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Rapid Report

Group II metabotropic glutamate receptor activation is required for normal hippocampal mossy fibre development in the rat

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Glutamate is the main neurotransmitter at hippocampal mossy fibre (MF) terminals. Because neurotransmitters have been proposed as regulating factors of neural network formation and neurite morphogenesis in the developing CNS, we examined the possible contribution of glutamate to MF pathfinding. Entorhino-hippocampal slices prepared from early postnatal rats were cultivated in the presence of glutamate receptor antagonists. Timm histochemical staining revealed that pharmacological blockade of metabotropic glutamate receptors (mGluR), but not of ionotropic glutamate receptors, induced abnormal outgrowth of the MFs. When slices were cultured in the presence of mGluR antagonists, DiI-labelled MF axons displayed a great degree of defasciculation, and MF-mediated EPSPs in the CA3 pyramidal cells were altered. Similar results were obtained for a selective antagonist of group II mGluR, but not of group I or III mGluR. Glutamate is, therefore, likely to regulate MF outgrowth via activation of group II mGluR. The present study may provide a novel role of glutamate in hippocampal development.

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The hippocampal mossy fibres (MFs), axons projecting from dentate granule cells to the apical dendrites of CA3 pyramidal cells, undergo a continuous turnover over a period of weeks (Gould *et al.* 1999), because the granule cells have the unusual property of prolonged postnatal neurogenesis that persists into adulthood (Altman & Das, 1965; Kaplan & Hinds, 1977). Recently, Shors *et al.* (2001) indicated that such newly generated granule cells in the adult are involved in the formation of hippocampal-dependent memory, suggesting that continuous MF pathfinding is important in maintaining normal hippocampal function. In temporal lobe epilepsy of human or experimental animals, the MFs abnormally innervate the inner molecular layer of the dentate gyrus (DG) and the basal dendrites of CA3 pyramidal cells (McNamara, 1994). This pathological sprouting may be attributable, at least in part, to aberrant MF guidance (Parent *et al.* 1997; Ikegaya, 1999). Therefore, elucidating the mechanisms of MF development is of particular importance in understanding the physiological and pathological roles of the hippocampus.

Establishing precise neural connections in the CNS depends on correct pathfinding by growth cones, which recognize appropriate guidance cues present in the environment (Tessier-Lavigne & Goodman, 1996). Accumulating evidence suggests that neurotransmitters serve as guidance cues and key regulators of neurite morphogenesis (Lipton & Kater, 1989; Lauder, 1993; Zheng *et al.* 1996; Song *et al.* 1997; Chang & De Camilli, 2001). Hannan *et al.* (2001) showed that loss of metabotropic glutamate receptor (mGluR) type 5 in mutant mice disrupts barrel pattern formation of thalamic innervation in the somatosensory cortex, suggesting that glutamate is involved in network formation during brain development. Likewise, Ichise *et al.* (2000) reported that mGluR type 1-deficient mice show deficits in the regression of multiple climbing fibre innervation onto Purkinje cells during cerebellar development. Hirai & Launey (2000) indicated that pharmacological blockade of both non-NMDA receptors and mGluRs causes a significant decrease in the number of branch points and in the diameter of Purkinje dendrites. Therefore, it is also possible that glutamate, a major neurotransmitter at MF synapses (Terrian *et al.* 1990), participates in the formation of the MF pathway. Using organotypic cultures of hippocampal slices, the present study shows for the first time that group II mGluR activation is required for normal MF development. This finding represents a novel role for glutamate in hippocampal function.

METHODS

The animal experiments were performed according to the Japanese Pharmacological Society guide for the care and use of laboratory animals.

Materials

Pharmacological agents used in this study were as follows: (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD), 1-aminoindan-1,5-dicarboxylic acid (AIDA), 2-amino-5-phosphonovalerate (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (*S*)-α-ethylglutamic acid (EGLU), (*RS*)-α-methyl-4-carboxyphenylglycine (MCPG), and α-methyl derivatives of serine-*O*-phosphate (MSOP). All these drugs were purchased from Tocris Cookson (Bristol, UK).

Organotypic cultures of hippocampal slices

Hippocampal slice cultures were prepared from 6-day-old Wistar/ST rats (SLC, Shizuoka, Japan), as previously described (Ikegaya, 1999). Animals were deeply anaesthetized by hypothermia, and their brains were aseptically removed and cut into transverse slices (300 μm thick) in aerated, ice-cold Gey's balanced salt solution supplemented with 25 mM glucose using a vibratome (DTK-1500; Dosaka, Kyoto, Japan). The entorhino-hippocampi were dissected out under stereomicroscopic control. Then, selected slices were cultured using a membrane interface technique. Briefly, slices were placed on 30-mm sterile membranes (Millicell-CM, Millipore, Bedford, MA, USA), and transferred into six-well tissue culture trays. Cultures were fed with 1 ml of culture medium consisting of 50 % minimal essential medium (Life Technologies, Grand Island, NY, USA), 25 % horse serum (Cell Culture Lab, Cleveland, OH, USA), and 25 % Hanks' balanced salt solution containing 25 mM glucose, 50 u ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin. The cultures were maintained in a humidified incubator at 37 °C in 5 % CO_2. The medium was changed every 3.5 days. Experiments were performed after 10 days *in vitro*.

Timm staining

After rinsing with PBS, slices were immersed in 0.4 % sodium sulfide solution at 4 °C for 15 min, and fixed with 10 % (v/v) formaldehyde for 15 min. After being washed in PBS, they were dehydrated with 70 % and 96 % ethanol twice for 30 min and then dried. To perform silver sulfide staining, the slices were incubated with citrate-buffered solution containing 20 % gum arabic , 2.1 % $AgNO_3$ and 0.085 % hydroquinone in a dark room at 26 °C for 50 min. The slices were washed with distilled water at the end of the reaction. To quantify MF terminals, the images were digitized with FinePix S1Pro (Fuji Photo Film, Tokyo Japan) equipped with a bright-field microscope. Average pixel intensity (eight-bit intensity levels) was estimated in each slice by acquiring intensity values in five different areas (5 μm × 400 μm) within the stratum lucidum, the CA3 stratum radiatum, the CA3 stratum pyramidale, the CA3 stratum oriens and the dentate hilus. Timm grain intensity in each subregion was determined by (values of the subregion − the mean value of the stratum radiatum in control slices)/values of the dentate hilus × 100.

Iontophoretic MF labeling

Slices were fixed with PBS containing 4 % paraformaldehyde for 24 h. A glass micropipette (1 MΩ resistance) filled with the fluorescent carbocyanine dye 1,1′-dioctadecyl 3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR, USA) (0.5 % in ethanol) was inserted into the dentate hilus, and a single negative pulse (100 V, 10 s) was applied through the pipette. After 7 days of incubation in the same fixative at room temperature, the labelled MFs were observed by using the confocal imaging system MRC-1000 (Bio-Rad) with a × 20 objective.

Extracellular recording

Cultures were transferred to a recording chamber mounted on a stereoscopic microscope, and continuously superfused with warmed (32 °C) artificial cerebrospinal fluid consisting of 124 mM NaCl, 26 mM $NaHCO_3$, 10 mM glucose, 5 mM KCl, 1.3 mM $MgSO_4$, 1.24 mM KH_2PO_4 and 2.4 mM $CaCl_2$, adjusted to pH 7.4. To record field responses, a glass micropipette filled with 0.15 M NaCl was placed in the stratum pyramidale of the CA3 region, and a bipolar tungsten stimulating electrode was placed along the stratum granulosum of the DG. The positive potential reflected a field EPSP (fEPSP) because it was completely blocked by 10 μM CNQX, a non-NMDA receptor antagonist (Ikegaya, 1999). The intensity of the stimulation (a rectangular pulse of 50 μs duration) was adjusted to produce fEPSPs with the maximum amplitude. Test stimuli were delivered every 30 s. The maximal size of the fEPSPs was used as an index of the number of functional synaptic contacts of the MFs.

Assessment of cell death

Cell death was assessed by uptake of propidium iodide (PI) (Molecular Probes). PI is a polar compound that only enters cells with damaged membranes and emits red fluorescence after binding to nucleic acids (Macklis & Madison, 1990). PI was added to the culture medium at a final concentration of 10 μg ml^{-1}, and the cultures were kept at 37 °C for 24 h. PI fluorescence images were obtained with the confocal imaging system MRC-1000 (Bio-Rad).

Statistics

Data are expressed as means ± S.E.M. values. Tests of variance homogeneity, normality and distribution were performed to ensure that the assumptions required for standard parametric ANOVA were satisfied. Statistical analysis was performed by one-way repeated-measures ANOVA and *post hoc* Tukey's test for multiple pairwise comparisons. Significance was set at the $P < 0.05$ level.

RESULTS

Because the MFs develop mainly in the postnatal second week in rodents, hippocampal slices were prepared from 6-day-old rats, which allow us to observe *de novo* MF development *in vitro* (Ikegaya, 1999). MF terminals contain a high concentration of Zn^{2+}, and thereby their spatial distribution can be reliably assessed by Timm's silver-sulfide method, a histochemical technique (Gähwiler, 1984; Ikegaya, 1999). Hippocampal slices were prepared from early postnatal rat pups, and organotypically cultured in the presence of MCPG, a broad-spectrum antagonist of mGluRs. At 10 days *in vitro*, they were subjected to the Timm staining. Timm signals in intact slices were detected predominantly in the stratum lucidum and the dentate hilus (Fig. 1*A* and *C*). In MCPG-treated slices, however, Timm intensity in the stratum lucidum was dramatically reduced, and a complementary increase in Timm signals was found in the stratum radiatum and the stratum pyramidale of the CA3 region (Fig. 1*B* and *C*),

which suggests that blockade of mGluR activation results in ectopic MF synaptogenesis. Timm intensity in the dentate hilus was not apparently changed (Fig. 1*A* and *B*). The MCPG effects were also observed for re-elongating MFs after the tracts were once transected at 0 days *in vitro* (Fig. 1*D*).

The MF axonal pathway was visualized by iontophoretic labelling with the fluorescent tracer DiI. In control slices, the MFs travelled within the stratum lucidum and were well fasciculated (Fig. 1*E*). In MCPG-treated slices, however, a few MFs extended through the stratum lucidum and the

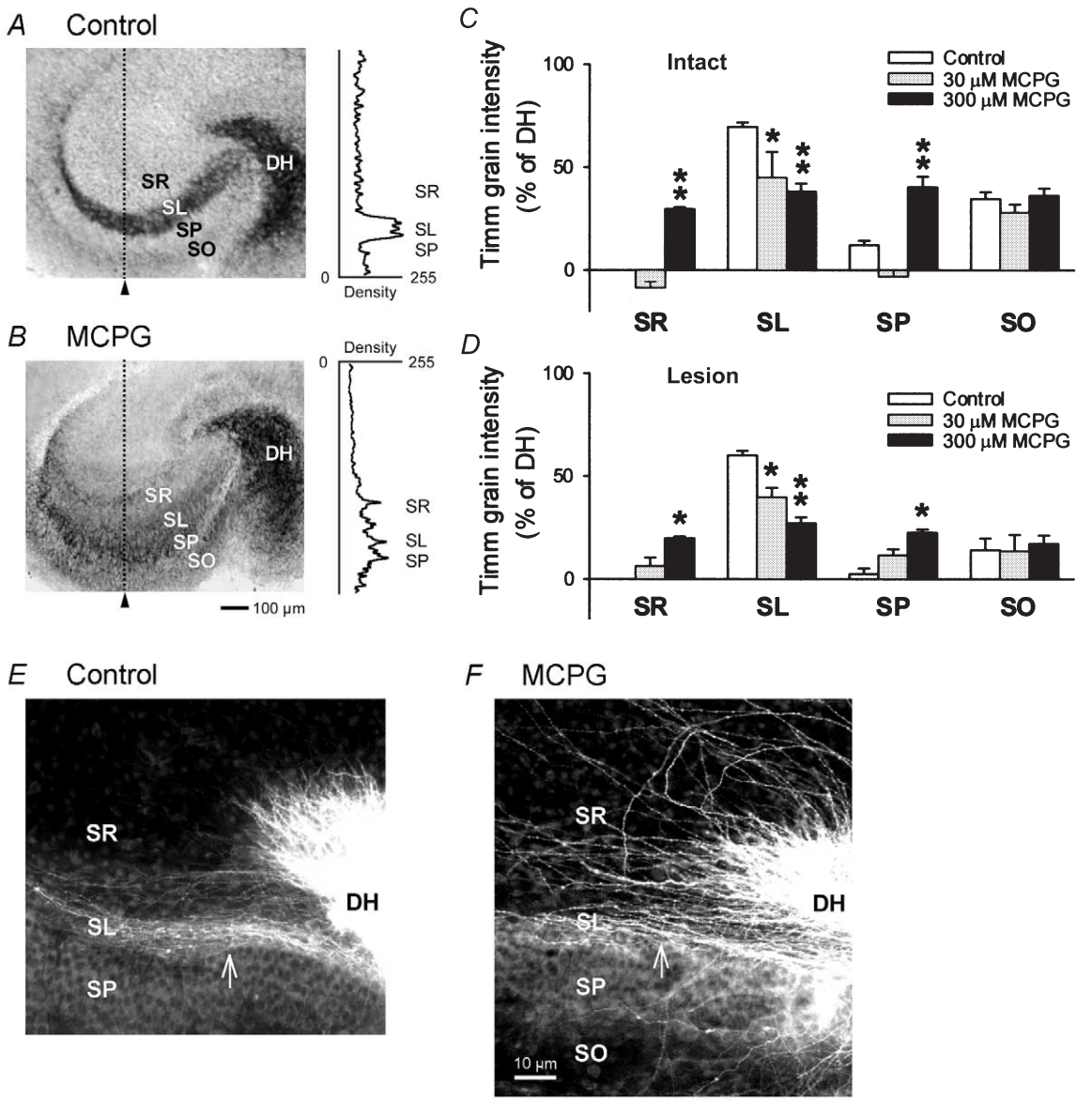

Figure 1. mGluR antagonist induces aberrant MF outgrowth in hippocampal slice cultures

A and *B*, representative Timm staining images of slices cultured for 10 days in the absence (*A*) or presence (*B*) of 300 μM MCPG. Right graphs show Timm signals that were scanned pixel by pixel along the transverse axis of CA3 to CA1 (indicated by black dotted lines in the photographs). *C*, Timm grain intensity was quantitatively analysed in control slices (open columns), 30 μM MCPG-treated (grey columns), or 300 μM MCPG-treated slices (filled columns). MF terminals in control slices were observed predominantly in the stratum lucidum (SL), but rarely in the stratum radiatum (SR), the stratum pyramidale (SP), or the stratum oriens (SO), while MCPG-treated cultures showed almost equiprobable distribution of MF terminals over all these four subregions. *D*, the same analysis was performed in cultures that had received a lesion of the MFs when the slices were prepared (Mizuhashi *et al.* 2001). * $P < 0.05$, ** $P < 0.01$ *versus* corresponding subregions of control slices; Tukey's test after ANOVA. Data are means ± S.E.M. of 5 slices each. Experiments were repeated with at least 3 different series of cultures, producing the same results. *E* and *F*, the MFs in hippocampal slices cultured for 10 days in the absence (*E*) or presence (*F*) of 300 μM MCPG were visualized by iontophoretic DiI injection into the dentate hilus (DH). Arrows indicate the MFs running through the stratum lucidum, a normal MF trajectory. In MCPG-treated slices, the MFs showed a great degree of defasciculation and formed synapse-like varicosities within aberrant target subregions. Similar results were obtained in every such experiment conducted ($n = 18–20$).

remaining invaded the stratum radiatum, the stratum pyramidale and the stratum oriens (Fig. 1*F*). MF synaptic responses after single-pulse stimulation of the stratum granulosum were extracellularly recorded from the CA3 stratum pyramidale. Consistent with the aberrant development of mossy fibre axons, the responses were dramatically altered in MCPG-treated cultures as compared to control slice cultures; the average fEPSP amplitude was 1.26 ± 0.03 mV (control), 0.85 ± 0.06 mV (30 μM MCPG) and 0.445 ± 0.04 mV (300 μM MCPG) (means ± S.E.M. of 10 slices each). On the other hand, fEPSPs evoked by stimulation of the CA3 stratum radiatum, i.e. associational recurrent afferents, were unaffected by MCGP treatment (data not shown), which indicates that CA3 pyramidal cells *per se* were electrophysiologically normal. These results suggest that prolonged treatment with MCPG selectively reduces the total number of proper MF synapses and also that the aberrant MF development is not attributable to a hypofunction of the target neurons.

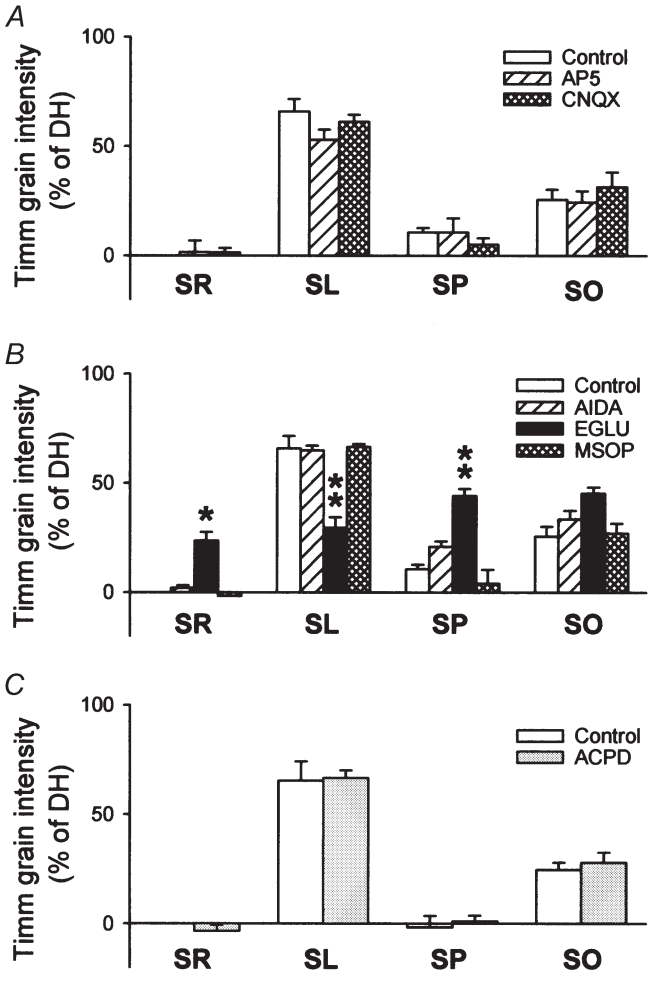

Figure 2. Group II mGluR activation is required for MF development

Experimental procedures were the same as in Fig. 1*C*. *A*, Timm grain intensity was analysed in control slices (open columns), 100 μM AP5-treated slices (hatched columns) and 10 μM CNQX-treated slices (cross-hatched columns). Neither AP5 nor CNQX affected Timm stain pattern. *B*, slices received 10 days treatment with three selective mGluR antagonists and were stained with Timm method. Characteristic Timm patterns were severely disrupted by treatment with 300 μM EGLU (filled column), but not with 300 μM AIDA (hatched column) or 300 μM MSOP (cross-hatched column). *C*, Timm staining was not affected by 10 days treatment with 300 μM ACDP. * $P < 0.05$, ** $P < 0.01$ versus control; Tukey's test after ANOVA. Data are means ± S.E.M. of 5 slices each. Experiments were repeated twice, producing the same results.

A normal Timm stain pattern was observed in slices treated with either the NMDA receptor antagonist AP5 or the non-NMDA receptor antagonist CNQX for 10 days (Fig. 2*A*). These results suggest that the activation of mGluRs, but not of ionotropic glutamate receptors, is important for normal MF development.

On the basis of sequence homology, effector coupling and pharmacological properties, mGluRs are conventionally subdivided into three groups, i.e. G_q protein-coupled group I and G_i protein-coupled groups II and III (Pin & Duvoisin, 1995), and all three types of receptors are expressed in the hippocampus (Shigemoto *et al.* 1997). To elucidate which group of mGluRs is involved in MF development, hippocampal slices were cultivated for 10 days in the presence of the group I mGluR antagonist AIDA, the group II mGluR antagonist EGLU, or the group III mGluR antagonist MSOP, and the distribution of MF terminals was assessed by the Timm method. EGLU reproduced the effect of MCPG, but AIDA or MSOP was virtually ineffective (Fig. 2*B*). Thus, group II mGluRs are likely to play a pivotal role in MF growth. Incidentally, the group I and II mGluR agonist ACPD at concentrations as high as 300 μM had no effect on Timm pattern formation (Fig. 2*C*).

In all experiments, we simultaneously monitored cell viability by assessing PI uptake, and found that 10 days exposure to any drug did not induce apparent neuronal death. Thus, the possibility that the disturbance of MF outgrowth was merely due to a massive loss of the CA3 pyramidale cells could be ruled out.

DISCUSSION

Although glutamate is well known as a major excitatory neurotransmitter in the mature CNS, its role in CNS development is not fully understood. The present study has shown that glutamate regulates hippocampal MF guidance via group II mGluR activation.

It has recently become clear that glutamate signalling is not only used for classical neurotransmission but is also likely to be functional in immature brains as well as non-neuronal tissues (Skerry & Genever, 2001). In the developing CNS, mGluRs are involved in network maturation in the somatosensory cortex (Hannan *et al.* 2001) and the cerebellum (Ichise *et al.* 2000). Furthermore, several

studies *in vitro* have presented direct evidence for a morphogenetic role of glutamate. Chang & De Camilli (2001) showed that glutamate inhibits the motility of axonal filopodia of cultured hippocampal neurons through non-NMDA receptor activation. Zheng *et al.* (1996) indicated that a gradient of glutamate concentration induces chemotropic turning responses of the growth cones of cultured *Xenopus* spinal neurons through NMDA receptor activation. In the light of these studies, our finding is the first evidence that glutamate contributes to determining the route of axon outgrowth in the CNS, and thus provides a novel role of endogenous glutamate as a regulating factor of neural network formation in the premature hippocampus.

Yokoi *et al.* (1996) reported that mGluR2-deficient mice showed no alternations in basal MF synaptic transmission, suggesting normal MF development without mGluR2 activity. The data apparently conflict with our result indicating that group II mGluR activation is required for MF pathfinding. However, the dentate granule cells express a significant amount of mRNA for mGluR3, another member of group II mGluRs (Ohishi *et al.* 1993*b*). The lack of mGluR2 may be compensated by the mGluR3 activity. Another possible explanation is that abnormal MF growth could not be detected by a simple electrophysiological experiment because mGluR2-deficient mice lack mGluR-dependent tonic inhibition of MF neurotransmission and thereby basal MF neurotransmission might be apparently unchanged (Yokoi *et al.* 1996). Further histochemical analyses on the mutant mice are required to clarify this discrepancy.

Although the present study indicates a crucial role of mGluRs in MF development, our pharmacological experiments could not identify the sites where the mGluRs act, i.e. MF growth cones, their target cells, or elsewhere. Interestingly, MF guidance was unaffected by chronic application of the mGluR agonist. If a glutamate gradient works in MF pathfinding, such non-specific, widespread mGluR activation would cause a collapse of characteristic formation of MF trajectories. Therefore, global activation, rather than site-specific, local activation, of mGluRs may mediate MF development. On the basis of the location where the mGluRs work, we can formulate at least two hypotheses for the mechanisms underlying mGluR-meditated MF pathfinding. First, MF terminals are a prominent site of group II mGluR expression in the mature hippocampus (Shigemoto *et al.* 1997), and mRNAs for group II mGluRs (mGluR2 and 3) are abundant in dentate granule cells (Ohishi *et al.* 1993*a,b*). Therefore, if mGluRs work on developing MFs, they perceive a spillover of glutamate from pre-existing MF synapses or receive glutamate released from the growing MFs themselves, and thereby the MFs may fasciculate with extant axons and find their correct pathway (Fig. 3*A*). However, the immunoreactivity for group II mGluRs is also found in glial cells throughout the stratum oriens, pyramidale, lucidum and radiatum of the CA1–CA3 regions (Petralia *et al.* 1996). In addition, precise patterns of group II mGluR expression in immature MFs remain to be fully elucidated. It is therefore possible that mGluRs work in surrounding glial cells and target pyramidal cells. In this case, the mGluRs may stimulate expression of soluble or membrane-bound attractant/repellant in these cells and indirectly support MF pathfinding (Fig. 3*B*).

Group II mGluRs are coupled to G_i protein, and their activation induces a decrease in cAMP level via inhibition of adenylate cyclase (Pin & Duvoisin, 1995). Several studies have suggested that cAMP level is a key factor controlling axon guidance. In cultured *Xenopus* spinal

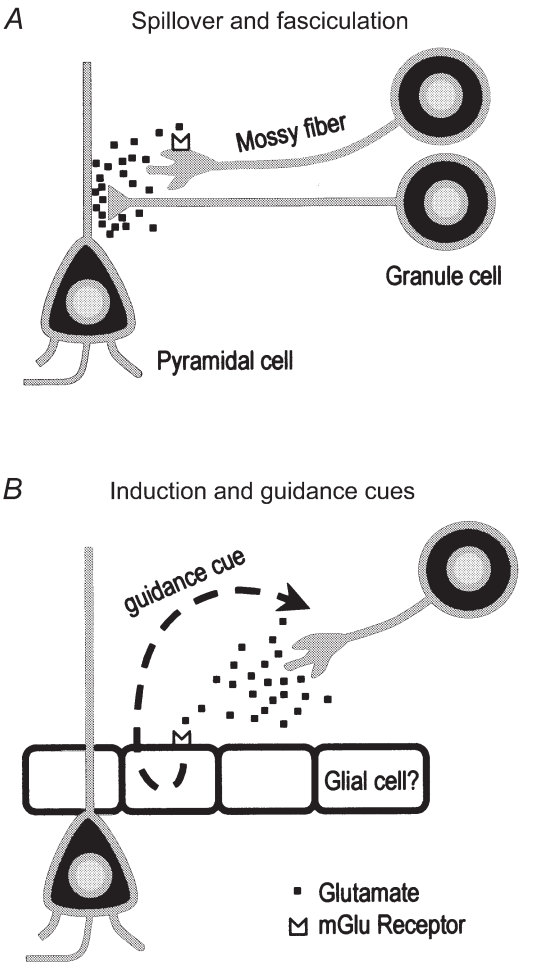

Figure 3. Schematic illustrations of possible mechanisms of mGluR-regulated axon guidance of the MFs

A, mGluRs work on MF growth cones. They detect glutamate that may spill over from axon terminals of pioneer MFs and cause MF fasciculation within the stratum lucidum, leading to appropriate pathfinding. *B*, mGluRs work in surrounding cells, e.g. glial cells and target neurons, and regulate MF growth indirectly. They detect glutamate that may be spontaneously released from growing MFs, which in turn induce the presentation of soluble or membrane-bound attractant/repellant to guide the MFs.

neurons, cAMP level definitively influences the turning responses of the growth cones (Lohof *et al.* 1992; Ming *et al.* 1997; Song *et al.* 1997, 1998; Höker *et al.* 1999). Our recent study also indicated that cAMP and cGMP signalling cascades mediate the pathfinding by the MFs (Mizuhashi *et al.* 2001). Therefore, we consider that the glutamate/mGluR system is a potential candidate for extracellular mechanisms for regulating cAMP level in MF guidance.

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