Rab3B protein is required for long-term depression of hippocampal inhibitory synapses and for normal reversal learning

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Rab3B, similar to other Rab3 isoforms, is a synaptic vesicle protein that interacts with the Rab3-interacting molecule (RIM) isoforms RIM1α and RIM2α as effector proteins in a GTP-dependent manner. Previous studies showed that at excitatory synapses, Rab3A and RIM1α are essential for presynaptically expressed long-term potentiation (LTP), whereas at inhibitory synapses RIM1α is required for endocannabinoid-dependent long-term depression (referred to as "i-LTD"). However, it remained unknown whether i-LTD also involves a Rab3 isoform and whether i-LTD, similar to other forms of long-term plasticity, is important for learning and memory. Here we show that Rab3B is highly enriched in inhibitory synapses in the CA1 region of the hippocampus. Using electrophysiological recordings in acute slices, we demonstrate that knockout (KO) of Rab3B does not alter the strength or short-term plasticity of excitatory or inhibitory synapses but does impair i-LTD significantly without changing classical NMDA receptor-dependent LTP. Behaviorally, we found that Rab3B KO mice exhibit no detectable changes in all basic parameters tested, including the initial phase of learning and memory. However, Rab3B KO mice did display a selective enhancement in reversal learning, as measured using Morris water-maze and fear-conditioning assays. Our data support the notion that presynaptic forms of long-term plasticity at excitatory and inhibitory synapses generally are mediated by a common Rab3/RIM-dependent pathway, with various types of synapses using distinct Rab3 isoforms. Moreover, our results suggest that i-LTD contributes to learning and memory, presumably by stabilizing circuits established in previous learning processes.

neurotransmitter release | synaptic plasticity | extinction learning | active zone | GABAergic synapse

Although the role of postsynaptic forms of long-term plasticity in learning and memory has been characterized more extensively than that of presynaptic forms, presynaptic forms of long-term synaptic plasticity are observed widely throughout the brain at both excitatory and inhibitory synapses (1–3). Presynaptic forms of long-term plasticity include long-term potentiation (LTP) in hippocampal mossy-fiber synapses (4, 5), cerebellar parallel-fiber synapses (6, 7), corticostriatal and corticothalamic synapses (8, 9), and cortico–lateral amygdala synapses (10). Moreover, presynaptic forms of long-term depression (LTD) have been observed at synapses in the striatum (11), nucleus accumbens (12), amygdala (13), and prefrontal cortex (14). Most presynaptic forms of LTD throughout the brain are mediated by the retrograde actions of endogenous cannabinoids (2). Endocannabinoids are produced postsynaptically and act transsynaptically to depress neurotransmitter release for short and/or long time periods. For example, synapses made by a subset of hippocampal inhibitory interneurons onto pyramidal excitatory cells express a form of endocannabinoid-mediated LTD referred to as "i-LTD" (1–3).

Synaptic vesicles contain several Rab proteins, including four Rab3 isoforms: Rab3A, Rab3B, Rab3C, and Rab3D (15). KO of individual Rab3 isoforms does not impair survival, whereas KO of multiple Rab3 isoforms is lethal (16, 17), arguing for functional redundancy among Rab3 proteins. Studies using quadruple Rab3 KO mice revealed that Rab3 is essential for the normal dynamics of neurotransmitter release (17, 18). Rab3 proteins, among others, act by binding to their effector proteins RIM1α and RIM2α (for Rab3-interacting molecule-1α and -2α). Thus far, all presynaptic forms of long-term plasticity tested, including all endocannabinoid-mediated forms of presynaptic long-term plasticity, require the Rab3 effector protein RIM1α; specifically, this requirement was shown for regular LTP at hippocampal mossy-fiber synapses (19–21), cerebellar parallel-fiber synapses (19), and cortico–lateral amygdala synapses (10), late LTP at CA3–CA1 hippocampal synapses (22), and i-LTD in the hippocampus and basolateral amygdala (23). In addition, Rab3A itself was shown to be essential for presynaptic mossy-fiber LTP (24) and LTD (25) and for late LTP (22). It is unclear, however, whether a Rab3 isoform also mediates other presynaptic forms of long-term plasticity, in particular i-LTD.

Here, we have examined the role of Rab3B in synaptic transmission and behavior. We show that Rab3B is enriched in inhibitory synapses of the CA1 region of the hippocampus. KO of Rab3B in mice significantly impaired i-LTD while preserving basal inhibitory and excitatory synaptic transmission. Rab3B KO mice do not exhibit abnormalities in locomotor or anxiety-like behaviors but display a significant enhancement in reversal learning and fear extinction. Our data suggest that Rab3B is a key player in the presynaptic machinery that modulates long-term plasticity in inhibitory synapses and provide a potential link between LTD of inhibitory inputs and learning and memory.

Results

Rab3B Is Enriched in Inhibitory Synapses in the Hippocampus. Examination of in situ hybridization patterns for Rab3B published in the Allen Brain Atlas (http://www.brain-map.org) suggests that in the hippocampus Rab3B is expressed preferentially in inhibitory interneurons. To investigate whether this expression pattern correlates with the localization of Rab3B protein, we examined cryostat sections from the mouse hippocampus by double immunofluorescence labeling with a polyclonal antibody to Rab3B (26) and a monoclonal antibody to vesicular glutamate transporter

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1 (vGlut1), as a marker of excitatory glutamatergic synapses, or glutamic acid dehydrogenase 65 (GAD65), as a marker for inhibitory GABAergic synapses. As negative controls, we used sections from Rab3B KO mice. Rab3B antiserum produced punctate staining throughout the CA1 region of the hippocampus, consistent with a localization of Rab3B to synaptic vesicles (Fig. 1). Staining was absent in sections from Rab3B KO mice (Fig. S1). Examination of the Rab3B staining pattern showed that the pyramidal cell body layer contained abundant Rab3B$^+$ puncta, suggesting a localization of Rab3B to inhibitory synapses. Moreover, a large degree of overlap in the staining of Rab3B with GAD65 was observed, with much less overlap in the staining with vGlut1 (Fig. S1). These results argue for a predominant localization of Rab3B in inhibitory synapses of the hippocampal CA1 region.

Rab3B KO Does Not Detectably Alter Excitatory Synaptic Transmission and Plasticity. We next asked whether the deletion of Rab3B alters excitatory synaptic transmission at Schaffer collateral synapses in the CA1 region of the hippocampus. We first measured the amplitude and frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs) by whole-cell recordings but saw no differences between Rab3B WT and KO mice (Fig. 2*A*). We then recorded excitatory field potentials (fEPSP) in the stratum radiatum by stimulating presynaptic fibers with increasing intensity. Plots of the resulting input/output curves again did not reveal a significant difference between WT and Rab3B KO mice (Fig. 2*B*). We next examined short-term synaptic plasticity by monitoring paired-pulse ratios (PPR) but again found no significant difference in PPR between genotypes (Fig. 2*C*). Moreover, classical NMDA receptor-dependent LTP monitored by field recordings was unchanged in Rab3B KO mice compared with their littermate WT controls (Fig. 2*D*). Thus, deletion of Rab3B does not affect basal excitatory synaptic transmission or LTP in the CA1 region of the hippocampus.

Rab3B KO Does Not Change Baseline Inhibitory Synaptic Transmission Significantly. Because our immunohistochemical data showed an enrichment of Rab3B in inhibitory synapses in the CA1 region of the hippocampus, we next tested whether the Rab3B KO affects inhibitory synaptic transmission. Analysis of the amplitude and frequency of miniature inhibitory postsynaptic currents (mIPSCs) in acute brain slices failed to uncover differences between Rab3B WT and KO mice (Fig. 3*A*). We next analyzed the amplitudes and decay kinetics of evoked IPSCs. Input/output curves again revealed no changes in IPSC amplitudes at all stimulus intensities (Fig. 3*B*). Likewise, KO of Rab3B did not affect the IPSC decay time constant (WT: 80 ± 4 ms, 30 cells, seven animals; KO: 79 ± 4 ms, 24 cells, seven animals, Student's unpaired *t* test, $P = 0.95841$) or spontaneous, action potential-driven IPSC activity (WT: 11 ± 1 Hz, 18 cells, seven animals; KO: 10 ± 1 Hz, 19 cells, seven animals, Student's unpaired *t* test, $P = 0.23255$). We then measured short-term synaptic plasticity in inhibitory synapses of Rab3B KO mice but found no differences between littermate Rab3B KO and WT control mice in PPR (Fig. 3*C*) or short-term depression during a 14-Hz stimulus train (Fig. 3*D*). These results indicate Rab3B is not required for baseline transmission and short-term plasticity at inhibitory synapses.

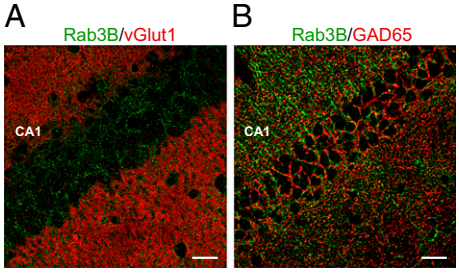


Fig. 1. Localization of Rab3B in the CA1 region of the hippocampus. (*A* and *B*) Merged images of coronal sections of the CA1 region of the hippocampus from WT mice labeled by double immunofluorescence with antibodies to Rab3B (green) and vGluT1 (red) (*A*) or Rab3B (green) and GAD65 (red) (*B*) as markers for excitatory and inhibitory synapses, respectively. (Scale bars: 20 μm.)

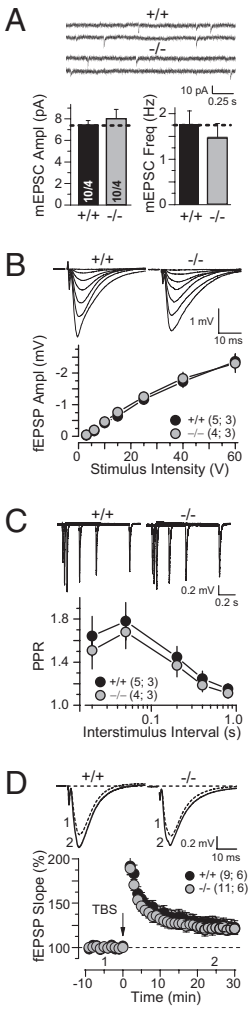


Fig. 2. Excitatory synaptic transmission in Rab3B KO mice. (*A*) Spontaneous mEPSCs monitored by whole-cell recordings in the CA1 region of the hippocampus in the presence of TTX. Example traces are shown on top, and summary graphs of the amplitudes and frequencies are shown on the bottom. (*B*). Input/output curves of synaptic transmission obtained by extracellular field recordings. Representative traces (averaged over 5–10 sweeps) for each stimulus intensity (3, 6, 10, 15, 25, 40, and 60 V) are shown on top, and summary graphs of the amplitudes of evoked fEPSPs plotted as a function of stimulus intensity for littermate WT ($^{+/+}$) and Rab3B KO ($^{-/-}$) mice are shown on the bottom. (*C*) Paired-pulse facilitation. Representative traces (averaged over 5–10 sweeps) for paired-pulse stimulations with interstimulus intervals (ISI) of 20, 50, 200, 400, and 800 ms are shown on top, and summary graphs for paired-pulse ratios (PPR) plotted as a function of the ISI are shown at the bottom. (*D*) LTP at Schaffer collateral–CA1 pyramidal cell synapses. (*Upper*) Representative fEPSP traces for slices from WT ($^{+/+}$) and Rab3B KO ($^{-/-}$) mice at the time points indicated. (*Lower*) The graph depicts relative synaptic strength, measured as the fEPSP slope, before and after LTP was triggered using TBS (arrow). Data shown are mean ± SEM (number of slices or cells and mice are indicated in parentheses).

KO of Rab3B Severely Impairs i-LTD. Because all tested forms of presynaptic plasticity require the Rab3 effector RIM1α, we next

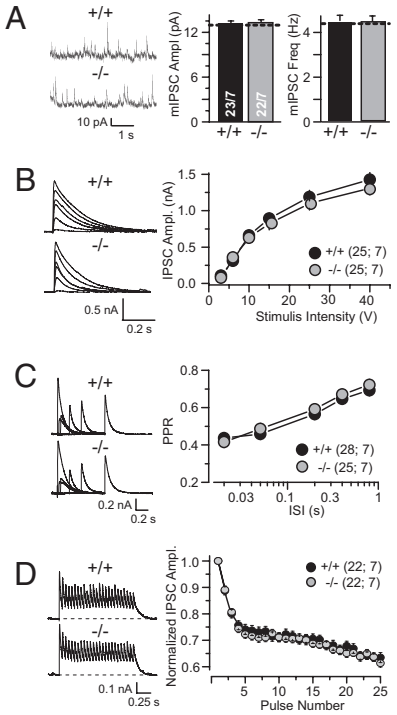


Fig. 3. Inhibitory synaptic transmission. (*A*) Spontaneous mIPSCs recorded in the presence of 1 μM TTX. Example traces are shown on the left, and summary graphs of the amplitudes and frequencies are shown on the right. (*B*) Input/output curves analyzed by whole-cell recordings. Representative traces (averaged over three to six sweeps) for each stimulus intensity (3, 6, 10, 15, 25, and 40 V) are shown on the left, and summary graphs of the IPSC amplitudes plotted as a function of stimulus intensity are shown on the right. (*C*) Paired-pulse depression. Representative traces (averaged over 4–11 sweeps) for paired-pulses at 20-, 50-, 200-, 400-, and 800-ms ISIs are shown on the left, and summary graphs for PPR are shown on the right. (*D*) Use-dependent synaptic depression. Representative IPSCs (averaged over three to five sweeps) evoked by a 14-Hz stimulus train (25 pulses) are shown on the left, and a plot of the IPSC amplitudes (normalized to the first pulse in the train) is shown on the right. Data shown are mean ± SEM (number of cells and mice are indicated in parentheses).

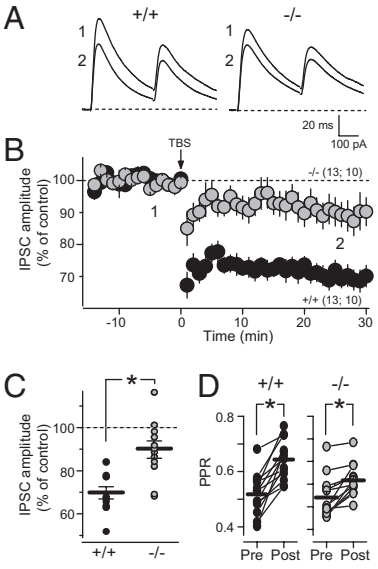


Fig. 4. Impaired i-LTD in Rab3B KO mice. (*A*) Representative IPSC traces taken before (1) and after (2) induction of i-LTD in slices from WT ($^{+/+}$) and Rab3B KO ($^{-/-}$) mice. (*B*) Summary plot of IPSC amplitudes as a function of time, normalized to 15-min baseline. i-LTD was triggered by TBS at time 0 (arrow). Data shown are mean ± SEM (number of cells and mice are indicated in parentheses). The magnitude of i-LTD was reduced significantly in Rab3B KO slices compared with WT (Student's unpaired *t* test, $P = 0.00025$). (*C*) i-LTD magnitude plotted for individual WT and KO experiments (*$P < 0.001$ by Student's unpaired *t* test). (*D*) PPRs, determined using a 100-ms ISI, before (Pre) and after (Post) i-LTD in WT ($^{+/+}$) and Rab3B KO ($^{-/-}$) slices. Consistent with a presynaptic locus of i-LTD expression, the PPR was increased significantly in WT mice (Student's paired *t* test, $P = 0.00004$), with Rab3B KO mice exhibiting a relatively smaller increase ($P = 0.0053$). Each pair of circles and the corresponding connecting line represent a single experiment; group means are indicated also.

examined whether Rab3B affects inhibitory synaptic plasticity. i-LTD is a presynaptic form of inhibitory LTD that depends on endocannabinoids and on the presynaptic active zone protein RIM1α (23). i-LTD was induced readily by theta burst stimulation (TBS) in WT mice but was severely impaired in slices from Rab3B KO mice (Student's unpaired *t* test, $P = 0.00025$; Fig. 4 *A* and *B*). Furthermore, i-LTD induction was associated with an increase in PPR in both WT and Rab3B KO slices, consistent with the presynaptic nature of i-LTD (Fig. 4 *C* and *D*). Paralleling impaired i-LTD in Rab3B KO mice, the relative change in PPR was less in Rab3B KO mice than in controls ($P = 0.0485$; Fig. 4*D*). These findings suggest that Rab3B is required for normal i-LTD in the CA1 region of the hippocampus.

Rab3B KO Mice Exhibit Selective Facilitation of Reversal Learning. Given the selective electrophysiological phenotype of Rab3B KO mice in i-LTD, we performed a battery of behavioral assays to determine whether this synaptic phenotype was associated with behavioral changes in these mice. We first tested baseline locomotion parameters using a force-plate actometer, which, based on the algorithms applied to the data, can measure a variety of attributes of locomotor behaviors reliably (27). No differences in baseline locomotion were found between WT and Rab3B KO littermates in all parameters analyzed (Fig. S2 *A–D*). Motor coordination and motor learning were assessed by means of an accelerating Rota-Rod and were indistinguishable between genotypes (Fig. S2*E*). Rab3B KO mice also displayed normal levels of anxiety, because the amount of time they spent in the center of the force plate arena (Fig. S2*B*) and in the light compartment of a light–dark box was similar to that of WT mice (Fig. S2*F*). Moreover, in a cookie-finding test, Rab3B KO mice exhibited no phenotype (Fig. S2*G*). These data suggest that the Rab3B KO does not cause major impairments in locomotion, motor and sensory functions, or anxiety in mice.

It generally is thought that postsynaptic, NMDA receptor-dependent LTP of synaptic transmission plays a crucial role in hippocampal-dependent learning and memory formation (28–30), but the contribution of presynaptic forms of long-term plasticity to learning and memory remains unclear. We tested littermate WT and Rab3B KO mice in the cue navigation version of the Morris water-maze task (31). During acquisition, we observed no differences between genotypes in how fast the mice learned to reach the hidden platform (genotype effect, $F_{[1,94]} = 2.667$, $P = 0.1058$; Fig. 5*A*). During the probe trial that followed acquisition training, both control and littermate Rab3B KO mice preferentially spent more time in the target quadrant than in the other three quadrants ($P = 0.0009$ for WT mice; $P = 0.0032$ for KO mice; Fig. 5*B*).

Interestingly, however, when the position of the platform was reversed, Rab3B KO mice showed significantly faster escape latencies than their control littermates throughout the trial (genotype effect, $F_{[1,64]} = 4.976$, $P = 0.0292$; Fig. 5*C*). At the end of the reversal trial, both genotypes exhibited similar reversal learning and displayed a strong preference for the new target quadrant ($P = 0.0159$ for WT mice; $P = 0.0008$ for KO mice; Fig. 5*D*). These data suggest that although Rab3B deletion does

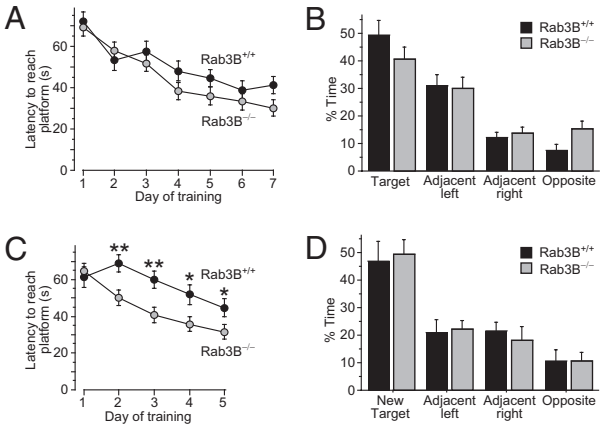


Fig. 5. Facilitation of reversal learning in Rab3B KO mice. (*A*) Latency to reach the hidden platform during the acquisition phase of the Morris water-maze task for WT and Rab3B KO mice. No differences in latency were observed between genotypes. Data shown are means of four trials/d. (*B*) Learning performance at the end of the acquisition phase (probe trial) expressed as percentage of time spent in each of the water-maze quadrants. Both WT and KO mice spent more time in the target quadrant during a single probe trial. (*C*) Latency to reach the platform during the reversal phase of the Morris water-maze task. Values are means of four trials/d. Rab3B-deficient mice showed faster reversal learning than their WT littermates. $*P < 0.05$; $**P < 0.01$. (*D*) Percentage of time spent in each quadrant during a single probe trial following reversal training. Animals of both genotypes spent more time in the new target quadrant, suggesting that they were able to reverse their learning of the platform location. All values are mean ± SEM. (WT, $n = 9$; KO, $n = 13$).

not alter spatial learning capabilities, it seems to facilitate the reversal of spatial learning in mice.

Rab3B Deletion Enhances Extinction of Contextual Fear. To test whether these results could be extended to another hippocampus-dependent learning paradigm, Rab3B KO mice were examined in a contextual fear-conditioning task. It is known that the hippocampus is required for formation and retrieval of context-fear associations (32) as well as for context-dependent encoding of fear extinction (33), which is considered a new learning process. During the initial preexposure to the training context (2 min before the first shock presentation), there were no differences in baseline freezing between Rab3B KO and control males (Fig. 6*A*). Assessment of freezing during reexposure to the training context 24 h after fear conditioning revealed no significant differences between genotypes (Fig. 6*A*). Two stimulus intensities were tested during fear conditioning to assure that the lower stimulus (0.5 mA) does not saturate the mouse's response. All mice then were subjected to repeated extinction trials by placement in the training context 2, 3, and 5 d after conditioning; freezing responses were normalized to those of the first reexposure (24 h after conditioning). During the extinction trials, Rab3B KO mice exhibited a significantly enhanced extinction rate ($F_{[1,23]} = 5.738$, $P = 0.0251$) compared with WT littermates, and this rate was most profound 5 d after initial conditioning (Fig. 6*B*). These results indicate that, in addition to enhanced reversal learning, Rab3B KO mice also exhibit a facilitation of extinction, suggesting that their context-dependent fear memories are more vulnerable to erasure than those of WT mice.

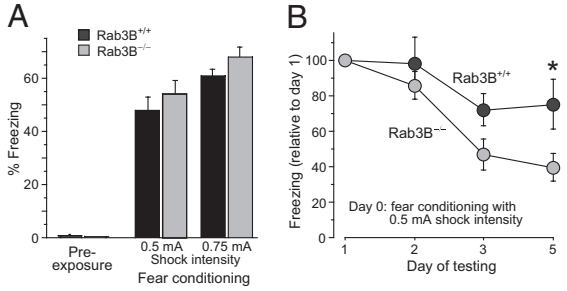
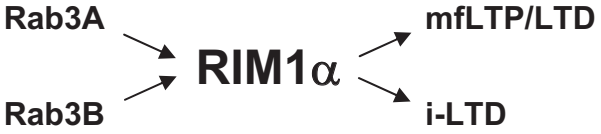


Fig. 6. Enhanced fear extinction in Rab3B KO mice. (*A*) Measurement of contextual fear expressed as percentage of time spent freezing during the 2-min period before shock initiation (preexposure) and 24 h after mice were conditioned with two different shock intensities (0.5 and 0.75 mA). (*B*) Fear extinction expressed as percentage of freezing time relative to that 24 h after conditioning with a 0.5-mA shock (day 1). All values are mean ± SEM. $*P < 0.05$ (WT, $n = 11$; KO, $n = 14$).

Discussion

Rab3 proteins are the most abundant GTP-binding Rab proteins in brain, but their functions have remained poorly defined. The most striking functional requirement for a Rab3 isoform has been described for Rab3A in mossy-fiber LTP and LTD (24, 25). Subsequent studies revealed that the Rab3A effector RIM1α also is essential for different types of mossy-fiber LTP (19–21) and is required for LTP at cerebellar parallel fiber synapses (19) and at cortico–lateral amygdala synapses (10), as well as for late LTP at CA3–CA1 hippocampal synapses (22) and for i-LTD in the hippocampus and the lateral amygdala (23). Overall, these results suggested a presynaptic pathway that mediates long-term plasticity whereby GTP-bound Rab3A activates RIM1α, which in turn causes a change in the efficacy of action potential-triggered neurotransmitter release. We now extend these findings by demonstrating that in inhibitory synapses of the hippocampus capable of undergoing i-LTD, a different Rab3 isoform, Rab3B, is required for long-term presynaptic plasticity. Immunohistochemical analysis of the hippocampal CA1 region showed that Rab3B is localized predominantly in inhibitory synapses. Acute slice recordings from Rab3B KO mice revealed that baseline transmission, presynaptic short-term plasticity, and postsynaptic LTP were normal in these mice, but i-LTD was severely compromised.

These findings suggest a general pathway mediating excitatory or inhibitory presynaptic long-term plasticity, which involves RIM1α binding to distinct Rab3 isoforms in different synapses:



In this scheme, mfLTP/LTD refers to mossy fiber LTP/LTD. Our finding that the Rab3B KO selectively impairs i-LTD raised the question whether this electrophysiological perturbation was associated with a behavioral phenotype. Rab3B KO mice showed normal locomotion, motor coordination, and anxiety-like behaviors in a battery of paradigms. Long-term plasticity in the hippocampus has been correlated extensively with cognition and the process of learning and memory (30). For this reason, we next examined whether Rab3B KO caused a change in a mouse's ability to perform in hippocampal-dependent learning and memory tasks. When tested in the cued version of the Morris water maze, Rab3B KO mice exhibited intact spatial learning indistinguishable from that of WT littermates. During the reversal phase of the water-maze task, however, Rab3B KO mice were more efficient than WT littermate mice in learning the new platform position, suggesting enhanced reversal learning and/or a faster erasure of the previous memory of the platform's location. Consistent with these results, Rab3B KO mice displayed no deficits in associative learning when tested in a hippocampal-dependent contextual fear-conditioning paradigm but exhibited

accelerated extinction of the fear memory compared with WT mice. According to these data, the contribution of Rab3B to i-LTD in the CA1 region of the hippocampus appears to be important for the stability of acquired memories. Although spatial navigation in the water maze and fear extinction are hippocampus-dependent behaviors, we cannot rule out the possibility that Rab3B also is essential for i-LTD in other areas of the brain and that impairments in i-LTD in these areas induced by the Rab3B KO contributes to the behavioral phenotype.

The induction of i-LTD in the hippocampus is mediated by retrograde signaling of endocannabinoids that are released postsynaptically and act presynaptically by binding type 1 cannabinoid receptors (CB1 receptors) (2, 34, 35). The role of endocannabinoid signaling and of i-LTD in learning and memory remains unclear, even though CB1 receptors are among the most abundantly expressed G protein-coupled receptors in the brain. Activation of CB1 receptors triggers multiple downstream events and controls short- and long-term inhibitory synaptic plasticity. During short-term plasticity, brief CB1 receptor activation directly reduces presynaptic voltage-gated Ca^{2+} channel activity, thereby transiently suppressing GABA release (36). On the other hand, long-term plasticity appears to require several minutes of CB1 receptor activation, suggesting that other presynaptic signals need to be integrated for endocannabinoid-dependent forms of long-term plasticity, including i-LTD (23, 37, 38).

One direct downstream target of CB1 receptor activation is adenylyl cyclase, which is inhibited upon CB1 receptor activation, resulting in a reduction in cAMP levels and protein kinase A (PKA) activity (39, 40). A reduction in PKA activity is important for the manifestation of i-LTD (23). Is the Rab3B/RIM1α complex a direct target of PKA? Although RIM1α is required for i-LTD, it is unclear whether RIM1α is activated directly by PKA-dependent phosphorylation (41). Moreover, disruption of spontaneous interneuron activity has been shown to block i-LTD through a pathway that involves the Ca^{2+}-activated phosphatase calcineurin (38). In the present study, however, KO of Rab3B had no effect on spontaneous action potential-driven inhibitory synaptic events, suggesting that the Rab3B KO probably does not impair i-LTD by disrupting spontaneous interneuron activity. Thus, alternative cascades or processes likely cause i-LTD via a Rab3B/RIM1α-dependent pathway.

Many studies have examined the effects of pharmacological and genetic disruptions of endocannabinoid signaling on behaviors. Use of CB1 receptor agonists or antagonists as well as generation of CB1-receptor KO mice has produced a variety of behavioral phenotypes that include large changes in locomotion (42–44), anxiety (45–48), and hippocampus- and amygdala-dependent memory tasks (13, 49–52). Interestingly, one of the major findings from studies of CB1 KO mice was that perturbations in endocannabinoid signaling cause defects in reversal learning and extinction of conditioned fear (13, 50). These results are in dramatic contrast to our present observation: Instead of major general changes, we found no phenotype in locomotion, anxiety, and memory-acquisition behaviors in Rab3B KO mice; instead of a defect, we observed a significant facilitation of reversal learning and fear extinction. CB1-receptor manipulations alter all properties of endocannabinoid-sensitive synapses throughout the brain, not only endocannabinoid-dependent forms of long-term plasticity but also generally neurotransmitter release and short-term synaptic plasticity at many excitatory and inhibitory synapses. Rab3B KO, in contrast, exerts a relatively selective effect, at least in the CA1 region of the hippocampus, on inhibitory long-term plasticity. It is possible that in other brain regions Rab3B KO also alters other forms of long-term synaptic plasticity, but this scenario seems unlikely given the distribution of Rab3B expression in comparison with that of other Rab3 isoforms. The more restricted synaptic changes in Rab3B KO mice may account for its more selective behavioral phenotype. Consistent with this notion, earlier studies on RIM1α KO mice—which suffer from the same changes in long-term plasticity but also exhibit massive changes in neurotransmitter release in all synapses tested (53)—noted more severe behavioral impairments (54).

In addition to providing a further definition of the RIM1α/Rab3 pathway as a critical component of presynaptic long-term plasticity, our data raise many new questions. How can the RIM1α/Rab3 pathway produce both LTP and LTD, depending on the type of synapse examined? Because RIM1α is essential for organizing presynaptic terminals to allow priming of synaptic vesicles and recruitment of Ca^{2+} channels to the active zone (53, 55–57), the involvement of Rab3 in long-term plasticity indicates that the actual switch does not operate by activating Rab3 but instead operates on machinery that contains the Rab3/RIM complex as an essential component. Our data provide a potential link between i-LTD and the modulation of reversal learning but also raise the question of specifically how i-LTD might stabilize memories to dampen reversal learning and whether i-LTD has other roles in learning and memory (e.g., in implicit learning paradigms). Addressing these questions will require development of additional tools to manipulate i-LTD even more specifically and reversibly than is possible with Rab3B KO.

Materials and Methods

Mouse Breeding, Genotyping, and Immunohistochemistry. Mouse breeding, genotyping, and immunohistochemistry were performed using standard procedures (17, 26, 56). All experiments in this paper analyzed littermate WT and Rab3B KO mice on a hybrid C57BL6;129sv background. All animal-handling procedures followed National Institutes of Health guidelines and were approved by Animal Care and Use Committees. Rab3B KO and littermate WT control mice were shipped to the Albert Einstein College of Medicine, and experimenters were blind to genotype.

Electrophysiology. Acute slices were analyzed by field and whole-cell recordings essentially as described (14, 23, 37, 38). Extracellular field potentials were recorded in the CA1 region of the hippocampus by stimulating Schaffer collaterals in stratum radiatum. For whole-cell recordings, CA1 pyramidal cells were voltage-clamped at +10 mV when monitoring IPSCs. NMDA receptors were blocked with 25 μM D-2-amino-5-phosphonovalerate (D-APV), and AMPA receptors and kainate receptors were blocked with10 μM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX). CA1 cells were voltage-clamped at −65 mV when monitoring EPSCs, and $GABA_A$ receptors were blocked with 100 μM picrotoxin. i-LTD and LTP were induced by TBS (10 bursts of five stimuli applied at 100 Hz with 200-ms interburst intervals repeated four times, 5 s apart). For long-term plasticity experiments, the last 5 min of baseline was compared with the last 5 min of recording. Stimulation and acquisition were controlled by custom software. Output signals from whole-cell and field recordings were filtered at 2.4 kHz, acquired at 5 kHz, and stored online in IgorPro (Wavemetrics).

Behavioral Analyses. Behavioral analyses were performed using male littermate mice at 2–4 mo of age. Procedures were approved by Stanford's Administrative Panel on Laboratory Animal Care. For details of the behavioral analysis, see *SI Materials and Methods*.

Force plate actometry. A custom-made force plate actometer (28 cm × 28 cm) was used to measure various parameters of locomotor activity in 30-min recording sessions (27).

Rota-Rod tests. Motor coordination and motor learning were assessed using a Rota-Rod (Med Associates. Inc.) that accelerated slowly from 3 to 30 rotations/min over 5 min. Three trials/d were given to each mouse for 3 d with intertrial intervals of 30–40 min.

Light–dark transition tests. Light–dark transition tests were performed in a transparent, lit, open-topped 42 × 22 × 30 cm chamber connected to an enclosed 28 × 22 × 30 cm black box by a 5 × 5 cm opening.

Morris water-maze experiments. Morris water-maze experiments were performed in an indirectly illuminated room (four 40-W bulbs, 12 lx) with salient cues located on the walls and using a circular water pool (1.7 m in diameter, 60 cm in height) with white nontoxic paint at 24–26 °C.

Contextual fear conditioning and extinction experiments. Contextual fear conditioning and extinction experiments were performed using two conditioning chambers (18 cm wide × 17 cm deep × 21 cm high) (Coulbourn Instruments)

housed in sound-attenuating boxes (70 × 50 × 50 cm). To avoid ceiling effects, extinction was assessed after fear conditioning using the lower shock intensity (0.5 mA). To exclude experimental bias, freezing behavior was scored automatically using Freezeframe software (Coulbourn Instruments), with comparable thresholding criteria for all mice.

Statistical Analyses. All data are shown as mean ± SEM. Electrophysiological data were analyzed by student's *t* test using OriginPro (Origin Lab), and behavioral data were analyzed by ANOVA followed by post hoc comparisons using the Fisher's test in cases of significance ($P < 0.05$) using StatView 5.0 (SAS Institute Inc.).

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