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MAP1B Is Required for Netrin 1 Signaling in Neuronal Migration and Axonal Guidance

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Summary

Background: The signaling cascades governing neuronal migration and axonal guidance link extracellular signals to cytoskeletal components. MAP1B is a neuron-specific microtubule-associated protein implicated in the crosstalk between microtubules and actin filaments.
Results: Here we show that Netrin 1 regulates, both in vivo and in vitro, mode I MAP1B phosphorylation, which controls MAP1B activity, in a signaling pathway that depends essentially on the kinases GSK3 and CDK5. We also show that *map1*B-deficient neurons from the lower rhombic lip and other brain regions have reduced chemoattractive responses to Netrin 1 in vitro. Furthermore, *map1*B mutant mice have severe abnormalities, similar to those described in *netrin* 1-deficient mice, in axonal tracts and in the pontine nuclei.
Conclusions: These data indicate that MAP1B phosphorylation is controlled by Netrin 1 and that the lack of MAP1B impairs Netrin 1-mediated chemoattraction in vitro and in vivo. Thus, MAP1B may be a downstream effector in the Netrin 1-signaling pathway.

Introduction

Ordered neural migration and axonal guidance are two essential steps in neural development. These processes are governed by specific guidance cues [1, 2]. Both in experimental animals and in humans, disruption of neural migration and aberrant axonal growth lead to structural and functional defects that are associated with neurological abnormalities.

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Netrin 1 is a chemoattractive or chemorepellent cue necessary for the formation of many commissural pathways through the brain including commissural connections in the spinal cord, the anterior commissure, the corpus callosum, and the hippocampal commissure in the forebrain [3, 4, 5, 6]. Netrin 1 also regulates the formation of other connections including the thalamocortical and corticothalamic pathways, the entorhinohippocampal connections, as well as the circumferential migration of precerebellar neurons in the hindbrain [7, 8, 9, 10, 11]. Deleted in colorectal cancer (DCC) and the UNC5H-family proteins are Netrin 1 receptors that mediate chemoattraction or chemorepulsion, respectively [6, 12, 13]. It has recently been shown that Netrin 1 signaling activates PI3K and MAP kinases [14, 15] and that the responses to Netrin 1 are regulated by the concentration of cAMP and cGMP and by the activation of L-type Ca^{2+} channels [16, 17, 18]. It is believed that Netrin 1 signaling is linked to cytoskeletal rearrangement by the small GTPases Cdc42 and Rac1 [19].

MAP1B is a neuron-specific microtubule-associated protein (MAP), which is expressed in all developing neurons in neurites and axonal growth cones [20]. Its function is regulated at the post-translational level by phosphorylation [21]. Mode I phosphorylation is mediated by the serine/threonine kinases glycogen synthase kinase 3 (GSK3) and cyclin-dependent kinase 5 (CDK5) [22, 23], which are dynamically regulated by functional stimuli such as lysophosphatidic acid (LPA) [24]. Mode II phosphorylation is catalyzed by casein kinase II and appears to be activated constitutively [25].

MAP1B has been implicated in neurite extension, the dynamic stability of microtubules and the crosstalk between microtubules and actin microfilaments, in a mode I phosphorylation-dependent manner [22, 23, 26]. *map1*B-deficient mice have variable degrees of axonal-tract abnormalities, which are believed to be a consequence of decreased capacity for axonal elongation in these mice [27, 28, 29]. A role in synaptogenesis has also been proposed for the *Drosophila* MAP1B ortholog Futsch [30]. Here we examine whether MAP1B participates in signaling pathways linked to extracellular guidance cues controlling neural migration and axonal navigation. We show that Netrin 1 controls mode I phosphorylation of MAP1B in a GSK3- and CDK5-dependent manner. We also show that the lack of MAP1B markedly reduces Netrin 1-evoked chemoattraction of both axons and migrating neurons. Lastly, *map1*B mutant mice display deficits in the pontine nuclei and abnormalities in brain axonal tracts; these results are similar to those described in *netrin* 1-deficient mice.

Results

Netrin 1 Stimulates MAP1B Mode I Phosphorylation In Vitro and In Vivo

The response of developing neurons to Netrin 1 is mediated through a complex signaling pathway, which is believed to transduce the Netrin 1 signal into cytoskele-

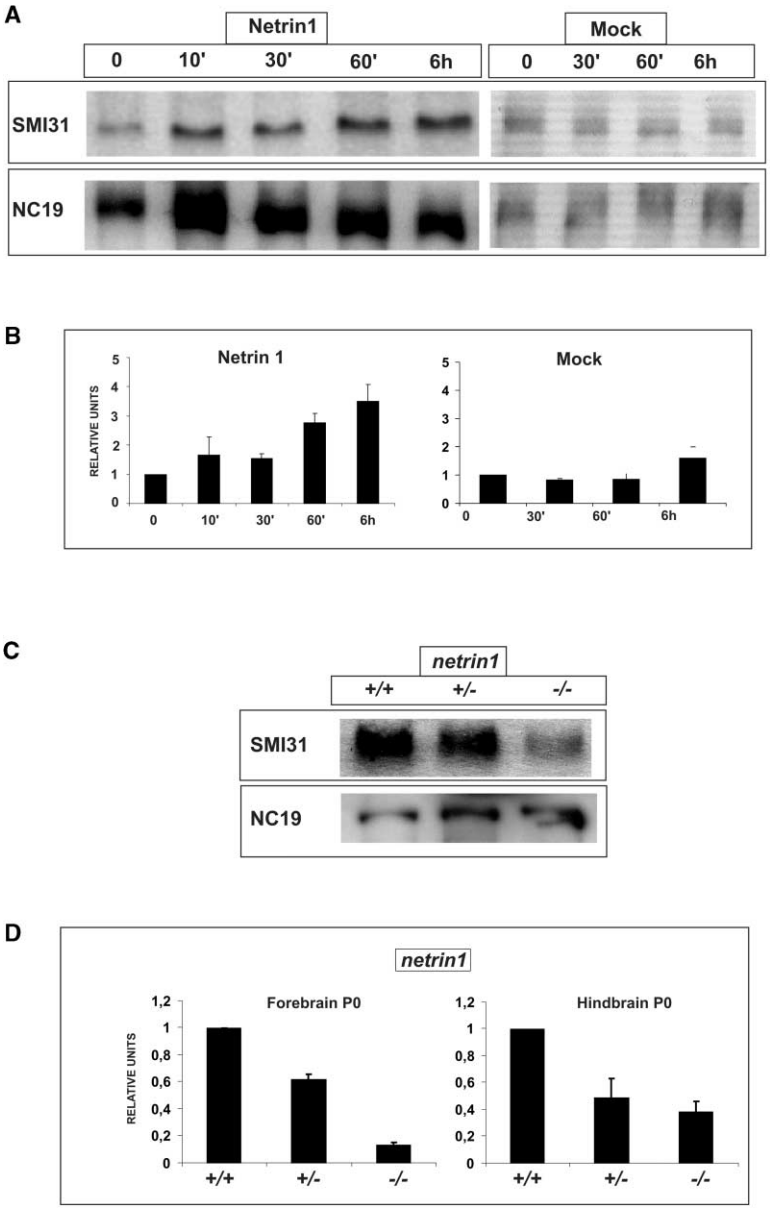

Figure 1. Netrin 1 Increases the Levels of Mode I MAP1B Phosphorylation in Cortical Neurons Cultured for 48 hr

Netrin 1-conditioned medium or control mock medium was added for different times (0, 10, 30, and 60 min; and 6 hr).

(A) Western blots of soluble-cell extracts were incubated with antibodies against mode I phosphorylated MAP1B (SMI31) and MAP1B protein (NC19), as a loading control.

(B) Diagrams showing mean-normalized densitometric values of SMI31 immunoreactivity (and standard deviations; n = 4) after Netrin 1 (left) or mock (right) treatments. The degree of phosphorylation of MAP1B protein in the SMI31 epitope was determined by densitometry and normalized with respect to the values corresponding to total MAP1B protein (NC19). Data are the means ± SD from four separate experiments.

(C) Western blots of brain extracts from the forebrains (P0) of *netrin* 1 mutant mice (−/−), and of heterozygous (+/−) and wild-type littermates. The blots were incubated with the SMI31 mAb (top line) and with the NC19 antibody (bottom). They show decreased levels of phosphorylation in mutant brains.

(D) Histograms showing levels of mode I MAP1B phosphorylation in the forebrain and hindbrain of newborn *netrin* 1-deficient and wild-type mice. The phosphorylation levels of MAP1B protein in the SMI31 epitope were normalized with respect to total MAP1B (NC19). The data from wild-type was referred to as one relative unit (mean ± SD).

tal changes. MAP1B binds microtubules and actin microfilaments, contributing to their stabilization in a process that is believed to depend on mode I MAP1B phosphorylation [23, 31]. To examine whether MAP1B is regulated by the Netrin 1-signaling pathway, we analyzed mode I MAP1B phosphorylation levels in response to Netrin 1 by using Western blots. We cultured neurons from murine, embryonic day 15 (E15) hippocampi, a region that responds at this stage to Netrin 1 [9]. After 2 days in vitro (DIV), neurons were treated with conditioned media from either 293T cells expressing recombinant Netrin 1 or control 293T cells. We analyzed the pattern of mode I phosphorylation by using a Western blot with the mAb SMI31. At 10 min, incubation with Netrin 1 had already increased mode I MAP1B phosphorylation levels, which remained elevated for up to 6 hr (Figures 1A and 1B). In contrast, mode II MAP1B phosphorylation was not altered by Netrin 1 (data not shown). Incubation with conditioned media from control 293T cells (mock cells) did not increase mode I phosphorylation of MAP1B (Figures 1A and 1B).

To examine the effects of Netrin 1 on MAP1B phosphorylation in vivo, we analyzed brain extracts from *netrin* 1-deficient mice (P0). The forebrains and hindbrains of newborn, homozygous mutant mice had a 2.5–7-fold reduction of mode I MAP1B phosphorylation levels compared to wild-type mice, whereas intermediate levels of phosphorylation were detected in heterozygous mice (Figures 1C and 1D). Together, these results support the idea that Netrin 1 triggers an intracellular cascade that increases mode I MAP1B phosphorylation.

Involvement of GSK3 and CDK5 Kinases in Netrin 1-Dependent MAP1B Phosphorylation

MAP1B is phosphorylated in two different ways by serine/threonine protein kinases; whereas GSK3 and CDK5 induce mode I phosphorylation, casein kinase II leads to mode II phosphorylation [22, 23, 25]. In order to iden-

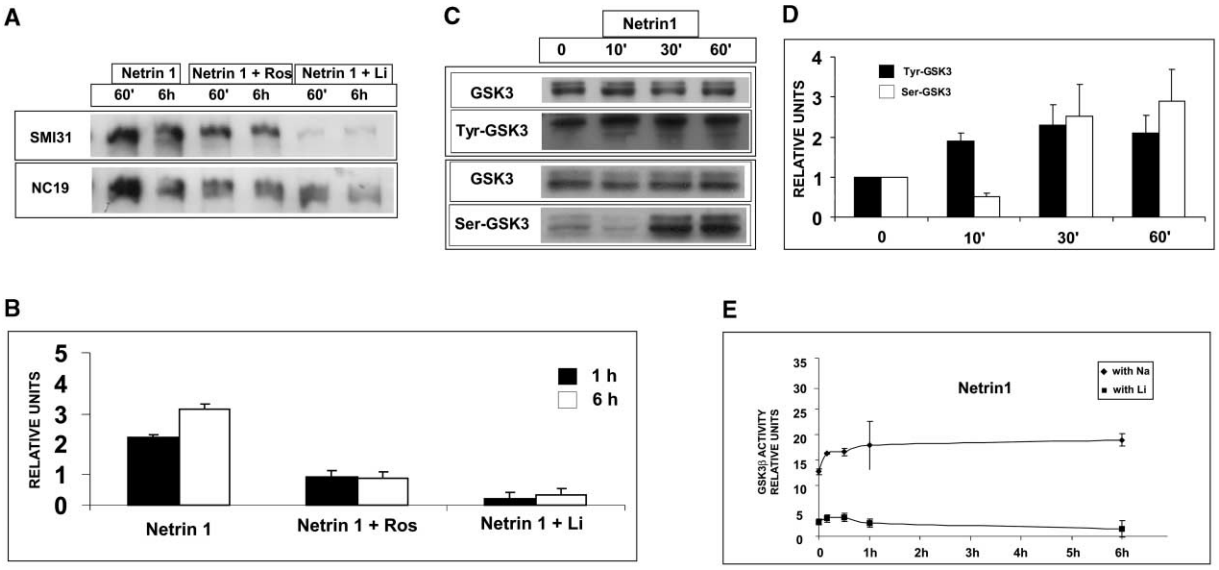

Figure 2. Contribution of GSK3 and CDK5 in the Mode I MAP1B Phosphorylation Induced by Netrin 1

(A and B) Western blots (A) of extracts from cortical neurons treated with Netrin 1 alone for 1 or 6 hr, or with Netrin 1 and lithium chloride (Li), or with Roscovitine (Ros). Membranes were immunoreacted with the SMI31 mAb or with the NC19 antibody. (B) Densitometric analysis of three Western blots (mean ± SD). The data were normalized with respect to total MAP1B levels (NC19 antibody). Inhibition of either CDK5 (Ros) or GSK3 (Li) activities greatly reduces Netrin 1-induced MAP1B phosphorylation levels.

(C) Western blots illustrating total GSK3 protein, P-Tyr-GSK3 levels, and P-Ser-GSK3 levels in cortical cultures treated with Netrin 1-conditioned media for 0–60 min, showing a raise in phosphorylation levels.

(D) Histograms showing levels of P-Tyr-GSK3 and P-Ser-GSK3 normalized with respect to total GSK3, after treatment of cortical cultures with Netrin 1. The data from time zero was referred to as one relative unit (mean ± SD).

(E) Plots illustrating the phosphorylation levels of a GSK3-specific substrate in neuronal cultures treated with Netrin 1-conditioned media for 0–6 hr, in the presence or absence of LiCl, a GSK3 inhibitor. GSK3 kinase activity is considered to be the difference between the kinase activities in the presence or absence of LiCl. GSK3 activity rises shortly after Netrin 1 incubation, and the increased activity lasts for 6 hr.

tify whether these kinases are responsible for Netrin 1-induced mode I phosphorylation of MAP1B, we used inhibitors of GSK3 and CDK5. Forebrain embryonic neurons treated with recombinant Netrin 1 in the presence of the GSK3 inhibitor LiCl (10 mM) [22, 32], showed a dramatic decrease of MAP1B phosphorylation at both 1 and 6 hr, supporting the idea that Netrin 1 triggers GSK3 activation (Figures 2A and 2B). Finally, inhibition of CDK5 with Roscovitine (250 nM, Calbiochem) halved mode I MAP1B phosphorylation levels induced by Netrin 1 (Figures 2A and 2B). We conclude that the Netrin 1-signaling cascade activates MAP1B mode I phosphorylation via a process that depends on GSK3 and CDK5 activation.

GSK3 activity is believed to be negatively regulated by serine phosphorylation [33]. However, GSK3 is also phosphorylated at its Tyr 216, which is believed to increase GSK3 activity [24]. Thus, we thus examined whether Netrin 1 altered the phosphorylation of GSK3. We found that Netrin 1 increased both serine and tyrosine phosphorylation levels of GSK3 (Figures 2C and 2D). Because serine and tyrosine phosphorylation levels may have opposite effects on GSK3 activity, we investigated the net effect of Netrin 1 on GSK3 activity. Indeed, we found that Netrin 1 increased GSK3 enzymatic activity after only 10 min, an effect that lasted over 6 hr (Figure 2E). We conclude that Netrin 1 increases the phosphorylation of GSK3 at both serines and tyrosines, and this in turn results in a marked rise in GSK3 enzymatic activity.

MAP1B Is Required for Netrin 1-Dependent Migration and Axonal Guidance In Vitro

The above-mentioned experimental results suggest that MAP1B might be required for the transduction of Netrin 1 in the regulation of neural migration and axonal guidance. A tangential migratory route is the circumferential pontine migratory stream (PMS), with neurons migrating from the lower rhombic lip (lRL) at the roof of the fourth ventricle toward the ventral midline to constitute the pontine nuclei [8, 11]. This migratory process depends upon Netrin 1, expressed at the midline, and on its receptor DCC, expressed in migrating neurons. To investigate whether MAP1B is required for Netrin 1-dependent migration, we compared the chemoattractive effect of Netrin 1 on migrating neurons from wild-type and mutant lRL by using collagen gel cocultures. E14 lRL explants cocultured with aggregates of control 293T cells showed a radial pattern of migrating neurons in both wild-type and *map1*B mutant tissues, as already described (data not shown) [8,11]. In these cultures cocultured with control cells, migrating neurons were arranged in characteristic chains of cells, which were of similar length in both groups (Figure 3K), indicating that the lack of MAP1B does not affect the capacity of pontine neurons to migrate. Whereas wild-type lRL explants cocultured with Netrin 1-expressing cells showed a strong chemoattractive response (18 out of 18 explants, Figures 3E–3G), *map1*B mutant tissue only exhibited a decreased chemoattractive response in 50% of cases (13 out of 26 explants, Figures 3H–3J) with the remaining explants

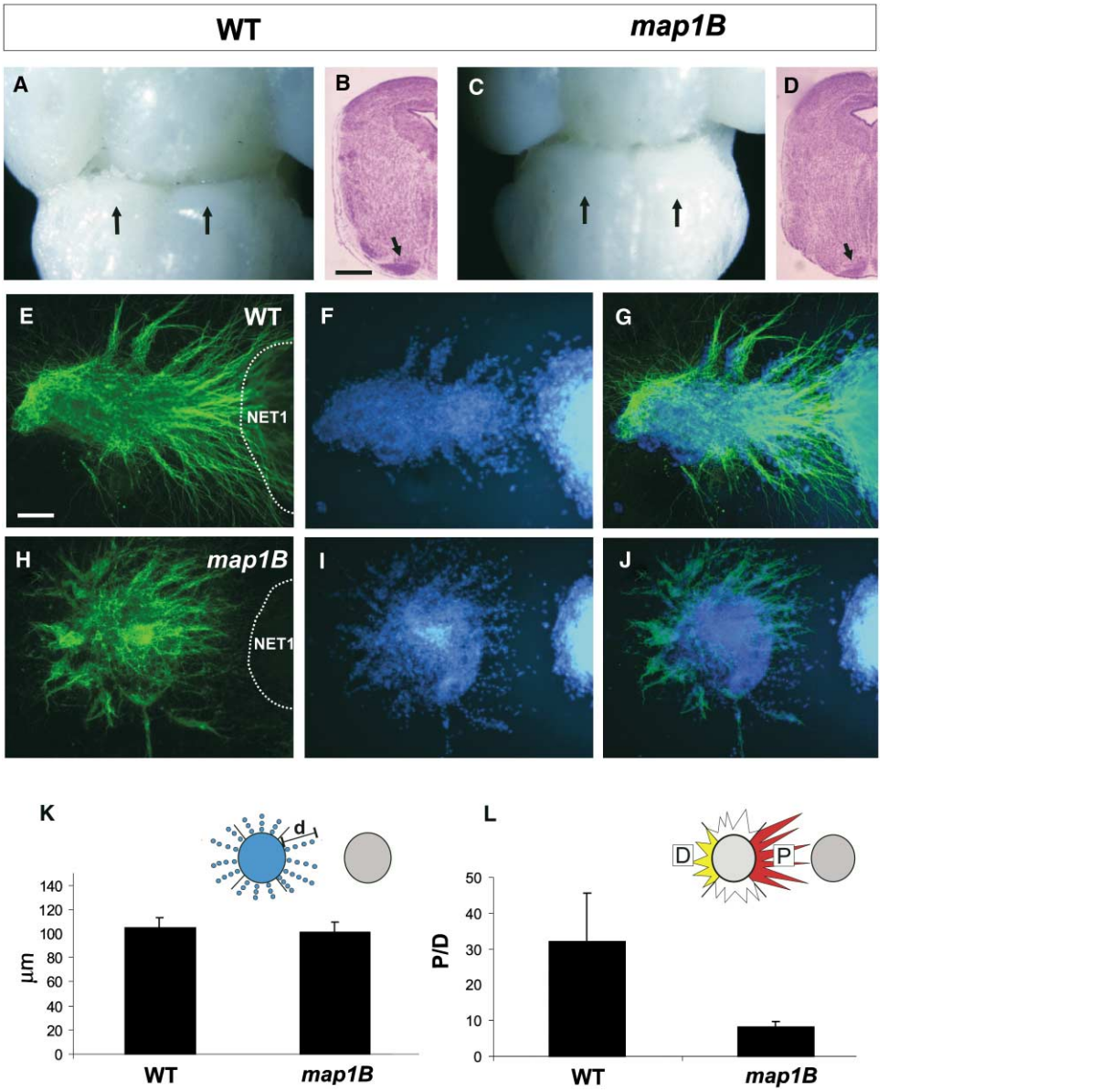

Figure 3. The Circumferential Migration of PMS-Migrating Neurons Is Impaired in the Absence of MAP1B

(A–D) Photomicrographs illustrating a lack of anatomical protuberances (arrows) at the base of the mesencephalon, corresponding to the pontine nuclei in *map1*B mutant embryos (C) and compared to wild-type littermates (A). Cross-sections of the mesencephalon reveal that the pontine nuclei (arrows) display smaller sizes in *map1*B mutant embryos (D) than in wild-type littermates (B). The scale bars represent 500 μm.

(E–J) Pattern of outgrowth of migrating neurons from E14 lRL explants cocultured with aggregates of Netrin 1-expressing cells. Whereas wild-type explants (n = 18) show clear chemoattractive responses (E–G), *map1*B mutant explants (n = 26) show impaired chemoattraction (H–J). Explants are immunostained for βIII-tubulin (E and H) and counterstained with bisbenzimide (F and I). (G and J) Merged photomicrographs. The scale bar represents 100 μm.

(K) Length of chains of migrating neurons (mean ± SD) in wild-type and *map1*B mutant lRL explants cocultured with aggregates of control 293T cells. No differences exist between both groups. (n = 14–23 explants per group).

(H) Chemoattraction of wild-type and *map1*B mutant lRL explants cocultured with aggregates of 293T cells expressing Netrin 1. Data is expressed as the P/D ratio, i.e., the ratio between the surface areas in the proximal and distal quadrants (mean ± SD). The strong chemoattractive response of wild-type explants is severely impaired by the lack of MAP1B. ($p \leq 0.03$).

showing radial outgrowth. Moreover, the overall degree of chemoattraction was 4-fold lower in *map1*B-deficient explants than in control tissue (Figure 3L). These results show that, although the lack of MAP1B does not completely abolish the chemoattractive response of lRL explants to Netrin 1, chemoattraction is severely impaired. This indicates that MAP1B is required for the correct transduction of the Netrin 1 signal in the guidance of pontine migrating neurons.

To substantiate the idea that MAP1B is required for Netrin 1-mediated chemoattraction of developing axons, we cultured explants from brain regions responding

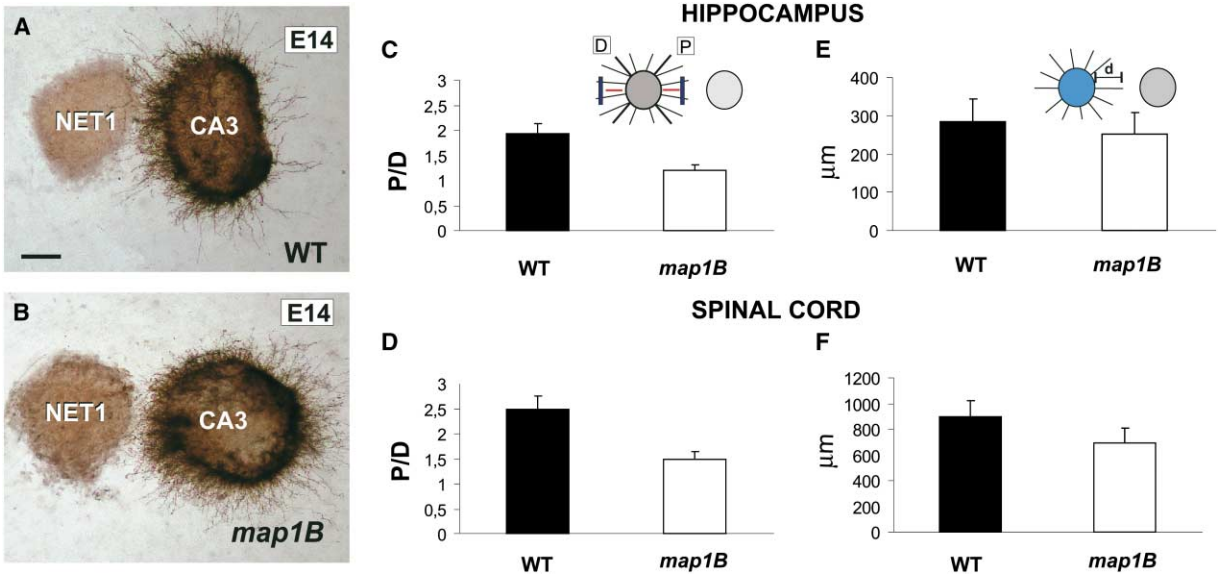

Figure 4. *map1*B-Deficient Axons Respond to Netrin 1 by Reduced Chemoattraction

(A and B) Photomicrographs illustrating axonal growth in wild-type (A) and *map1*B-deficient (B) CA3-hippocampal explants cocultured with aggregates of 293T cells expressing Netrin 1. Whereas wild-type explants show chemoattraction, *map1*B mutant explants show a radial pattern of growth. The scale bar represents 100 μm.

(C and D) Quantification of the chemoattractive response of wild-type and *map1*B axons from E15 hippocampal and E10 spinal cord explants cocultured with cells secreting Netrin 1 (P/D ratio; mean ± SD). The chemoattractive response of wild-type explants (black bars) is severely impaired in *map1*B-deficient explants (white bars). ($p \leq 0.05$).

(E and F) Axonal length (mean ± SD) in wild-type and *map1*B mutant explants from the CA3 region and the spinal cord, cocultured with aggregates of control 293T cells, and showing no differences between both groups.

to Netrin 1 in collagen gels. In agreement with previous studies [4, 9], explants from the dorsal spinal cord (E10) and CA3 hippocampal region (E15) of wild-type embryos exhibited clear chemoattractive responses after being cocultured with aggregates of Netrin 1-expressing cells. In contrast, explants from mutant embryos showed diminished chemoattractive responses to Netrin 1. For instance, *map1*B-deficient hippocampal explants exhibited a marked decrease in their chemoattractive response (Figures 4A, 4B, and 4C). A significant, although less severe, decrease was observed in the chemoattractive response of mutant dorsal spinal cord explants (Figure 4D). Finally, cocultures with aggregates of control 293T cells showed radial axonal growth and similar fiber lengths in both wild-type and *map1*B mutant explants (Figures 4E and 4F), indicating that axonal outgrowth is not impaired by the lack of MAP1B. These results indicate that the chemoattractive response of cultured axons to Netrin 1 depends upon MAP1B.

*map1*B-Deficient Mice Show Migration and Axonal Growth Deficits

We next examined possible in vivo abnormalities in the PMS of *map1*B-deficient embryos. Because *map1*B-deficient mice die soon after birth, the phenotype of mutant embryos was analyzed at E18. The pontine nuclei in wild-type embryos were recognized in toto preparations as protuberances, which correspond in cross-sections to large, well-developed nuclei, at the ventral surface of the mesencephalon (Figures 3A and 3B). In contrast, homozygous mutants lacked anatomical protuberances, and histological examination confirmed that the pontine nuclei were smaller and less clearly delimited than in control embryos (Figures 3C and 3D). Although less dramatic, these abnormalities are reminiscent of those in *netrin* 1- and *dcc*-deficient mice [8, 34].

Because Netrin 1 is also essential for axonal guidance, we examined alterations in fiber tracts, which are disrupted by *netrin* 1 and *dcc* mutations [5, 6, 9, 10], in the forebrain of *map1*B-deficient embryos. Immunostaining for the cell adhesion molecules TAG1 and L1 at E18 labeled many fiber tracts in the forebrain including the major commissures, the reciprocal thalamocortical and corticothalamic connections, and several hippocampal pathways. We found that the corpus callosum and the hippocampal commissure were severely disrupted in mutant mice (Figures 5A and 5B). Thus, the corpus callosum was absent in 5 out of 7 mutant embryos (Figure 5B), whereas the thickness of the corpus callosum was reduced by 70% in the remaining mutants (2 out of 7 cases), compared to controls. Similar phenotypic changes were observed for the hippocampal commissure (e.g., 55% thickness reduction). Other midline tracts, such as the anterior commissure (Figure 5A), were completely absent in *map1*B mutant embryos. These fiber-tract alterations are reminiscent, with some differences (see Discussion), of those in *netrin* 1 and *dcc* mutant mice. Moreover, the major hippocampal axonal tracts in the entorhinohippocampal termination zone, in the white matter, and in the fimbria were impaired in mutant mice, which showed ectopic and disorganized fiber bundles (Figures 6A–6C) in a similar way to those in *netrin* 1-deficient mice [9].

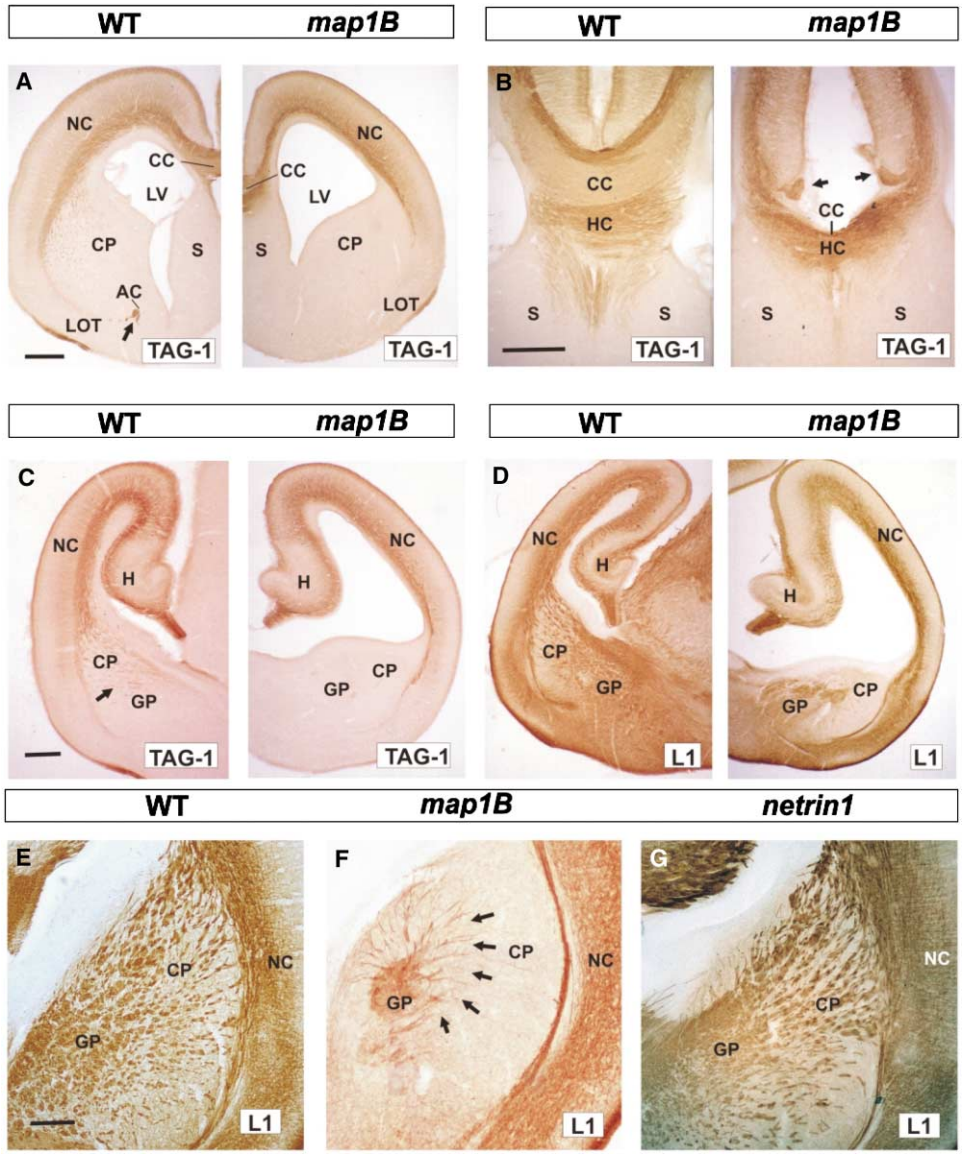

Figure 5. *map1*B Mutant Mice Display Abnormalities in Major Fiber Tracts of the Forebrain

(A and B) Coronal sections from wild-type and mutant forebrains (E18), at the level of the corpus callosum, were immunostained with anti-TAG1 antibodies. Whereas the thickness of the *map1*B mutant corpus callosum is reduced in (A), the corpus callosum is virtually absent in the example shown in (B). Instead, callosal fibers appear to terminate in the ipsilateral cortex (arrows in [B]). Note also the lack of anterior commissure (AC) in the mutants (arrows in [A]) and that the hippocampal commissure (HC) is thinner in *map1*B mutants (B). The scale bar represents 500 μm.

(C and D) Coronal sections from wild-type and *map1*B mutant forebrains at the level of the dorsal hippocampus immunostained with TAG1 and L1 antibodies. (C) TAG1-immunoreactive corticothalamic axons traversing the striatum (arrows, left) are completely absent in *map1*B mutants (right). Thalamocortical axons immunostained with L1 antibodies (D), appear to stop near the caudate putamen in *map1*B mutant mice (right panel). The scale bar represents 200 μm.

(E–G) High-power photomicrographs illustrating the pattern-TAG1 immunostaining in the striatum of wild-type, *map1*B-deficient, and *netrin* 1 mutant embryos. In wild-type and *netrin* 1-deficient embryos (E and G), reciprocal corticothalamic and thalamocortical axonal bundles cross the caudate putamen (CP). In *map1*B mutant embryos (F), cortical axonal bundles do not enter into the striatum. Thalamocortical axons reach the Globus Pallidus (GP), from where they radiate in several directions (arrows) with no invasion of the neocortex. The scale bar represents 500 μm. Abbreviations: NC = neocortex; CP = caudate putamen; GP = globus pallidus; H = hippocampus; HC = hippocampal commissure; S = septum; AC = anterior commissure; LOT = lateral olfactory tract; LV = lateral ventricle.

Reciprocal thalamocortical and corticothalamic connections develop in a "hand-shake" process that depends upon Netrin 1 expression in the ganglionic eminence [7, 35, 36]. At E18 in wild-type and *netrin* 1-deficient embryos, these reciprocal connections were already formed, appearing as tight L1-positive bundles that perforated the striatum to reach the neocortex and dorsal thalamus, respectively (Figures 5D, 5E, and 5G). TAG-1 antibodies labeled corticothalamic axons, but not thalamocortical axons (Figure 5C). In mutant embryos,

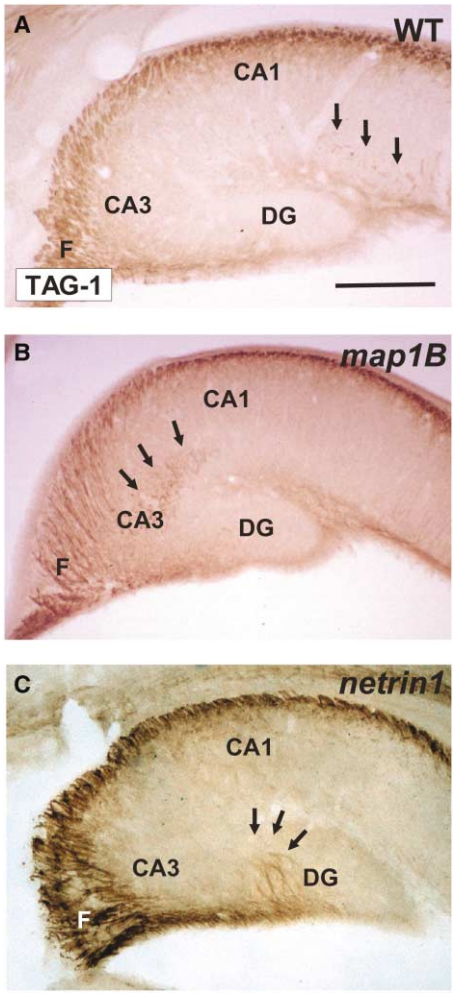

Figure 6. Pattern of TAG-1 Immunostaining in the Hippocampus

(A and B) TAG1 immunopositive fiber bundles in the hippocampus of *map1*B mutant embryos (B) are disorganized in the fimbria (F) and are ectopically located in several layers (arrows) (compare with A), reminiscent to the fiber abnormalities observed in *netrin* 1-deficient embryos (C). The scale bar represents 200 μm.
Abbreviations: DG = dentate gyrus; CA1-CA3 = hippocampal regions.

thalamocortical fibers, visualized with L1 immunostaining, successfully reached the internal capsule and the Globus Pallidus, but stopped abruptly within the striatum itself and failed to invade the neocortex (Figures 5D and 5F). Conversely, corticothalamic fibers visualized with TAG-1 immunolabeling were confined to the intermediate zone of the neocortex and did not enter the adjacent striatum (Figure 5C).

To confirm that reciprocal thalamocortical connections were disrupted in *map1*B mutants, we injected DiI into the dorsal thalamus. DiI-traced thalamocortical axons in wild-type embryos entered the striatum to reach the cortical intermediate zone, the subplate, and the cortical plate (Figures 7A and 7B). In contrast, most thalamocortical axons in *map1*B mutants turned ventrally before reaching the striatum (Figures 7C and 7D), and only a few axons entered the striatum. No thalamic axons were observed in the cerebral cortex (Figure 7D). Conversely, DiI injections in wild-type embryos labeled corticothalamic axonal bundles traversing the striatum and retrogradely-labeled thalamocortical neurons (Figure 7E). Injections in the neocortex of mutant embryos stained fiber bundles that were restricted to the intermediate zone. These fibers coursed mediolaterally through the developing white matter and terminated in growth cones near the piriform cortex (Figure 7F). No retrogradely-labeled neurons were observed in the thalamus of *map1*B-deficient embryos (Figure 7F).

These findings show that thalamocortical and corticothalamic connections are severely disrupted in *map1*B mutant mice in a more dramatic way than in *netrin* 1- and *dcc*-deficient mice [36]. Because the tangential PMS and the axonal pathways that depend on Netrin 1/DCC signaling are disrupted in *map1*B-deficient embryos, we conclude that MAP1B is also required for Netrin 1-dependent migration and axonal guidance in vivo.

Discussion

Netrin 1 Signaling Phosphorylates MAP1B through the GSK3 and CDK5 Kinases

The present analyses in vivo and in vitro indicate that MAP1B may be essential for correct transduction of Netrin 1. First, both in vivo and in vitro Netrin 1 stimulate mode I, but not mode II, phosphorylation of MAP1B and triggers GSK3 activation; second, *map1*B mutant explants show decreased chemoattractive responses to Netrin 1; and third, *map1*B mutant mice display abnormalities reminiscent of those of *netrin* 1 mutant mice.

Microtubules and actin filaments, which are both concentrated at the leading process of migrating neurons and in growing axons, are essential for neuronal migration and axonal growth [37]. MAP1B is believed to control the dynamic properties of microtubules [31, 38]. For instance, *map1*B-deficient neurons contain fewer microtubules than wild-type cells. Moreover, MAP1B binds actin filaments in vitro, suggesting that it may control actin dynamics [31, 38, 39]. Consistent with this vision, *map1*B-deficient neurons in vitro show aberrant actin distribution and polymerization, abnormal number of filopodia-like structures, and decreased levels of GTP-Rac1 [40]. These data strongly support the idea that MAP1B controls actin and microtubule dynamics/stability, and the crosstalk between these cytoskeletal components.

We show here that mode I MAP1B phosphorylation is activated by Netrin 1 in vivo *and in vitro*. Mode I phosphorylated MAP1B is upregulated during development and is enriched at the distal ends of growing axons [20, 26, 41]. Inactivation of GSK3 or CDK5 kinases induces a neural phenotype in vitro that resembles that of *map1*B-deficient neurons [22, 23, 42]. Ablation by microCALI (chromophore-assisted laser inactivation) experiments of mode I MAP1B phosphorylation affects growth-cone tuning in cultured neurons [26]. All these data suggest that most functions of MAP1B are regulated by mode-I phosphorylation, which is likely to increase microtubule and actin instability and crosstalk [23, 26].

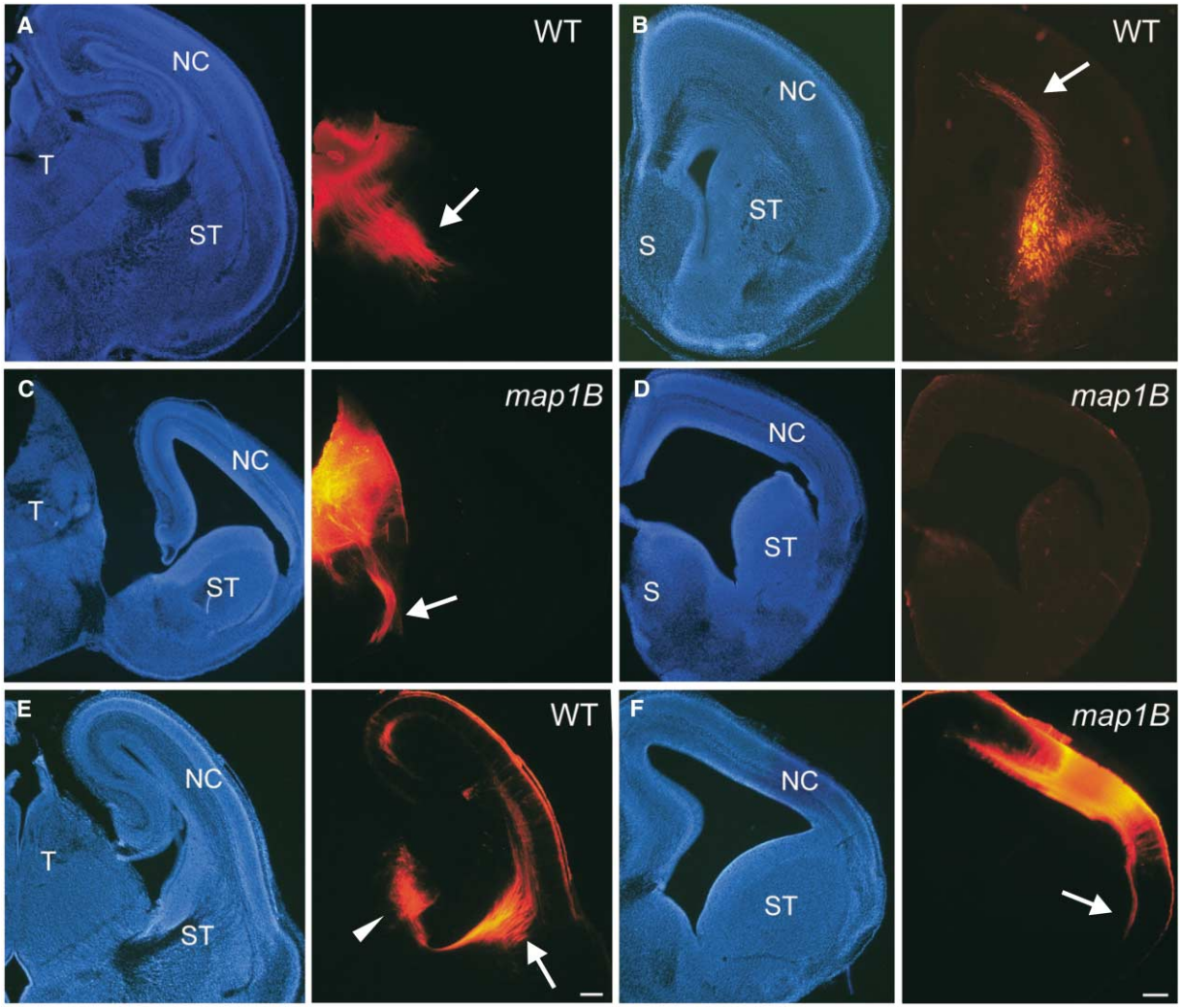

Figure 7. DiI Labeling of Thalamocortical and Corticothalamic Axons

The same sections are shown for bisbenzimide staining (left panels) and DiI labeling (right panels).

(A and B) Coronal sections from wild-type forebrains injected with DiI in the dorsal thalamus (A). Thalamic fibers reach the striatum (arrow in [A]) and invade the neocortex (arrow in [B]).

(C and D) In *map1*B mutant embryos, thalamic axons (arrow) do not enter the striatum (C) and no axons are seen in the neocortex (D).

(E and F) DiI injections in the neocortex of wild-type embryos (E) labeled corticothalamic fibers that traverse the striatum (arrow) and reach the thalamus (arrowhead). (C) In contrast, neocortical injections in *map1*B mutants (F) labeled fibers that do no enter the striatum. Axons run in the cortical white matter (arrow in [F]).

Abbreviations are the same as in Figure 5. The scale bars represent 200 μm.

Several kinases, including GSK3 and CDK5, have been implicated in mode I MAP1B phosphorylation [22, 23]. Our experiments show that blockading GSK3 reduces mode I MAP1B phosphorylation induced by Netrin 1. These results indicate an essential role of this kinase in mode I phosphorylation, and this role is further supported by the rise in GSK3 kinase activity detected in the present study. Inhibition of CDK5 also yielded a decrease in Netrin 1-induced phosphorylation of MAP1B, indicating that both GSK3 and CDK5 cooperate in vitro and in vivo in the Netrin 1-dependent mode I phosphorylation of MAP1B.

GSK3 activity is downregulated by phosphorylation of serines and upregulated by phosphorylation on tyrosines [43]. Several pathways have been proposed to inhibit GSK3 activity, including the Reelin pathway through activation of the PI3K-Akt/PKB pathway [33] and the Wnt/Wingless signaling cascade [44]. The present data show that Netrin 1 increases phosphorylation of both serines and tyrosines of GSK3, which results in a marked raise in GSK3 enzymatic activity. This notion is also consistent with the finding that mode I phosphorylation of MAP1B, a GSK3 substrate, increases upon exposition to Netrin 1.

A recent study reports that the chemorepellent Semaphorin 3A decreases Serine phosphorylation of GSK3, although tyrosine phosphorylation was not reported [32]. Moreover, pharmacological blockading of GSK3 prevents Semaphorin 3A-induced growth-cone collapse, but activation of GSK3 alone does not induce

growth-cone collapse [32]. GSK3 could be equally activated by Semaphorin 3A and Netrin 1, and the distinct ouputs (collapse versus growth) may be regulated by the activation of other signaling cascades. However, the fact that Netrin 1 increases both serine and tyrosine phosphorylation indicates that the mechanisms that regulate GSK3 via Netrin 1 and Semaphorin 3A are distinct. In line with this, hippocampal *map1*B mutant axons are chemorepelled by Semaphorin 3A (unpublished observations).

CDK5 has been implicated in axonal growth [45]. Thus, the present data implicate both GSK3 and CDK5 activities in the intracellular response triggered by Netrin 1 in neuronal migration and axonal guidance. Furthermore, the in vitro assays showing that MAP1B becomes phosphorylated in mode I by Netrin 1, and the finding that *netrin* 1 mutant mice have decreased levels of MAP1B phosphorylation in vivo, indicate that Netrin 1 signaling controls the phosphorylation state of MAP1B. This suggests that this protein is a downstream effector in the Netrin 1-signaling cascade

MAP1B Is Required for Netrin 1-Dependent Axonal Growth and Migration

It has been suggested that MAP1B could be involved in neurite extension in cultured neurons [40]. However, the present results in vivo showing that many axonal tracts extend for long distances in *map1*B mutants do not support this view. In contrast, most of the axonal pathways are abnormal either in their trajectory (e.g., corticothalamic and thalamocortical pathways, and the anterior commissure) or in their termination pattern (e.g., the entorhinohippocampal pathway), suggesting that MAP1B is required for transduction of extracellular guidance cues. The explant assays show that the lack of MAP1B impairs the chemoattractive response of developing axons and IRL-migrating neurons to Netrin 1. Taken together the data show that MAP1B is required in cultured neurons for the response to Netrin 1.

We show that the lack of MAP1B in vivo leads to dramatic abnormalities in major axonal tracts such as the corpus callosum, the hippocampal commissure, the anterior commissure, and the reciprocal corticothalamic pathway. Most of these deficits are reminiscent of those in *netrin* 1- and *dcc*-deficient mice [5, 6, 9, 36], again supporting the hypothesis that MAP1B plays a role in Netrin 1-mediated axonal guidance in vivo.

Whereas the defects in some axonal tracts, such as the anterior commissure and the entorhinohippocampal pathway, are almost identical to those in *netrin* 1- and *dcc*-mouse mutants, other connections (e.g., the corpus callosum and the hippocampal commissure) show less dramatic alterations, which suggests that there is functional redundancy of cytoskeletal-associated proteins. Conversely, the phenotype of the reciprocal corticothalamic pathway, with a complete absence of corticothalamic and thalamocortical projections, is much more dramatic in *map1*B mutants than in *netrin* 1 and *dcc* mutant mice [36]. In addition to Netrin 1, an uncharacterized chemoattractive factor has been implicated in the guidance of the reciprocal corticothalamic pathway [10, 36]. Thus, the severe phenotype of the *map1*B mutant suggests that several signaling cascades governing axonal guidance may converge on the MAP1B protein for the development of the reciprocal thalamocortical projections.

The notion that *map1*B is a downstream effector in the Netrin 1 pathway is supported by the findings that the PMS, a Netrin 1-dependent migratory pathway disrupted in *netrin* 1- and *dcc*-deficient mice [8, 11, 34], is also affected in *map1*B mutant mice and that Netrin 1 decreases chemoattraction in mutant IRL explants.

Conclusions

The present phosphorylation assays and in vitro explant experiments, as well as the phenotype of *map1*B mutant mice, strongly support the idea that MAP1B is an important downstream effector of Netrin 1-dependent axonal guidance and neuronal migration in the developing CNS. This may be achieved by direct regulation of mode I MAP1B phosphorylation in a signaling cascade that depends on both GSK3 and CDK5 kinases.

Experimental Procedures

Stimulation of Dissociated Neuronal Cultures

Netrin 1-conditioned media was obtained from a stable 293T cell line overexpressing Netrin 1 [46]. Cells were grown for four days in DMEM containing 0.5% serum, and the medium was concentrated about 20× with Millex GV filters (Millipore). For dissociated cortical cultures, E15–E16 embryos were dissected out. Their brains were dissected in PBS containing 0.6% glucose, and the neocortex and hippocampus were dissected out. After trypsin (Gibco-BRL) and DNAse (Roche Diagnostics) treatments, tissue pieces were gently swept to dissociate them. Cells were then counted and seeded onto poly-D-lysine coated dishes in neurobasal medium containing 1% horse serum and B27 supplement (Gibco-BRL). In most experiments, cells were seeded in 6-well dishes at 1 million cells per well.

After 2 DIV, conditioned medium containing Netrin 1 was diluted with fresh culture medium and added to the neuronal cultures for different time periods. Control experiments included incubation with conditioned media from control cells. After stimulation, cells were collected in 2× loading-sample buffer for SDS-PAGE (0.15 M Tris [pH 6.5], 1 mM 2-mercaptethanol, 1% SDS, 10% glycerol, and 0.025% bromophenol blue), boiled for 10 min, and subjected to SDS-PAGE.

Protein-Brain Extracts

The brains of *netrin* 1 mutant mice were collected at postnatal day zero (P0). In brief, the hindbrains and forebrains of mutant mice and control littermates were homogenized in: 20 mM HEPES (pH 7), 150 mM NaCl, 5 mM EGTA, 1 mM $MgCl_2$, 10% glycerol, 1 mM aprotinin, 1 mM leupeptin, 0.2 mM PMSF, 0.1 M NaF, 10 mM sodium pyrophosphate, and 0.2 mM sodium orthovanadate. After centrifugation, supernatants were analyzed by Western blot.

Western Blot

Samples were loaded and run in 6% polyacrylamide gels at 150 V. After running, transfer to nitrocellulose membranes was performed in 120 mM glycine, 125 mM Tris, 0.1% SDS, and 20% methanol. Transfer was performed at 300 mA for 2 hr. Then, filters were saturated in 3% BSA in TBS and incubated with the following antibodies: 1/1000 SMI 31 MAb (Sternberger Monoclonals), 125 MAb [40], 1/1000 anti-tubulin (Roche Diagnostics), 1/1000 NC19 antibody (Santa Cruz Biotechnology), 1/1000 antiactin (Sigma), 1/1000 anti-GSK3 (Transduction Laboratories), and with 1/1000 anti-P-Tyr-GSK3 and anti-P-Ser-GSK3 (Bioscience International). Secondary antibodies were used at a concentration of 1/2000 in TBS containing 3% powder milk. Labeling was visualized with ECL plus (Amersham). For densitometric analyses, the Quantity One (Bio-Rad) program

was used. Densitometric analyses were normalized to total protein levels by the detection of tubulin, actin, or total MAP1B (NC19 antibody). All the Western blot data represent a minimum of four separate experiments.

Determination of GSK3 Activity

GSK3 assays were carried out as previously described [24]. Cultured cell extracts were prepared at 0–6 hr after incubation with Netrin-containing media, or with mock supernatant. Cells were collected with a scraper and homogenized in a buffer containing 20 mM HEPES (pH 7.4), 100 mM NaCl, 100 mM NaF, 1 mM sodium ortohovanadate, and 5 mM EDTA. The soluble fraction was immunoprecipitated with a GSK3 antibody (Transduction Laboratories). Samples of 10 μl were incubated in a buffer containing 25 mM HEPES (pH 7.5), 1 mM DTT, 10 mM $MgCl_2$, and a specific GSK3-substrate peptide [24] at a final concentration of 0.75 mg/ml, in the presence of γ^{32}P-ATP. After 30 min, the reaction was stopped with 1% H_3PO_4. The difference between the kinase activities in the presence or absence of the GSK3 inhibitor LiCl (20 mM) was considered to reflect GSK3 kinase activity [24].

Pharmacological Inhibition of Protein Kinases

Neuronal cultures were prepared and treated with Netrin as described above. Cultures were supplemented with 10 mM lithium chloride and 20 mM myo-inositol to inhibit GSK3 activity [32] or with 250 nM roscovitine to inhibit CDK5 activity [40]. In some experiments, cultures were incubated with both inhibitors.

*map1*B and *Netrin* 1 Mutant Embryos

Generation of *map1*B mutants was made via the gene trapping approach [47]. The gene-trapping vector contained a fused chimaeric gene composed of neomycine phosphotransferase (neo^p) and β-galactosidase (β-gal) controlled by the promoter of the endogenous trapped gene [47]. To genotype the mutant mice, genomic DNA was isolated from mice tails and analyzed by PCR with oligonucleotides corresponding to the neomycine phosphotransferase gene (neo^r) contained in the gene-trapping vector [27]. Heterozygous animals were bred to obtain homozygous *map1*B mutants. The genotype of homozygous mutants was indicated by abnormal posturing of limbs [27] and subsequently confirmed by Western blot analyses of spinal-cord protein extracts immunostained with anti-MAP1B (monoclonal antibody 125) and anti-β-galactosidase (Promega) antibodies, as previously described [27]. *netrin* 1 mutant mice were generously provided by Dr. M. Tessier-Lavigne (Stanford University, CA). Crossing of heterozygous mice and genotyping was done as previously described [9].

Histology

Embryos were transcardially perfused at E18 with 4% paraformaldehyde. Dissected brains were postfixed in the same fixative, cryoprotected, and frozen in dry ice. Coronal sections (40 μm thick) were Nissl-stained or immunostained with the following antibodies: anti-calbindin (Swant, Bellinzona, Switzerland), anti-TAG-1, anti-CSPG, and anti-L1. After incubation with biotinylated secondary antibodies and streptavidin-HRP complex, sections were developed with diaminobenzidine and hydrogen peroxide.

For tracing developing connections, the parietal neocortex and dorsal thalamus of E18 embryos were injected with DiI [9]. Seven *map1*B mutants and nine wild-type or heterozygous embryos were used for these studies. After some weeks in fixative, vibratome coronal sections were stained with bisbenzimide and viewed under epifluoresecence.

Explant Cocultures

Explants from the E15 CA3 hippocampal region, the E14 lRL, or the E10 dorsal spinal cord were dissected and cultured as previously described [9, 11, 48]. Explants were cocultured for 2 days in collagen gels with aggregates of Netrin 1-expressing 293T cells or aggregates of control 293T cells [9]. Explants were stained with the TUJ1 mAb, which was visualized with either immunofluorescence or an immunoperoxidase reaction. Some explants were also counterstained with bisbenzimide. Chemoattraction was quantified as previously described [11]. In brief, for axonal counts, the data were expressed as the ratio between the number of axons present in the proximal and distal quadrants (P/D ratio) at a distance of 100–150 μm [48]. For lRL explants, we measured the surface area covered by chains of migrating neurons stained by the TUJ1 antibody in the proximal and distal quadrants of explants cocultured with Netrin 1, and data were expressed as the P/D ratio [11] (mean ± SD). For the length of hippocampal axons and lRL chains we also used TUJ-1 staining. The extent of axons and chains were measured in explants cocultured with control cells. Data were analyzed with the t test.

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