K252a, an inhibitor of Trk, disturbs pathfinding of hippocampal mossy fibers

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Hippocampal mossy fibers, which are the axons of dentate granule cells, are continuously generated owing to adult neurogenesis of granule cells. They extend exclusively into the stratum lucidum, a proximal layer of the CA3 pyramidal cells. We visualized the mossy fiber tracts by Timm histochemical staining and DiI labeling in the cultured hippocampal slices from newborn rats. The fibers were abnormally expanded when the slices were cultured in the presence of K252a, an inhibitor of the neurotrophin receptor Trk. Similar defasciculation was observed with an inhibitor of MEK, which is one of the signaling molecules downstream of Trk. This study suggests for the first time that Trk and the MEK pathway are required for mossy fiber pathfinding. *NeuroReport* 17:481–486 © 2006 Lippincott Williams & Wilkins.

Keywords: brain-derived neurotrophic factor, granule cells, hippocampus, MAPK, nerve growth factor, organotypic culture, regeneration

Introduction

Mossy fibers are axons of dentate granule cells that undergo continuous turnover throughout life [1], and recent studies have suggested that the newly generated granule cells play some role in hippocampus-dependent memory [2,3]. These fibers extend into the stratum lucidum, a narrow region of the proximal apical dendrite of the CA3 pyramidal cells, and form giant synapses. Single mossy fibers synapse with as few as 14 CA3 pyramidal cells on an average and have a strong influence on the activity of the CA3 pyramidal cells [4]. Therefore, the mechanism by which these fibers select appropriate targets is critical for shaping hippocampal circuit functions.

Here, we report molecules required for the normal mossy fiber pathfinding, MEK and Trk, which is a group of receptors for brain-derived neurotrophic factor, nerve growth factor and other neurotrophins.

Materials and methods

Materials

The drugs used in this study were K252a (Wako, Osaka, Japan), PD98059 (Calbiochem Bad Soden, Germany) and Wortmannin and U73122 (Sigma, St Louis, Missouri, USA).

Organotypic cultures of hippocampal slices

The hippocampal slices were prepared as described previously [5]. In brief, postnatal day 6 Wistar/ST rats (SLC, Shizuoka, Japan) were deeply anesthetized by hypothermia, and 300-μm-thick horizontal sections of the brain were prepared using a vibratome (Zero 1; Dosaka EM, Kyoto, Japan). Ten animals were used in each independent experiment. The entorhinohippocampi were dissected and cultured on Millicell CM membranes (Millipore, Bedford, Massachusetts, USA) with medium consisting of 50% minimal essential medium (Sigma), 25% horse serum (Cell Culture Lab, Cleveland, Ohio, USA) and 25% Hanks' balanced salt solution containing 25 mM glucose, 50 U ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin. The medium was changed every 3.5 days.

Neo-Timm staining

Prior to staining, the slices were immersed in 0.4% sodium sulfide solution at 4°C for 15 min, fixed with 10% (v/v) formaldehyde for 15 min and dehydrated with 70% and 96% ethanol twice for 30 min. For Timm staining, silver sulfide was developed in 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 average pixel intensity of the eight-bit images of Timm-stained slices was quantified in five different areas (10 pixels × 10 pixels) of each subregion. Each subregion was defined on the basis of the distance from the cell layer, as shown in Fig. 1e and f. The raw intensity (I) was normalized using background value (B, values from the CA1 stratum pyramidale) and the maximum intensity value (M, values from the dentate hilus) as $I-B/M-B$. The average values of B and M of all the experimental groups showed no significant differences. At least two thirds of the samples were processed in a blind manner; the same results were obtained in both the conditions.

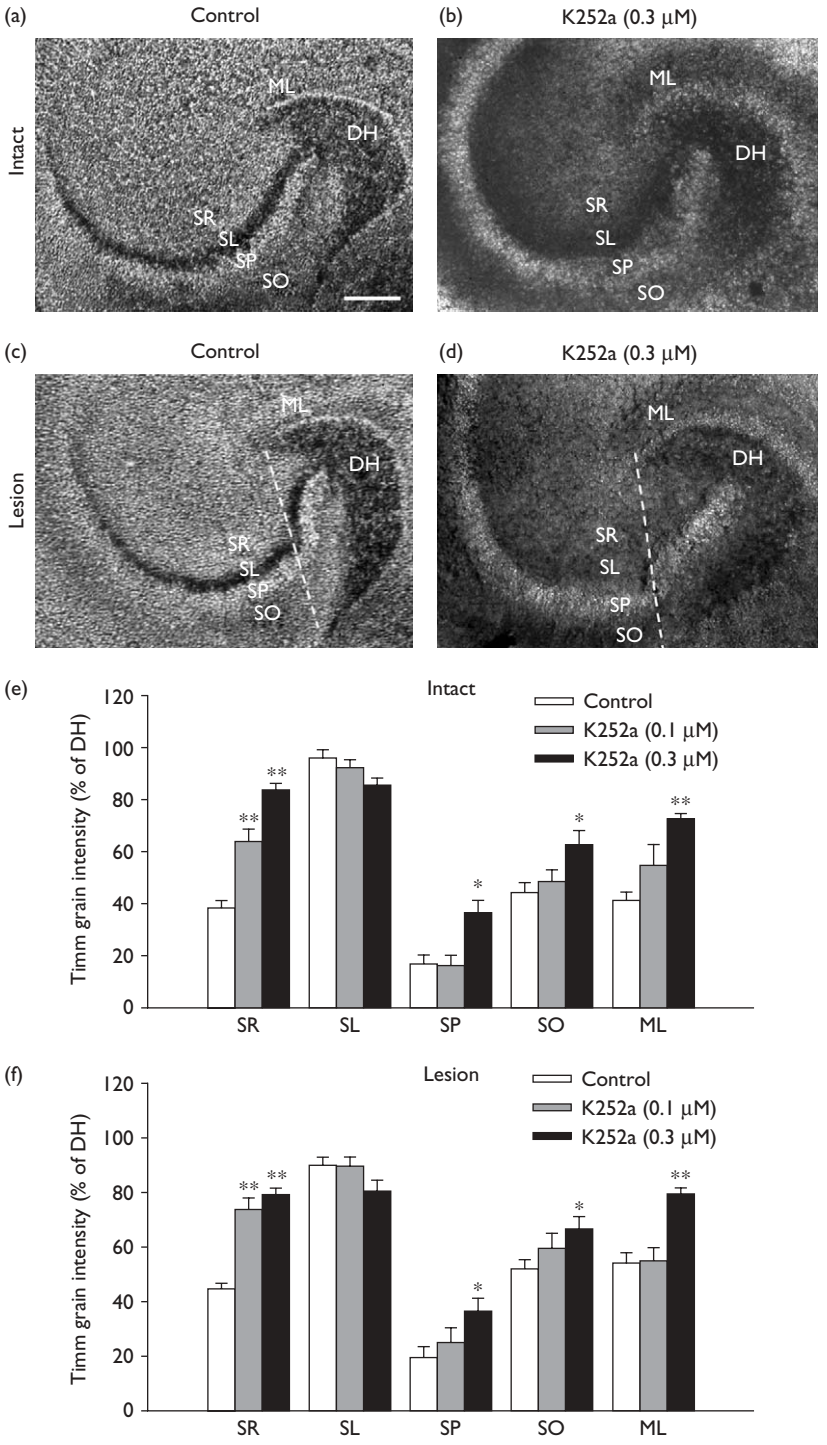

Fig. 1 K252a induced an abnormal distribution of mossy fibers. (a)–(d) Representative Timm images of intact (a, b) and once-transected (c, d) slices. The slices were cultured for 8 days in the absence (a, c) or presence (b, d) of 0.3 μM K252a. Brownish-black-stained spots at mossy fiber terminals in control slices were observed mainly in the stratum lucidum (SL), which is defined as the region within 70 μm from the midline of the cell layer. In the K252a-treated slices, the localization of the spots was expanded to the molecular layer (ML) and to the other CA3 areas including the stratum radiatum (SR), stratum lucidum (SL), stratum pyramidale (SP) and stratum oriens (SO). (e) and (f) Timm grain intensity that was quantified as described in the Materials and methods section. $*P < 0.05$ and $**P < 0.01$ versus control slices; Tukey's test after analysis of variance (n=8–16 obtained from four to five independent experiments). Data values are means ± SEM. Bars: 200 μm. DH, dentate hilus.

DiI labeling of mossy fibers

For 1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) labeling, the slices were fixed with 4% paraformaldehyde for 24 h, injected with DiI solution (Molecular Probes, Eugene, Oregon, USA; 0.5% in dimethylformamide) using a glass capillary under pressure with the aid of a picoinjector (PLI-100; Narishige, Tokyo, Japan). Then, these slices were incubated at 37°C for 7 days.

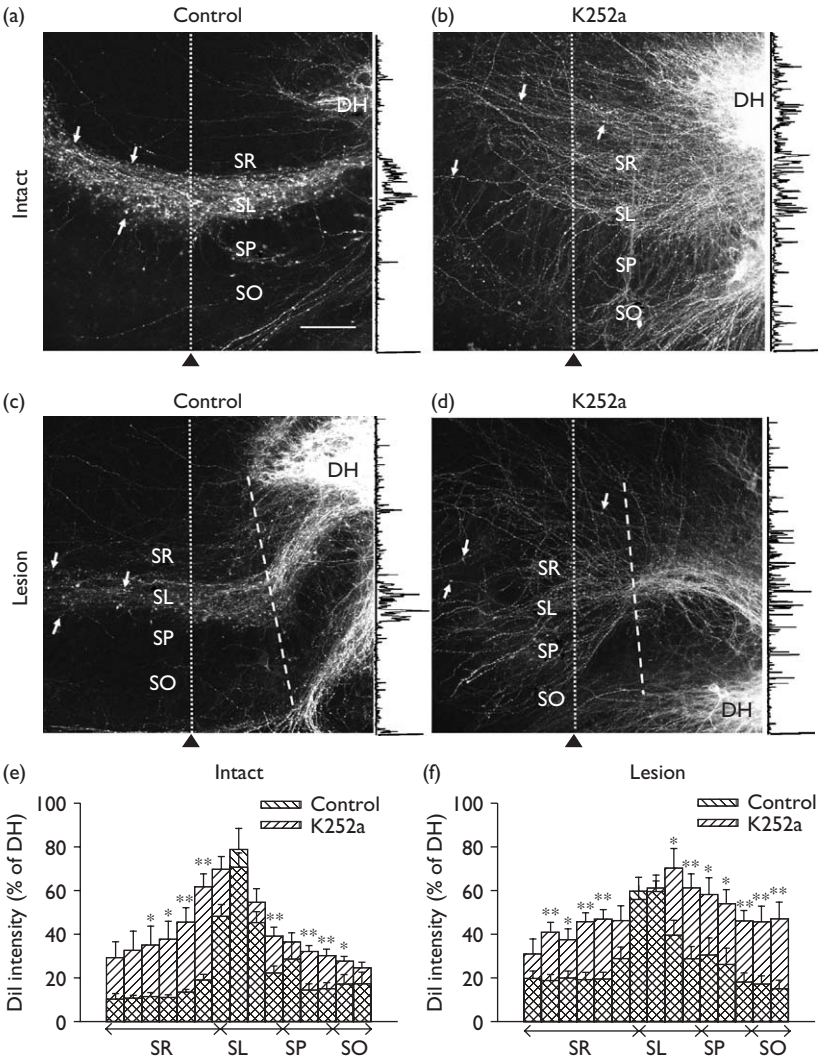

Fig. 2 K252a inhibits mossy fiber pathfinding during the development and regeneration in the hippocampal slice cultures. (a)–(d) Mossy fiber visualized by DiI of intact (a, b) and once-transected (c, d) slices. These slices were cultured for 8 days in the absence (a, c) or presence (b, d) of 0.3 μM K252a. The neighboring line graphs show DiI fluorescent intensity that was scanned pixel by pixel along the transverse axis (dotted lines). The mossy fibers in control slices run through the stratum lucidum (SL), which is the normal trajectory (a, c). In the K252a-treated slices, the mossy fibers showed defasciculation toward aberrant target subregions (b, d). Arrows show synapse-like varicosities. (e) and (f) DiI fluorescent intensities that were quantified in each 30-μm section from the midline of the cell layer. The subregions that had been defined by the distance are shown on the *x*-axis. (e) Derived from the averages of intact slices and (f) derived from those of once-transected slices. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control slices; paired Student's *t*-test after analysis of variance (n=8–10 obtained from four independent experiments). Data values are means ± SEM. Bars: 100 μm.

Average intensities of the images were evaluated by the confocal imaging system MRC-1000 (Bio-Rad, Hercules, California, USA) along with the CA3 stratum ($30 \times 100\,\mu m^2$, as seen in Fig. 2e and f).

Assessment of cell death

Cell death was assessed by prodium iodide (Molecular Probes) uptake and Nissl staining. The intensity in the cell layers was evaluated using the digitized images.

Results

To address the mechanisms underlying mossy fiber pathfinding, we cultured hippocampal slices from 6-day-old rats in the presence of K252a, an inhibitor of the neurotrophin receptor Trk. As mossy fibers mainly develop until the postnatal second week in rodents, hippocampal slices from rats of this age allow us to observe the mossy fiber development *in vitro*. At 8 days *in vitro*, the hippocampal slices were subjected to Timm staining. On Timm staining, brownish-black spots develop at mossy fiber terminals because these terminals contain a high concentration of Zn^{2+} vesicles. Timm staining in the control slices was restricted to the stratum lucidum and the dentate hilus; this staining pattern is similar to that observed *in vivo* (Fig. 1a). In the K252a-treated slices, however, images of the Timm-stained sections were dramatically altered. The intensity of Timm staining outside the stratum lucidum increased (Fig. 1b). The altered relative intensity of Timm staining demonstrated an abnormal distribution of mossy fiber trajectories (Fig. 1e). Next, in order to investigate the effect of K252a on the regenerated mossy fibers, we transected

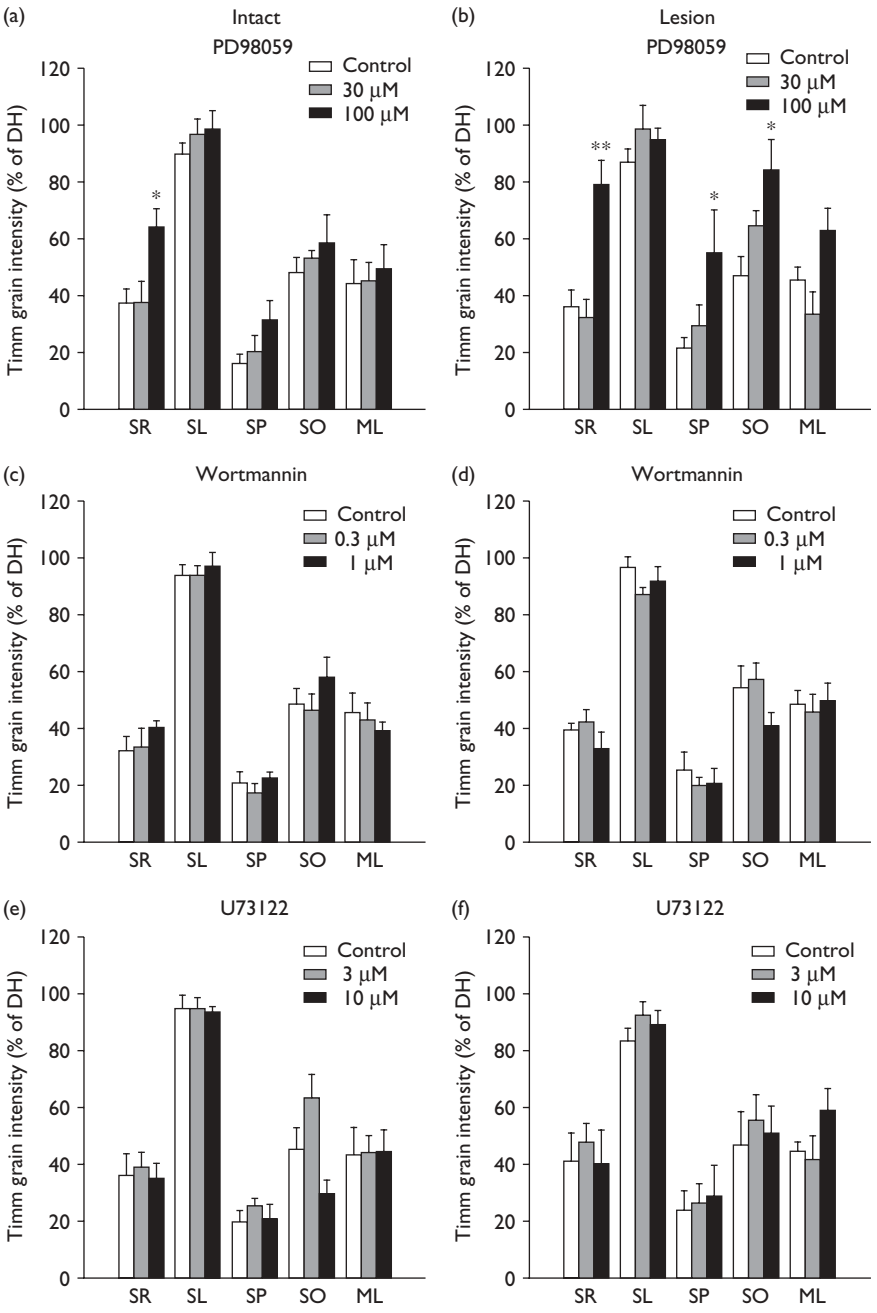

Fig. 3 MEK pathway activation is required for normal projection of mossy fibers in the hippocampal slice cultures. As seen in Fig. 1e, Timm grain intensity was quantified in PD98059 (a, b), Wortmannin (c, d) and U73122 (e, f)-treated slices. (a), (c) and (e) represent intact slices and (b), (d) and (f) represent slices with once-transected mossy fibers. SR, CA3 stratum radiatum; SL, stratum lucidum; SP, stratum pyramidale; SO, stratum oriens; ML, molecular layer; DH, dentate hilus. A significant difference was detected in Timm grain intensity only in slices treated with PD98059 (a, b). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control slices; Tukey's test after analysis of variance (n=5–8 obtained from three independent experiments). Data are means ± SEM.

them at the time the slices were prepared. As a result, the application of K252a also resulted in an aberrant Timm staining pattern (Fig. 1c, d and f).

To further confirm that the abnormal pattern observed in the images of Timm-stained slices was due to defasciculation of mossy fibers rather than merely the altered distribution of Zn^{2+} vesicles, they were visualized by DiI labeling. In control slices, most of the mossy fibers were bundled within the stratum lucidum (Fig. 2a and e). In contrast, the fibers in the K252a-treated slices defasciculated and extended outside the stratum lucidum (Fig. 2b and e). With regard to the hippocampal slices, including lesioned mossy fibers, the regenerated fibers also defasciculated from the lesioned point and distributed in abnormal regions (Fig. 2c, d and f). It was obvious that in the K252a-treated slices, the impairment of mossy fiber elongation did not occur. In addition, the mossy fibers traveling through aberrant target regions formed synapse-like varicosities (arrows in Fig. 2b and d).

We monitored cell viability by assessing prodium iodide uptake and Nissl staining under the same experimental conditions. On the basis of this, we confirmed that apparent

cell loss as a result of the K252a treatment was not observed in the CA3 region (data not shown). Therefore, K252a-induced abnormal mossy fiber distribution was not due to the neuronal cell death of CA3 neurons, which are targets of these fibers. These results demonstrated that chronic treatment with K252a inhibits normal mossy fiber pathfinding during both mossy fiber development and regeneration.

K252a has been used as an inhibitor of the neurotrophin receptor Trk. Pharmacological studies were carried out to examine whether signaling events downstream of Trk participate in mossy fiber pathfinding. The three main signaling cascades downstream of Trk are the phosphotidylinositol 3-kinase pathway, phospholipase Cγ pathway and MEK pathway [6]. The hippocampal slices were cultivated in the presence of the phosphotidylinositol 3-kinase inhibitor Wortmannin, the phospholipase Cγ inhibitor U73122 or the MEK inhibitor PD98059, and the distribution of mossy fiber terminals was evaluated by Timm staining. The doses of the above-mentioned inhibitors were determined on the basis of previous studies, in which similar doses were effective in hippocampal slice cultures [7–9]. Our study shows that the mossy fibers are abnormally distributed in the PD98059-treated slices (Fig. 3a and b). On the other hand, there was no significant difference in the Timm grain intensity between the control and the Wortmannin-treated slices or between the control and the U73122-treated slices (Fig. 3c–f). In the PD98059-treated slices, massive cell loss was not detected by assessing the prodium iodide uptake (data not shown). These results indicate that the activation of the MEK pathway plays an important role in the mossy fiber pathfinding.

Discussion

This study demonstrated that K252a inhibited the normal fasciculation of mossy fibers in the hippocampal slice cultures. In addition, we found that the MEK pathway contributes to mossy fiber guidance as PD98059 also induces widening of the fiber tracts.

K252a is a relative specific inhibitor of the tyrosine kinase activity of neurotrophin receptors [10]. Although K252a inhibits the activity of serine/threonine protein kinases, including that of cAMP-dependent protein kinase A and protein kinase C, it is a more effective inhibitor of the neurotrophin receptor Trk [11–13]. Further, many studies have reported the use of K252a as an inhibitor of Trk at the concentration used in this study; at these concentrations, K252a was found to inhibit exogenous neurotrophins [14,15]. Thus, we consider that K252a inhibited the action of endogenous neurotrophins and the activation of Trk, thereby resulting in the induction of abnormal mossy fiber pathfinding.

In the hippocampus, neurotrophins including brain-derived neurotrophic factor, nerve growth factor and neurotrophin-3 are most highly expressed in the stratum lucidum and dentate hilus, in which normal mossy fibers extend [16,17]. In addition, these neurotrophins are highly expressed during postnatal 2 weeks [18]. This expression timing corresponds with the major development of mossy fibers. As regards MEK signaling, although it can occur downstream of a number of receptors, neurotrophins are probable candidates because the phosphorylated extracellular-regulated kinase (ERK), the MEK substrate and neurotrophins colocalize mainly in the stratum lucidum [19]. These data corroborate our study, suggesting that K252a affects mossy fiber pathfinding by inhibiting the activation of neurotrophins via the MEK pathway.

The abnormal distribution of mossy fibers in the PD98059-treated slices was more obvious in the transected mossy fibers than in the intact ones. This might suggest the participation of another downstream pathway that functions together with MEK in the intact slices. A similar tendency, however, was observed in the K252a-treated slices, as shown in Fig. 2. This may be because the drugs were more accessible to fiber tips in the once-lesioned slices.

The possible mechanism for the neurotrophin action in this system can be explained as a direct guidance cue [20] or an inducer of other cues. Our observation of ectopic synapses suggests a possibility that neurotrophins serve as repulsive cues or induce other repulsive cues outside the stratum lucidum. Neurotrophins that regulate the expression of receptors for a repulsive cue [21] may also be involved.

As the mossy fibers are generated throughout life, their pathfinding and synaptogenesis lead to the formation of new neuronal circuits. Our data suggest the involvement of neurotrophins and MEK, which have been related to memory formation [22,23], in mossy fiber pathfinding. Although these data were obtained from the slices prepared from newborn rats, it is possible for the adult fibers to share the common molecular mechanisms. Thus, our finding might lead to the elucidation of a novel mechanism of memory consolidation that recruits mossy fibers.

Conclusion

Our present study showed that K252a, an inhibitor of the neurotrophin receptor Trk, and PD98059, an inhibitor of intracellular MEK that is downstream of Trk, disrupted normal mossy fiber pathfinding. These findings suggest a novel role of neurotrophins in controlling mossy fiber development.

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