receptors. *J. Neurosci.*, **10**, 2385–2399.

Wu, G., Malinow, R. & Cline, H.T. (1996) Maturation of a central glutamatergic synapse. *Science*, **274**, 972–976.

Yokoi, M., Kobayashi, K., Manabe, T., Takahashi, T., Sakaguchi, I., Katsuura, G., Shigemoto, R., Ohishi, H., Nomura, S., Nakamura, K., Nakoa, K., Katsuki, M. & Nakanishi, S. (1996) Impairment of hippocampal mossy fiber LTD in mice lacking mGluR2. *Science*, **273**, 645–647.

Zimmer, J. & Gahwiler, B.H. (1987) Growth of hippocampal mossy fibers: a lesion and coculture study of organotypic slice cultures. *J. Comp. Neurol.*, **264**, 1–13.