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Interactions between Plexin-A2, Plexin-A4, and Semaphorin 6A Control Lamina-Restricted Projection of Hippocampal Mossy Fibers

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SUMMARY

Hippocampal mossy fibers project preferentially to the stratum lucidum, the proximal-most lamina of the suprapyramidal region of CA3. The molecular mechanisms that govern this lamina-restricted projection are still unknown. We examined the projection pattern of mossy fibers in mutant mice for semaphorin receptors plexin-A2 and plexin-A4, and their ligand, the transmembrane semaphorin Sema6A. We found that *plexin-A2* deficiency causes a shift of mossy fibers from the suprapyramidal region to the infra- and intrapyramidal regions, while *plexin-A4* deficiency induces inappropriate spreading of mossy fibers within CA3. We also report that the *plexin-A2* loss-of-function phenotype is genetically suppressed by *Sema6A* loss of function. Based on these results, we propose a model for the lamina-restricted projection of mossy fibers: the expression of plexin-A4 on mossy fibers prevents them from entering the Sema6A-expressing suprapyramidal region of CA3 and restricts them to the proximal-most part, where Sema6A repulsive activity is attenuated by plexin-A2.

INTRODUCTION

In many regions of the vertebrate central nervous system, afferent projections are distributed in a precise laminar pattern (for a review, see Sanes and Yamagata, 1999). This targeting of axons to specific laminae or dendritic segments of the target neurons is fundamental to the physiology of neural circuits (for a review, see Förster et al., 2006). Therefore, one important issue to be addressed is how individual axons are instructed to invade, arborize, and terminate at particular laminae.

The hippocampus is an excellent experimental system in which to study cellular and molecular mechanisms that govern lamina-restricted termination of axons. Pyramidal cells, the principal neurons of the cornu ammonis (CA), receive inputs from a variety of sources in a lamina-specific manner. In CA3, the distal segments of the apical dendrites of pyramidal cells (the stratum lacunosum-moleculare) receive axons from the entorhinal cortex; the more proximal parts of the apical dendrites (the stratum radiatum) and the basal dendrites of pyramidal cells (the stratum oriens) are primarily innervated by axons of CA3 pyramidal cells from both the contralateral and ipsilateral sides (the commissural/associational afferent); and the proximal-most parts of the apical dendrites in CA3 (the stratum lucidum) and the basal dendrites in CA3c are occupied by axons from the dentate gyrus, the mossy fibers (Amaral and Witter, 1995; also see Figures 1A–1D).

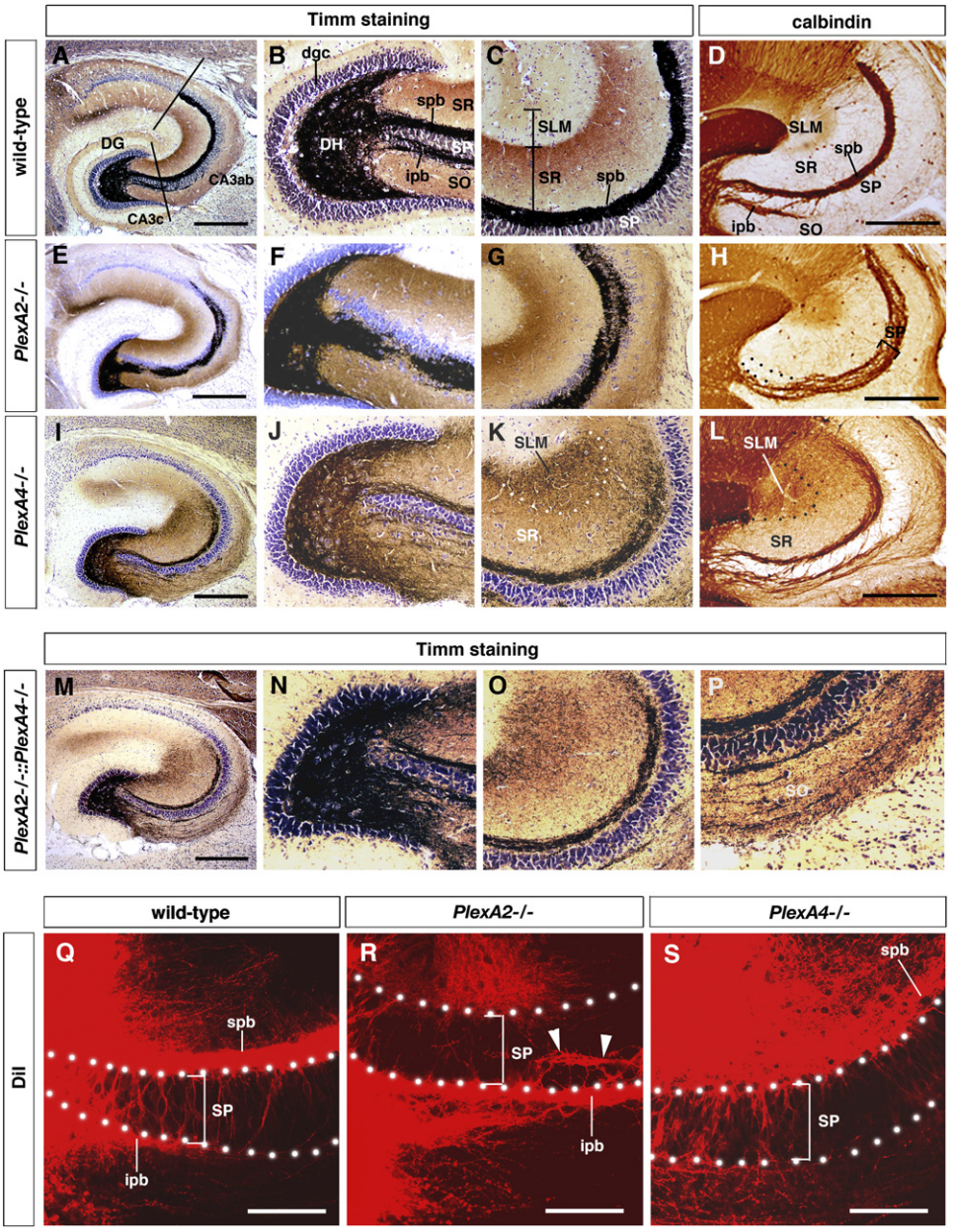

Figure 1. Mossy Fiber Projection in *Plexin* Mutant Mice

(A–P) Timm staining and immunostaining with calbindin of horizontal sections of the hippocampus of adult wild-type, *plexin-A2* homozygous (*PlexA2*$^{-/-}$), *plexin-A4* homozygous (*PlexA4*$^{-/-}$), and *plexin-A2/plexin-A4* double knockout (*PlexA2*$^{-/-}$*::PlexA4*$^{-/-}$) mice. The areas corresponding to CA3c and CA3ab in (A), (E), (I), and (M) are shown at a higher magnification in (B and C), (F and G), (J and K), and (N and O), respectively. (P) indicates the stratum oriens (SO) of CA3ab in (M) at a higher magnification. Dotted lines in (H) indicate the outline of the stratum pyramidalis (SP) in CA3c. Dotted lines in (K) and (L) indicate the boundary of the stratum lacunosum-moleculare (SLM) and the stratum radiatum (SR). DG, dentate gyrus; DH, dentate hilus; dgc, dentate granule cells; spb, suprapyramidal bundle of mossy fibers; ipb, infrapyramidal bundle of mossy fibers. The abbreviations also apply to other figures of this paper. Scale bar is 250 μm in (A), (D), (E), (H), (I), (L), and (M).

(Q–S) Mossy fiber projection in P5 wild-type, *PlexA2*$^{-/-}$, and *PlexA4*$^{-/-}$ mice, detected by DiI filling of the fibers. Dotted lines indicate the outline of the stratum pyramidalis. Arrowheads in (R) indicate DiI-filled mossy fibers within the stratum pyramidalis. The dentate hilus is on the left side of each figure. Scale bar is 100 μm in (Q) through (S).

It has been proposed that specific molecular cues must be involved in guiding the different hippocampal afferents along their correct pathways into their definitive target laminae during development (for reviews, see Skutella and Nitsch, 2001; Förster et al., 2006). Several extracellular molecules that can guide hippocampal afferent fibers

have been described. An extracellular matrix molecule, hyaluronic acid, is thought to play a role in the termination of entorhinal fibers to the outer molecular layer of the dentate gyrus (Zhao et al., 2003). Eph ligand ephrin-A3 (Stein et al., 1999) and RGMa (Brinks et al., 2004) have been shown to repel entorhinal fibers in vitro. Other repulsive guidance molecules, namely Slit-2 (Nguyen-Ba-Charvet et al., 1999) and class 3 semaphorins (Chédotal et al., 1998; Steup et al., 1999, 2000; Pozas et al., 2001), have been shown to act as repellents for pyramidal cell axons, mossy fibers, or entorhinal fibers in vitro. However, the contribution of these axon guidance molecules to the lamina-specific guidance of hippocampal projections has not been demonstrated in vivo, with the exception of the contribution of netrin-1. In *netrin-1*-deficient mice, lamina-specific targeting of entorhinal fibers to the hippocampus proper and the associational, CA3-to-CA1 Schaffer collaterals were defective (Barallobre et al., 2000). Therefore, the molecular mechanisms that govern the laminar projection of hippocampal afferents are still unknown.

Type A plexins can directly propagate repulsive activities of class 6 transmembrane semaphorins (Toyofuku et al., 2004; Suto et al., 2005) as well as class 3 secreted semaphorins by forming receptor complexes with neuropilins (Takahashi et al., 1999; Cheng et al., 2001; Suto et al., 2003; Yaron et al., 2005). All type A plexins (plexin-A1, -A2, -A3, and -A4) are expressed in the developing hippocampal system (Cheng et al., 2001; Murakami et al., 2001; Bagri et al., 2003; Suto et al., 2003), suggesting their involvement in neuronal wiring in the hippocampus. Plexin-A3 was shown to control the pruning of mossy fibers (Bagri et al., 2003; Liu et al., 2005), but the in vivo function of the other type A plexins has not been thoroughly studied.

To further elucidate the function of type A plexins in vivo, we generated protein null mutant mice for plexin-A2 (see Supplemental Material 1 in the Supplemental Data) and plexin-A4 (Suto et al., 2005), and examined the projection pattern of mossy fibers. We here report that plexin-A2 and plexin-A4, and their putative ligand Sema6A, a class 6 transmembrane semaphorin, play crucial roles in lamina-restricted projection of hippocampal mossy fibers, but are not a prerequisite for synaptic connectivity of the fibers.

RESULTS

Abnormal Projection of Mossy Fibers in *Plexin-A2* and *Plexin-A4* Mutant Mice

We first examined the projections of mossy fibers in the adult hippocampus by Timm staining and calbindin immunohistochemistry, which can visualize presynaptic terminals and trajectories of mossy fibers, respectively. In wild-type mice, mossy fibers first accumulated in the dentate hilus, and then invaded the stratum lucidum in the suprapyramidal region of CA3 to form the suprapyramidal bundle (Figures 1A–1D). Mossy fibers also invaded the infrapyramidal region of CA3c, forming the infrapyramidal bundle.

The lamina-restricted pattern of mossy fiber projection was disrupted in *plexin-A2*-deficient mice and *plexin-A4*-deficient mice. In all *plexin-A2* homozygous animals (*PlexA2*$^{-/-}$: n = 10), mossy fibers did not grow into the suprapyramidal region, but invaded the infrapyramidal region of CA3c and the stratum pyramidalis of CA3ab (Figures 1E–1H). In *plexin-A2* heterozygous animals (n = 5), mossy fibers projected to normal positions (data not shown). On the other hand, mossy fibers spread out inappropriately within CA3 in *plexin-A4* homozygous animals: in all *PlexA4*$^{-/-}$ mice (n = 8), mossy fibers invaded most parts of CA3, particularly the stratum lacunosum-moleculare and the stratum oriens, and to a lesser extent the stratum radiatum (Figures 1I–1L). Some mossy fibers also entered the proximal part of the suprapyramidal region of CA3, their normal target site. However, the suprapyramidal bundle was not as tightly packed as in the wild-type animals (compare Figures 1L and 1D). *Plexin-A4* heterozygous animals (n = 4) did not show obvious abnormalities of mossy fiber projections (data not shown).

To examine a possible functional correlation of plexin-A2 and plexin-A4 in mossy fiber projection, we generated *plexin-A2*/*plexin-A4* double knockout mice by crossing *plexin-A2* and *plexin-A4* single mutants. In *PlexA2*$^{-/-}$*::PlexA4*$^{-/-}$ mice (n = 5), mossy fibers were widely distributed within CA3 as in *PlexA4*$^{-/-}$ animals (Figures 1M–1P), suggesting a predominant role of plexin-A4 in the laminar projection of mossy fibers.

The first mossy fibers invade CA3 at around postnatal day 0 (P0), and their number gradually increases during postnatal development. Furthermore, the early formed mossy fibers serve as scaffolds for late-arriving mossy fibers. Therefore, we examined the projection pattern of mossy fibers in P5 *plexin* mutant mice by labeling the fibers with DiI. As in the adult animals, mossy fibers preferentially grew into the infrapyramidal region and the stratum pyramidalis of CA3 in *PlexA2*$^{-/-}$ animals (Figure 1R), and spread out within CA3 in *PlexA4*$^{-/-}$ animals (Figure 1S). These results indicate that the mossy fiber projection is defective from the beginning of its development.

Localization of the Plexin-A2 and Plexin-A4 Proteins in the Developing Hippocampus

In situ hybridization (ISH) analyses on P1 mice revealed strong signals for *plexin-A2* and *plexin-A4* transcripts in both dentate granule cells and CA3 pyramidal cells (Figures 2A and 2J). However, the distinct mossy fiber projection defects in *plexin-A2* and *plexin-A4* mutants suggested that the two plexin-A proteins might be differentially distributed in the developing hippocampus. Therefore, we generated plexin-A2-specific and plexin-A4-specific antibodies (see Supplemental Material 2 and 4) and examined the localization of these two plexins in the developing hippocampus.

In P1 mice a plexin-A2-specific monoclonal antibody, Mab-A2D3, stained the suprapyramidal and

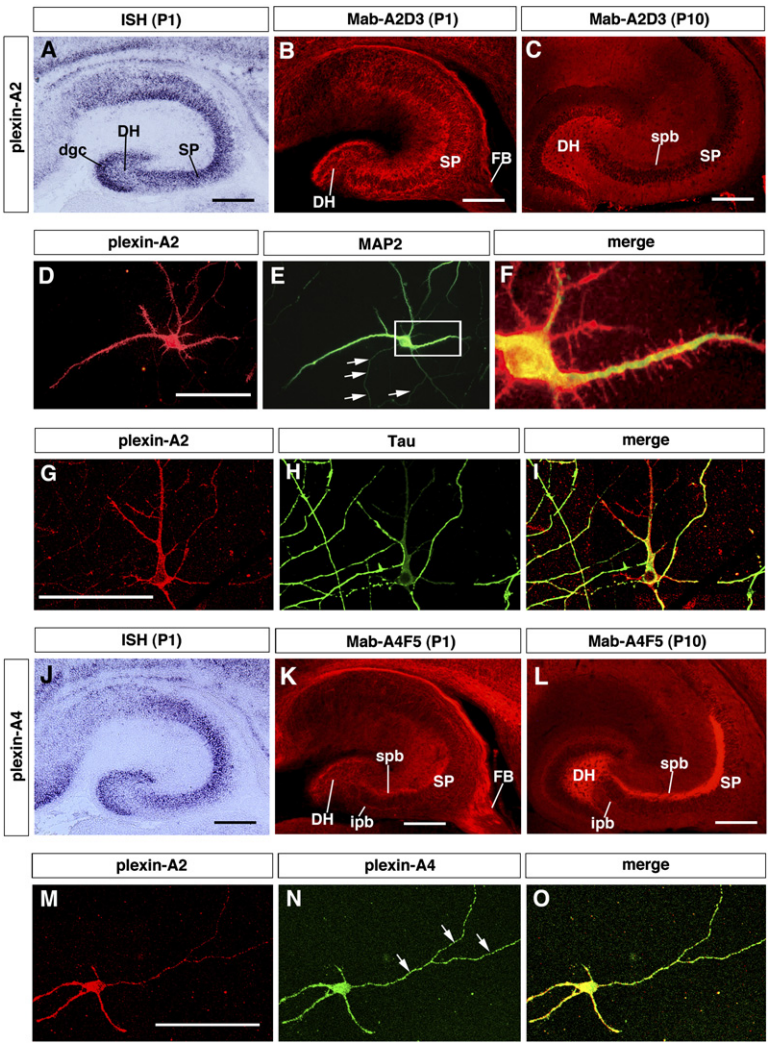

Figure 2. Expression of Plexin-A2 and Plexin-A4 in the Developing Hippocampus

(A–C) Expression of plexin-A2 in the hippocampus of P1 and P10 mice, detected by ISH analysis and immunostaining with a plexin-A2-specific monoclonal antibody, Mab-A2D3, in horizontal sections. (D–I) Cultured CA3 pyramidal cells coimmunostained with plexin-A2 (rabbit serum antibodies; see Supplemental Materials 2 and 4) and MAP2 antibody (a dendritic marker; D–F) or Tau antibody (an axonal marker; G–I). A merged figure corresponding to the region indicated by a square in (E) is given in (F) at a higher magnification. (J–L) Expression of plexin-A4 in the hippocampus of P1 and P10 mice, detected by ISH analysis and immunostaining with a plexin-A4-specific monoclonal antibody, Mab-A4F5, in horizontal sections. (M–O) A cultured dentate granule cell coimmunostained with the plexin-A2 serum antibodies and plexin-A4 antibody (Mab-A4F5). Arrows: axons. Scale bar is 200 μm in (A) through (C) and (J) through (L), and 100 μm in (D), (G), and (M).

infrapyramidal regions of CA3, the dentate hilus, and inner parts of the dentate molecular layer (Figure 2B). Interestingly, the plexin-A2 staining was strongest in the proximal part of the suprapyramidal region, the presumptive target area for mossy fibers. At P10, the plexin-A2 staining was still strong in the dentate hilus but became weaker in CA3 (Figure 2C). The suprapyramidal bundle of mossy fibers was mostly negative for the plexin-A2 staining (Figure 2C). Immunostaining of cultured CA3 pyramidal cells with rabbit serum antibodies to mouse plexin-A2 (see Supplemental Materials 2 and 4) revealed that the plexin-A2 protein was enriched in MAP2-positive dendrites and dendritic spines (Figures 2D–2F) and less abundant in Tau-positive axons (Figures 2G–2I). Furthermore, in cultured dentate granule cells, the plexin-A2 protein was abundant in dendrites but less so in axons (Figures 2M–2O). These results indicate that the plexin-A2 protein is enriched in dendrites but less so or nil in axons.

On the other hand, the plexin-A4 proteins were mainly distributed in mossy fibers. In P1 and P10 mice, a plexin-A4-specific monoclonal antibody, Mab-A4F5, labeled the suprapyramidal and infrapyramidal bundles of mossy fibers and the dentate hilus (Figures 2K and 2L). Axons of cultured granule cells were immunopositive for the antibody (Figures 2M–2O), confirming the distribution of the plexin-A4 protein on mossy fibers. As ISH signals for the *plexin-A4* transcripts were weak in the dentate hilus (see Figure 2J), the immunostaining in the dentate hilus most likely corresponds to plexin-A4 expression on mossy fibers. A weak expression of plexin-A4 was also detected in the suprapyramidal region of CA and in the fimbria.

Distinct Roles of Plexin-A2 and Plexin-A4 in Laminar Projection of Mossy Fibers

The preferential localization of plexin-A2 on pyramidal cell dendrites, but not on mossy fibers, and of plexin-A4 on mossy fibers suggests that plexin-A2 functions in CA3 pyramidal neurons, and plexin-A4 in mossy fibers, to regulate laminar projection of mossy fibers. To test this hypothesis, we cocultured slices of the dentate gyrus (DG slice) and CA3 (CA3 slice) of P5 to P8 wild-type or *plexin* mutant mice in various combinations (see

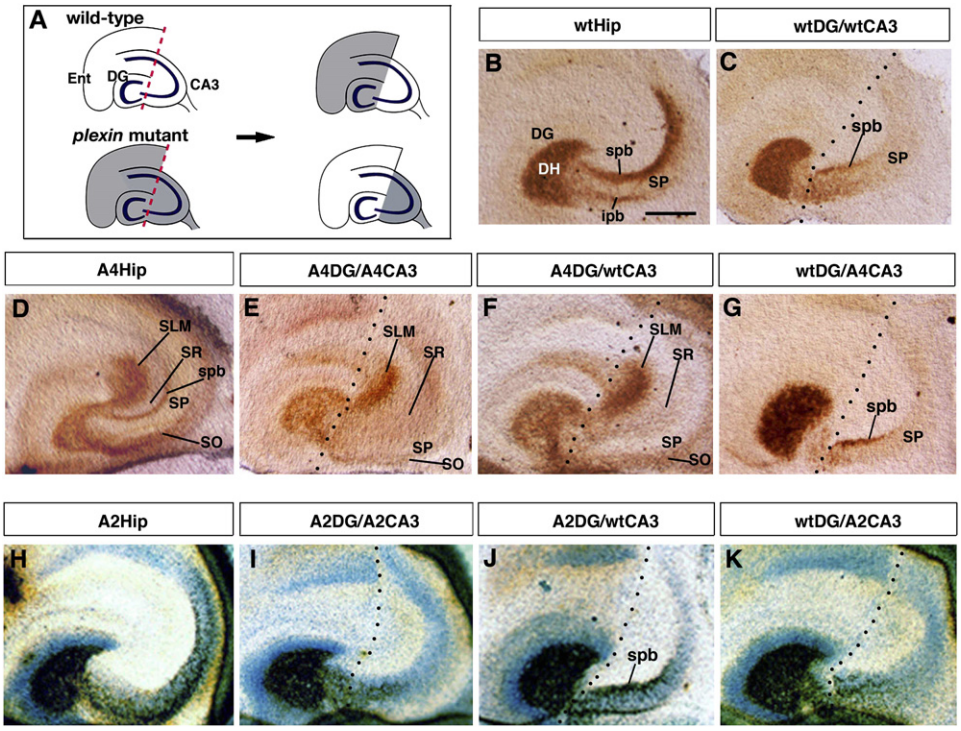

Figure 3. Mossy Fiber Projection in Cocultures of the Dentate Gyrus and CA3

(A) Schematic diagram of the coculture. Horizontal slices of the hippocampus of P5 to P8 wild-type or *plexin* mutant mice are divided into two pieces; a slice that contains the dentate gyrus and the entorhinal cortex (DG slice) and a slice that contains CA3 and CA1 (CA3 slice). The DG slices and the CA3 slices were combined in various combinations and cultured for 5 days.

(B–K) Timm staining of the cultures. The cultures in (H–K) were counterstained with toluidine blue. Dotted lines indicate the border of the DG slice and CA3 slice. Each slice is abbreviated as follows. wtHip, A2Hip, and A4Hip: slices of the hippocampus of wild-type, *PlexA2*$^{-/-}$, and *PlexA4*$^{-/-}$ mice, respectively; wtDG, A2DG, and A4DG: DG slices of wild-type, *PlexA2*$^{-/-}$, and *PlexA4*$^{-/-}$ mice, respectively; wtCA3, A2CA3, and A4CA3: CA3 slices of wild-type, *PlexA2*$^{-/-}$, and *PlexA4*$^{-/-}$ mice, respectively. Scale bar (in B) indicates 250 μm for (B) through (K).

Figure 3A), and examined the projection pattern of mossy fibers.

In slice cultures of the wild-type hippocampus (wtHip; Figure 3B) and cocultures of wild-type DG slices (wtDG slices) with wild-type CA3 slices (wtCA3 slices) (Figure 3C), mossy fibers projected to their normal positions, the proximal part of the suprapyramidal region of CA3 and the infrapyramidal region of CA3c. In slice cultures of the *PlexA4*$^{-/-}$ hippocampus (A4Hip; Figure 3D), mossy fibers massively invaded the stratum lacunosum-moleculare and the stratum oriens, and diffusely invaded the stratum radiatum. In the proximal part of the suprapyramidal region, mossy fibers formed the suprapyramidal bundle. In cocultures of *PlexA4*$^{-/-}$ DG slices (A4DG slices) with either *PlexA4*$^{-/-}$ CA3 slices (A4CA3 slices) (Figure 3E) or wtCA3 slices (Figure 3F), mossy fibers invaded the stratum lacunosum-moleculare and diffusely invaded the stratum oriens. The suprapyramidal bundle, however, was not formed. In contrast, in cocultures of wtDG slices with A4CA3 slices, mossy fiber projection was normal (compare Figures 3G and 3C). Collectively, if the *PlexA4*$^{-/-}$ DG slices were used in the coculture, the mossy fiber projection pattern was always similar to that of *PlexA4*$^{-/-}$ animals, irrespective of the genetic origin of the CA slices (see Table S1 in Supplemental Material 3). In slice cultures of the *PlexA2*$^{-/-}$ hippocampus (A2Hip; Figure 3H), mossy fibers projected to the infrapyramidal region or the stratum pyramidalis, but not to the suprapyramidal region. The *PlexA2*$^{-/-}$-like abnormal mossy fiber projection was always induced whenever the *PlexA2*$^{-/-}$ CA3 (A2CA3) slices were used in the coculture (Figures 3I and 3K), though the sprouting of mossy fibers was usually reduced and often absent (see Table S2 in Supplemental Material 3). In contrast, when A2DG slices were cocultured with wtCA3 slices, mossy fiber projection was normal: mossy fibers projected to the suprapyramidal region and formed the suprapyramidal bundle (Figure 3J).

Taken together, these results indicate that plexin-A4 functions in mossy fibers, while plexin-A2 acts in CA3 pyramidal cells to regulate the laminar projection of mossy fibers.

Sema6A Repels Mossy Fibers

The spreading out of *plexin-A4*-deficient mossy fibers within CA3 led us to speculate that *plexin-A4*-deficient mossy fibers lose their responsiveness to repulsive cues

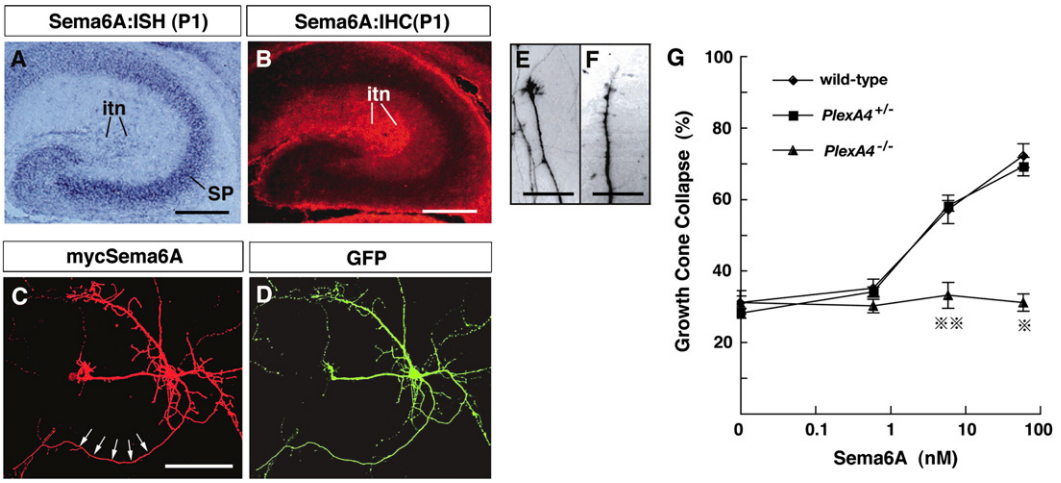

Figure 4. Expression and Functions of Sema6A

(A and B) Expression of Sema6A in the hippocampus of P1 mice, detected by ISH analysis and immunohistochemistry in horizontal sections. itn, interneurons in the stratum lacunosum-moleculare. Scale bar is 250 μm in (A) and (B).

(C and D) Coexpression of myc-tagged Sema6A and GFP in a cultured CA3 pyramidal cell. Note that myc-tagged Sema6A is localized on axons (arrows) and dendrites. Scale bar is 100 μm in (C).

(E–G) Effects of Sema6A on the morphology of growth cones in mossy fibers. Mossy fibers sprouted from explants of the dentate gyrus were visualized by immunostaining with calbindin. Note that Sema6A collapses growth corns of wild-type (F) but not *plexin-A4*-deficient (E) mossy fibers. Incidences of collapsed growth cones were plotted against different concentrations of Sema6A (G). The average percentage for growth cone collapse at each point was calculated for 10 to 20 explants (from six littermates). Vertical bars indicate SEM. *$p < 0.001$; **$p < 0.005$ (Student's t test). Scale bar is 10 μm in (E) and (F).

in CA3. As we have previously shown that Sema6A, a class 6 transmembrane semaphorin, repels sympathetic ganglion axons and that the repulsive activity of Sema6A is mediated by plexin-A4 (Suto et al., 2005), we asked whether Sema6A would function as a repellent for mossy fibers. We first examined Sema6A expression pattern in the hippocampus. In P1 mice *Sema6A* transcripts were detected in CA3 pyramidal cells and interneurons in the stratum lacunosum-moleculare (Figure 4A). Immunostaining for Sema6A revealed that Sema6A was widely expressed in the suprapyramidal and infrapyramidal regions of CA3 and interneurons of the stratum lacunosum-moleculare (Figure 4B). The absence of staining of the suprapyramidal bundle and the dentate hilus indicates that mossy fibers do not express Sema6A. Sema6A expression in CA3 was rapidly downregulated during postnatal development: at P10, Sema6A protein was absent from CA3 (data not shown). To examine subcellular localization of Sema6A, we transfected and expressed myc-tagged Sema6A and green fluorescent protein (GFP) in cultured pyramidal cells. The epitope-tagged Sema6A was distributed almost evenly in all parts of the cells, including their dendrites and axons (Figures 4C and 4D).

We next tested the repulsive activity of Sema6A against mossy fibers using the growth cone collapse assay. We cultured small dentate gyrus fragments from P6 wild-type and *PlexA4*$^{-/-}$ animals and applied recombinant proteins for the Fc-dimerized Sema6A ectodomain (Sema6A$_{ect}$-Fc; Suto et al., 2005). The recombinant proteins induced growth cone collapse of wild-type and *PlexA4*$^{+/-}$ mossy fibers, in a dose-dependent manner, but not of *PlexA4*$^{-/-}$ mossy fibers (Figures 4E–4G). These results show that Sema6A is a repellent of mossy fibers and that this repulsive activity is mediated by plexin-A4.

Plexin-A2 Attenuates Repulsive Activities of Sema6A

Next, we asked whether the Sema6A-induced repulsion of mossy fibers plays a role in the laminar projection of mossy fibers. To address this question, we examined mossy fiber projection pattern in *Sema6A* mutant mice (*Sema6A*$^{-/-}$) that carry a gene-trap insertion in the *Sema6A* gene and are phenotypically null (Leighton et al., 2001; Mitchell et al., 2001). *Sema6A*$^{-/-}$ mice did not show any obvious abnormality in the trajectory and termination of mossy fibers (Figures 5A and 5C). To our surprise, however, an almost normal mossy fiber projection was restored in *plexin-A2*/*Sema6A* double knockout animals (*PlexA2*$^{-/-}$*::Sema6A*$^{-/-}$; n = 5) (Figures 5B and 5D). The rescue of the *plexin-A2* loss-of-function phenotype by *Sema6A* loss of function suggests that during normal development, Sema6A-mediated mossy fiber repulsion is attenuated by plexin-A2 expressed in the proximal part of the suprapyramidal region of CA3, allowing mossy fibers to invade this area.

This result also suggests direct molecular interaction between Sema6A and plexin-A2. Accordingly, we showed that the Fc-dimerized recombinant proteins for the alkaline phosphatase (AP)-tagged ectodomain of Sema6A (AP-Sema6A$_{ect}$-Fc) bound with a high affinity to plexin-A2 expressed in L cells: the dissociation constant (Kd)

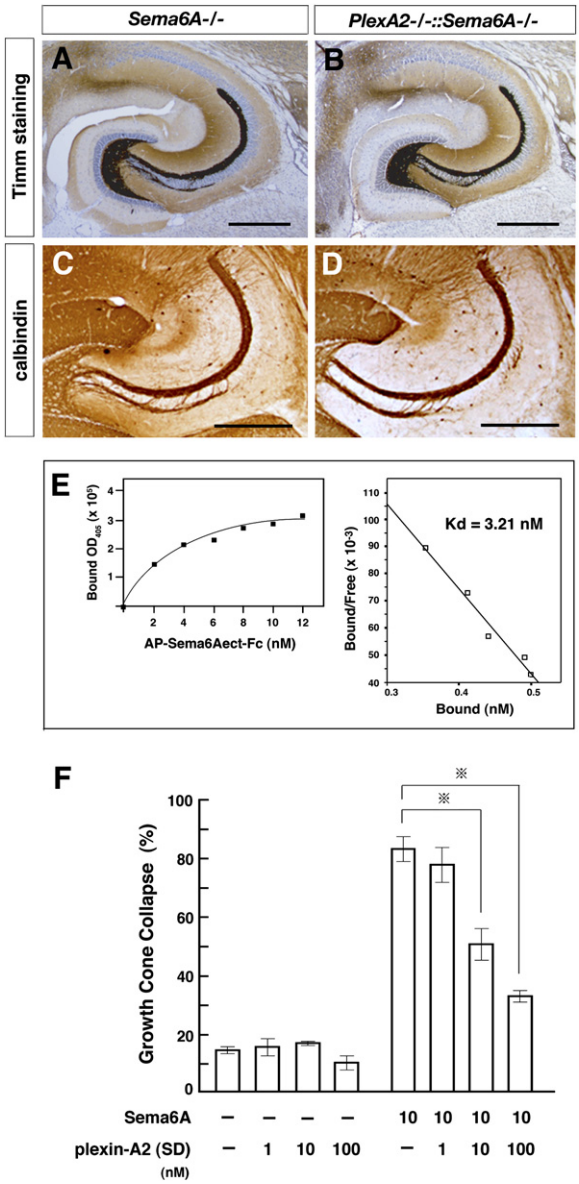

Figure 5. Genetic, Physical, and Functional Interaction between Sema6A and Plexin-A2

(A–D) Mossy fiber projection in the adult *Sema6A* homozygous animals (*Sema6A*$^{-/-}$) and *plexin-A2*/*Sema6A* double knockout animals (*PlexA2*$^{-/-}$*::Sema6A*$^{-/-}$), detected by Timm staining and immunostaining with calbindin in horizontal sections of the hippocampus. Note that mossy fiber projection defects in *plexin-A2* mutants are rescued in *Sema6A* loss-of-function animals. Scale bar is 250 μm in (A) through (D).

(E) Binding of recombinant proteins for the Fc-dimerized AP-tagged Sema6A ectodomain (AP-Sema6A$_{ect}$-Fc) to L cells that express the plexin-A2 proteins, detected by AP histochemistry (left), and Scatchard plots derived from the data (right). To obtain specific binding of the recombinant proteins to plexin-A2, the AP activities derived from the binding of AP-Sema6A$_{ect}$-Fc to parental L cells were subtracted. The protein concentration for AP-Sema6A$_{ect}$-Fc was converted as a monomer.

(F) Effects of plexin-A2 on Sema6A-induced growth cone collapse in mossy fibers. Sema6A (10 nM) was preincubated with plexin-A2 sema domain (SD) of given concentration, and then applied to the cultures. The average percentage for growth cone collapse at each column was calculated for 100 to 140 dentate gyrus explants in each independent experiment (n = 4). Vertical bars indicate SEM. *p < 0.005 (Student's t test).

value for the interaction of Sema6A with plexin-A2 was 3.21 nM (Figure 5E), and thus comparable to the Kd value for the interaction of Sema6A with plexin-A4 (3.56 nM; Suto et al., 2005). We then tested whether plexin-A2 attenuates Sema6A response by the growth cone collapse assay. Preincubation of Sema6A with the plexin-A2 sema domain significantly suppressed the growth cone collapse activity of Sema6 (Figure 5F). These results indicate that plexin-A2 competitively suppresses binding of Sema6A to plexin-A4 and attenuates Sema6A response.

Synapse Formation of Mossy Fibers in *Plexin* Mutant Mice

We next tried to determine whether mossy fibers that project to ectopic positions in *plexin* mutants make synapses. The strong intensity of Timm staining in the stratum lacunosum-moleculare and the stratum radiatum in *plexin-A4* mutants (Figure 1K) suggests that mossy fibers formed synapses at ectopic positions. This was further confirmed by double immunostaining of the stratum lacunosum-moleculare of *plexin-A4* mutants with calbindin and synaptophysin, a presynaptic marker (Figures 6A–6C).

As the deprivation of plexin-A2 from CA3 pyramidal cells induced the shift of mossy fibers from the suprapyramidal region to the infra- and intrapyramidal regions, we asked whether localization of plexin-A2 on the proximal dendritic segment of CA3 pyramidal cells is required for the synaptic termination of mossy fibers to this segment. To address this question, we generated chimeric mice with *plexin-A2*-deficient and wild-type cells (*PlexA2*$^{-/-}$/wt chimeas: n = 5). *Plexin-A2*-deficient cells were labeled with GFP by crossing *plexin-A2* mutant mice with *GFP* transgenic mice (Okabe et al., 1997).

In a chimera in which about 30% of pyramidal cells were GFP-positive, and thus *plexin-A2*-deficient, mossy fibers invaded not only the infrapyramidal region of CA3c but also the proximal part of the suprapyramidal regions of CA3c (Figure 6D) and CA3ab (Figure 6E), which exactly corresponds to the stratum lucidum in normal mice. As the repulsive activity of the transmembrane semaphorin Sema6A seems effective only at a short distance, mossy fibers might follow the wild-type pyramidal cell dendrites to invade the suprapyramidal region of CA3 in the chimera. Interestingly, several presynaptic boutons of mossy fibers were apposed to the dendritic shafts of wild-type, but also GFP-positive/*plexin-A2*-deficent, pyramidal cells (Figure 6F). Wild-type mossy fibers also formed synapses on the dendritic shafts of *plexin-A2*-deficent pyramidal cells (Figure 6G). Furthermore, the dendritic shafts of GFP-positive/*plexin-A2*-deficent pyramidal cells possessed thorny excrescences, the postsynaptic components for mossy fiber synapse (Figure 6H). Taken together, these

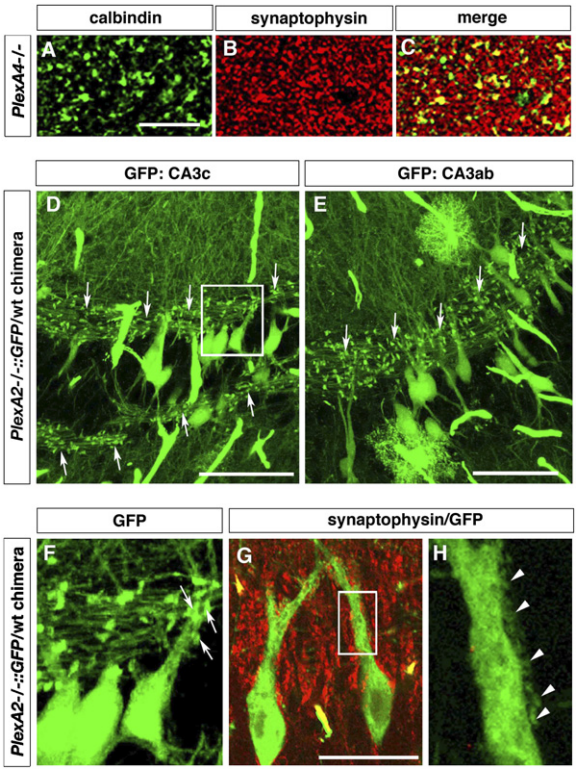

Figure 6. Formation of Mossy Fiber Synapses in *Plexin* Mutant Mice

(A–C) Presynaptic boutons of mossy fibers in the stratum lacunosum-moleculare of adult *PlexA4*$^{-/-}$ mice, detected by double immunostaining with calbindin and synaptophysin. Scale bar is 10 μm in (A).

(D and E) Projection and termination of mossy fibers in an adult wild-type/*plexin-A2* chimeric mouse. Note that several GFP-positive/*plexin-A2*-deficent mossy fibers and swellings that are characteristic of the presynaptic boutons of mossy fibers (arrows) are distributed in both the suprapyramidal and infrapyramidal regions of CA3c (D) and the suprapyramidal region of CA3ab (E). Scale bar is 30 μm in (D) and (E).

(F–H) Synaptic contacts of mossy fibers with pyramidal cells. In (F), the area indicated by a square in (D) is given at a higher magnification. Note that GFP-positive presynaptic boutons of mossy fibers (arrows) were apposed to the dendritic shaft of GFP-positive/*plexin-A2*-deficent pyramidal cells. In (G) and (H), CA3ab of the chimeric mouse was stained with synaptophysin antibodies. Note that synaptophysin-positive presynaptic boutons of wild-type mossy fibers were apposed to the dendritic shafts of GFP-positive/*plexin-A2*-deficent pyramidal cells. A region indicated by a square in (G) is shown at a higher magnification in (H). Note that spine-like structures (arrow heads) are formed on the dendritic shaft of a GFP-positive/*plexin-A2*-deficent pyramidal cell. Scale bar is 10 μm in (G).

results indicate that the proximal segments of the apical dendrite of *plexin-A2*-deficent pyramidal cells can make synaptic contacts with mossy fibers whenever they are accessed by the fibers.

Mossy Fiber Synapses Are Functionally Normal in *Plexin-A2* Mutant Mice

We next examined whether mossy fiber synapses in the hippocampus of *plexin-A2*-deficient mice were functional with an electrophysiological approach. One of the criteria for detecting mossy fiber synapses is profound paired-pulse facilitation (PPF) (Zalutsky and Nicoll, 1990). The PPF ratio at a 50 ms interstimulus interval in *PlexA2*$^{-/-}$ mice (335% ± 17%, n = 5) in the stratum lucidum of CA3 was comparable (p = 0.71) to that in wild-type mice (327% ± 9%, n = 4) (Figure 7A). Another critical feature of the mossy fiber synapse is that mossy fiber synaptic transmission is almost completely blocked by the group II metabotropic glutamate receptor (mGluR) agonists (Kamiya et al., 1996), which have little or no effect on the other types of excitatory synapse in the hippocampus. This property was also preserved in mossy fiber synapses of *PlexA2*$^{-/-}$ mice (Figure 7B). The application of 1 μM (2S,2′R,3′R)-2-(2′3′-decarboxycyclopropyl)glycine (DCG-IV), a group II mGluR agonist, inhibited excitatory synaptic responses by about 80% in *PlexA2*$^{-/-}$ mice (to 20% ± 8% of baseline, n = 8), which was comparable (p = 0.46) to the inhibition in wild-type mice (to 13% ± 5% of baseline, n = 8).

Long-term potentiation (LTP) in mossy fiber synapses involves the activation of a cAMP-protein A kinase pathway in presynaptic terminals (Weisskopf et al., 1994). The application of 10 μM forskolin, an activator of adenylate cyclase, induced large potentiation of excitatory synaptic transmission at mossy fiber synapses in wild-type mice (394% ± 19% of baseline, n = 6), as well as in *PlexA2*$^{-/-}$ mice (418% ± 22% of baseline, n = 6; p = 0.44) (Figure 7C). Furthermore, LTP in mossy fiber synapses of *PlexA2*$^{-/-}$ mice was intact: high-frequency stimulation of mossy fibers (four trains of 25 Hz/5 s stimulation repeated at 20 s intervals) caused large posttetanic potentiation (PTP: 539% ± 109% of baseline, n = 5) and the following LTP (154% ± 13% of baseline: 30 min after the tetanus), which were both indistinguishable from PTP (626% ± 37% of baseline, n = 6; p = 0.43) and LTP (136% ± 6% of baseline; p = 0.23) in wild-type mice (Figure 7D). These results indicate that the functional properties of mossy fiber synapses examined in this study are preserved in *plexin-A2* mutants.

DISCUSSION

Plexin-A4-Mediated Axon Repulsive Activities of Sema6A Prevent Invasion of Mossy Fibers to CA3

The present study shows that the deprivation of two semaphorin receptors, plexin-A2 and plexin-A4, induces distinctive defects in mossy fiber projection, as summarized in Figure 8A. Type A plexins, including plexin-A2 and plexin-A4, form receptor complexes with neuropilins to mediate chemorepulsive signals of class 3 semaphorins. However, mice lacking the class 3 semaphorin Sema3F (Sahay et al., 2003) or its receptors, neuropilin-2 (Chen et al., 2000; Giger et al., 2000) or plexin-A3 (Cheng et al., 2001), have no apparent defects in laminar projection of mossy fibers, although the infrapyramidal bundle of mossy fibers overshoots in these mutants (Bagri et al., 2003; Liu et al., 2005). Moreover, *Sema3A*-deficient mice

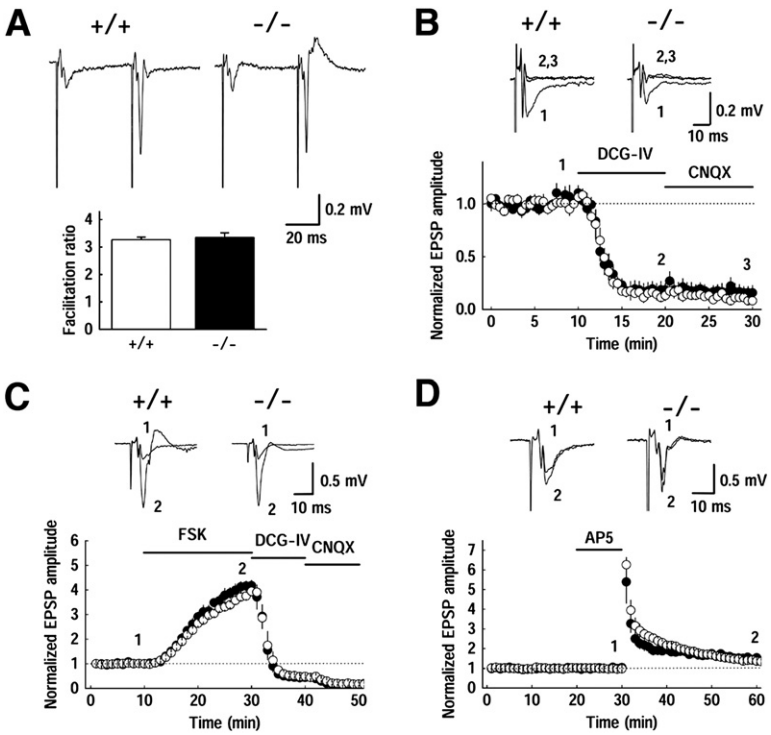

Figure 7. Normal Functional Properties of Hippocampal Mossy Fiber Synapses in *Plexin-A2*-Deficient Mice

(A) PPF (50 ms intervals) of excitatory synaptic transmission at hippocampal mossy fiber synapses. (B) Sensitivity of the mossy fiber synaptic response to the group II mGluR agonist DCG-IV (1 μM). Following the DCG-IV administration, the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX: 10 μM) was applied to isolate the presynaptic fiber volley component. (C) Synaptic facilitation induced by forskolin, an activator of adenylate cyclase. DCG-IV was applied to confirm that the recorded responses were mossy fiber synaptic responses. (D) Mossy fiber LTP. The NMDA receptor antagonist D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5: 25 μM) was applied to prevent contamination of NMDA receptor-dependent synaptic potentiation.

have a normal mossy fiber projection (data not shown). Therefore, it is likely that the disturbance of lamina-restricted projection of mossy fibers in *plexin-A2* and *plexin-A4* mutants is not due to an alteration of class 3 semaphorin signaling. Our previous study (Suto et al., 2005) has shown that plexin-A4 serves as a direct receptor for Sema6A, mediating its repulsive activity. The present study shows that Sema6A is expressed in CA3 and plexin-A4 on mossy fibers, and also that Sema6A induces growth cone collapse in vitro of mossy fibers from wild-type, but not *plexin-A4*-deficient, animals. Thus, our results suggest that Sema6A functions as a repellent for plexin-A4-expressing mossy fibers. Plexin-A4-expressing mossy fibers may primarily be prevented from invading the Sema6A-expressing CA3, while plexin-A4-deficient ones could grow freely into CA3.

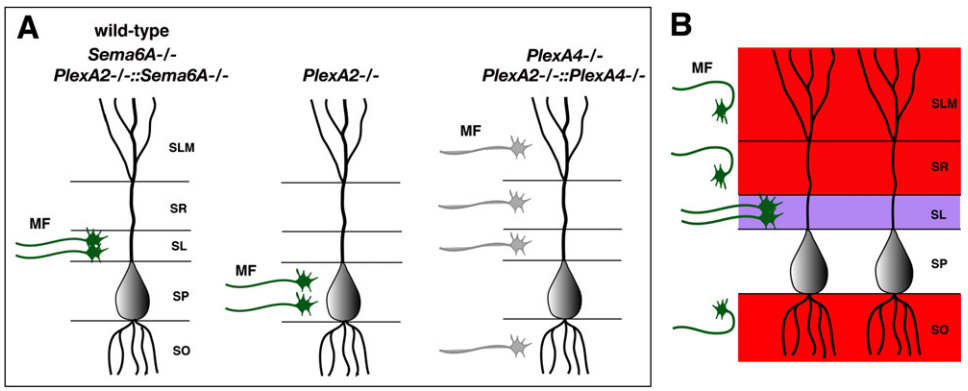

Figure 8. Summary of Functions of Plexin-A2, Plexin-A4, and Their Ligand, Sema6A, in Lamina-Restricted Mossy Fiber Projection

(A) Schematic diagrams of the mossy fiber projection patterns in CA3ab of *plexin* and *Sema6A* knockout mice. In *plexin-A2* mutants, mossy fibers (MF; green color) do not grow into the stratum lucidum (SL), the normal target lamina for the fibers, but invade the stratum pyramidalis (SP). Mossy fibers still project to the stratum lucidum in *Sema6A* and *plexin-A2*/*Sema6A* double knockout animals. By contrast, *plexin-A4*-deficent mossy fibers (gray color) spread out within CA3, even in *plexin-A2*/*plexin-A4* double knockout animals.

(B) A model for the lamina-restricted mossy fiber projection. Plexin-A4-expressing mossy fibers are primarily repelled by Sema6A (red color) expressed in CA3, but can invade the stratum lucidum, where the Sema6A repulsive activity is attenuated by plexin-A2 expressed in the proximal segments of pyramidal cell dendrites (blue color).

Mossy fibers projected normally in *Sema6A* mutant mice (see Figure 8A), however, in contrast to *plexin-A4* mutants. While Sema6A is not genetically required for repulsion of plexin-A4-positive mossy fibers in the normal situation, a direct requirement for both plexin-A4 and Sema6A is revealed in the absence of plexin-A2. Failure of mossy fiber projection to the stratum lucidum caused by removal of plexin-A2 (see Figure 8A) can be suppressed by removal of either plexin-A4 or Sema6A. As removal of either gene is sufficient to suppress this defect, this suggests that no other repulsive ligands are required to exclude axons from the stratum lucidum in this context. The spreading of *plexin-A4*-deficient mossy fibers toward the stratum radiatum and the stratum lacunosum-moleculare suggests the existence of additional plexin-A4-related mossy fiber repellents that function redundantly to Sema6A.

Attenuation of Repulsive Activities of Sema6A by Plexin-A2 Is Essential in Lamina-Restricted Projection of Mossy Fibers

Why can mossy fibers invade the stratum lucidum, the proximal-most lamina of the suprapyramidal region of CA3 where Sema6A is expressed? Our genetic analyses reveal that mossy fibers fail to invade the stratum lucidum in *plexin-A2*-deficent mice, a phenotype that is genetically rescued in *Sema6A*/*plexin-A2* double mutants (see Figure 8A). These results indicate that plexin-A2 is genetically required to allow mossy fibers to invade the stratum lucidum, but not required in the absence of Sema6A, suggesting that its function is to suppress or block the activity of Sema6A in this region (Figure 8B). The attenuation of Sema6A-induced growth cone collapse in mossy fibers by plexin-A2 (see Figure 5F) supports this interpretation.

The present study shows that plexin-A2 is localized on pyramidal cell dendrites while Sema6A is localized on both dendrites and axons. Therefore, how plexin-A2 can suppress Sema6A function in CA3 is still an open question. As proposed for Ephs and their ligand ephrins in retinal axons (Carvalho et al., 2006), plexin-A2 could interact laterally with Sema6A at the surface of the same cell, in *cis*, masking Sema6A to plexin-A4-expressing mossy fibers. As more plexin-A2 protein is expressed on the proximal dendritic segment of CA3 pyramidal cells, these segments may become permissive to mossy fibers. Alternatively, plexin-A2 could suppress Sema6A repulsive activity expressed on commissural/associational fibers, the axons of CA3 pyramidal cells, in *trans*. Mossy fibers are the last hippocampal afferents to invade CA3. When the first mossy fibers start to invade CA3, proximal parts of the suprapyramidal region have been fully occupied by the commissural/associational fibers (data not shown). Therefore, attenuation of repulsive activities of Sema6A in commissural/associational fibers would be necessary for the growth of mossy fibers into CA3. As proposed in the Eph-ephrin interactions in hippocampal axons (Zimmer et al., 2003) and motor axons (Marquardt et al., 2005), Sema6A might interact with plexin-A2 and be endocytosed as receptor/ligand complexes from the surface of commissural/associational fibers, thereby resulting in the growth of mossy fibers into proximal parts of the suprapyramidal region of CA3.

Whatever the mechanisms, the present results indicate a non-cell-autonomous function of a semaphorin receptor, plexin-A2, in axon guidance: plexin-A2 is expressed by mossy fiber target neurons and modulates the repulsive activity of a mossy fiber repellent to generate the lamina-restricted projection pattern. Axon guidance molecules, including semaphorins, Eph ligand ephrins, and slits, interact with multiple receptors. Two members of the class 3 semaphorins, Sema3B (SemaA) and Sema3C (SemaE), act as agonists to induce growth cone collapse at neuropilin-2 receptors, but act as antagonists to suppress the collapse activity of Sema3A at neuropilin-1 receptors (Takahashi et al., 1998). The physiological importance of having multiple receptors, however, is mostly unclear. The present study provides the evidence that one receptor serves as a modulator to attenuate the activity of the cognate ligand, thereby regulating the guidance of axons expressing the counter receptors.

Plexin-A4-deficient mossy fibers spread out inappropriately within CA3, but many still reach the proximal parts of the suprapyramidal region of CA3. In addition, in *plexin-A2* knockouts, mossy fibers always project to the stratum pyramidalis in CA3ab. These results suggest the existence of attractants that direct mossy fibers toward the stratum pyramidalis. Netrin-1 might be a candidate for such an attractant because the molecule is expressed in CA3 pyramidal cells and attracts neurites from the dentate gyrus in vitro (Steup et al., 2000). Together, the Sema6A-based repulsion and the attraction by factors secreted from CA3 pyramidal cells may delineate the layer permissive for mossy fiber growth. It is also an open question why mossy fibers can invade the infrapyramidal region in *plexin-A2*-deficient animals, since Sema6A is also present in this region. As some mossy fibers normally invade the infrapyramidal region of CA3c, the region may also contain attractants for mossy fibers.

Plexin-A2 Is Not Required for Synapse Formation of Mossy Fibers

Recent studies have shown that synaptic connections are specified by invariant molecular cues. For instance, the cell adhesion molecules Sidekicks are distributed in specific laminae of the inner molecular layer of the neural retina and determine lamina-specific synaptic connectivity (Yamagata et al., 2002). Likewise, neurofascin 186 is restricted to the initial segments of cerebellar Purkinje cell axons and directs the synaptic targeting of basket cell axons (Ango et al., 2004). By contrast, the localization of plexin-A2 on the proximal dendritic segments and the dendritic spines of CA3 pyramidal cells appears not to be an absolute prerequisite for mossy fiber connectivity: in the hippocampus of *PlexA2*$^{-/-}$/wt chimeras, wild-type and *plexin-A2*-deficient mossy fibers were able to form synapses on appropriate dendritic

segments of *plexin-A2*-deficient pyramidal cells that they contacted. This result seems to contradict the *cis*-suppression model discussed above: according to this, mossy fibers would not contact with *plexin-A2*-deficient, and thus Sema6A-active, dendrites. This discrepancy, however, may be explained by the replacement of mossy fibers and the downregulation of Sema6A expression in target neurons: the dentate mossy fibers are replaced continuously throughout life, while the expression of Sema6A in pyramidal cells ceases by P10. As the present chimeric analyses were done on the adult animals, it is likely that we observed the synapses of the late-arriving mossy fibers on pyramidal cell dendrites that had already ceased the Sema6A expression. In the chimeras in the neonatal period, the early formed mossy fibers might be suppressed from contacting the *plexin-A2*-deficient/Sema6A-active dendrites, but contact and follow the wild-type dendrites to invade the suprapyramidal region of CA3 and provide scaffolds for late-arriving mossy fibers. Our results imply, at least, that plexin-A2 is not required for the synapse formation of mossy fibers, but is required for the attenuation of Sema6A repulsive activity in target areas that determine the positions of mossy fiber projection.

Normal Electrophysiological Properties of Mossy Fiber Synapses in *Plexin-A2* Mutant Mice

The electrophysiological results support the idea that the attenuation of Sema6A repulsive activity by plexin-A2 is dispensable for synaptogenesis and further indicate that the lack of plexin-A2 has no apparent effect on synaptic function. Mossy fiber synaptic transmission is characterized by extremely large PPF (Zalutsky and Nicoll, 1990), which suggests that the neurotransmitter release probability is quite low (Manabe et al., 1993), and this property is preserved in *PlexA2*$^{-/-}$ mice. Thus, it is conceivable that the transmitter release mechanism at mossy fiber synapses is intact in *PlexA2*$^{-/-}$ animals. This is further confirmed by the forskolin experiments, which indicate that the sensitivity of mossy fiber terminals to forskolin (Weisskopf et al., 1994) is unaltered. In addition, the results of DCG-IV experiments (Kamiya et al., 1996) strongly suggest normal localization of functional molecules, such as the mGluR, in presynaptic terminals of mossy fibers in *PlexA2*$^{-/-}$ mice. Furthermore, mossy fiber LTP is intact in *PlexA2*$^{-/-}$ mice, confirming that the mossy fiber synapses are functionally normal.

EXPERIMENTAL PROCEDURES

Mice

Plexin-A2 null mutant mice were generated by targeted disruption of the *plexin-A2* gene (for details, see Supplemental Materials 1 and 4). Generation of *plexin-A4* mutant mice has been reported elsewhere (Suto et al., 2005). The day of birth corresponds to P0. All animal experiments and animal care was performed in accordance with the guidelines of the Animal Care and Experimentation Committee of each institution.

Generation of *Plexin-A2*$^{-/-}$/Wild-type Chimeric Mice

To label *plexin-A2*-deficient cells with GFP, *plexin-A2* mutant mice were first crossed with the "Green mice," a transgenic mouse line with an enhanced green fluorescent protein (EGFP) cDNA (Okabe et al., 1997). Cells of the *GFP::PlexA2*$^{-/-}$ morulae were microinjected into wild-type 8-cells stage embryos. The embryos were cultured in M 16 medium overnight to blastocysts and then transplanted into recipient mice.

In Situ Hybridization

ISH using the DIG-labeled probes for plexin-A2 and plexin-A4 was performed as described previously (Murakami et al., 2001; Suto et al., 2003).

Culture of Hippocampal Neurons and Slices

Hippocampal neurons were dissociated from E16.5 mouse embryos and cocultured with glial cells on poly-L-lysine (PLL)-coated glass coverslips, with Neurobasal (NB) medium (GIBCO) supplemented with B-27 Supplement (GIBCO) and 2 mM L-glutamine (NB/B-27 medium) for 4 days, following the procedures reported (Goslin et al., 1998). Cultures of hippocampal slices were prepared following the procedures reported (Mizuhashi et al., 2001) (for details, see Supplemental Material 4).

Growth Cone Collapse Assay

Explants of the dentate gyrus from P3 mice were cultured in PLL/laminin-coated 8-well chamber slides (Nunc) with NB/B-27 medium for 2 days. Recombinant proteins for the Fc-dimerized Sema6A ectodomain (Sema6A$_{ect}$-Fc-His$_6$; see Supplemental Material 4) were added to the cultures for 1 hr at 37°C. In the competition assay, mixtures of Sema6A$_{ect}$-Fc-His$_6$ and PlexA2$_{SD}$-Fc-His$_6$ were incubated for 30 min at 37°C, and then added to the cultures for 30 min. The cultures were fixed and processed for immunohistochemistry with calbindin antibodies to detect mossy fibers.

Binding of Sema6A to Plexin-A2

Binding of the recombinant proteins for Fc-dimerized AP-tagged Sema6A ectodomain (AP-Sema6A$_{ect}$-Fc) to the full-length mouse plexin-A2 proteins expressed in L cells (see Supplemental Material 4) was determined following the procedures reported previously (Suto et al., 2005).

Procedures for the production of antibodies, histology, DiI labeling, transfection, and electrophysiology are given in Supplemental Material 4.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/53/4/535/DC1/.

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