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Fear Learning and Extinction are Linked to Neuronal Plasticity Through Rin1 Signaling

Joanne M. Bliss[1], **Erin E. Gray**[1], **Ajay Dhaka**[2], **Thomas J. O'Dell**[1], and **John Colicelli**[1,*]
[1]David Geffen School of Medicine at UCLA, Los Angeles, California
[2]Department of Biological Structure, University of Washington, Seattle, Washington

Abstract

The amygdala is known to have a crucial role in both the acquisition and extinction of conditioned fear, but the physiological changes and biochemical mechanisms underlying these forms of learning are only partly understood. The Ras effector Rin1 activates Abl tyrosine kinases and Rab5 GTPases and is highly expressed in mature neurons of the telencephalon including the amygdala, where it inhibits the acquisition of fear memories (*Rin1*$^{-/-}$ mice show enhanced learning of conditioned fear). Here we report that *Rin1*$^{-/-}$ mice exhibit profound deficits in both latent inhibition and fear extinction, suggesting a critical role for Rin1 in gating the acquisition and persistence of cue-dependent fear conditioning. Surprisingly, we also find that depotentiation, a proposed cellular mechanism of extinction, is *enhanced* at lateral-basolateral (LA-BLA) amygdaloid synapses in *Rin1*$^{-/-}$ mice. Inhibition of a single Rin1 downstream effector pathway, the Abl tyrosine kinases, led to reduced amygdaloid depotentiation, arguing that proper coordination of Abl and Rab5 pathways is critical for Rin1-mediated effects on plasticity. While demonstrating a correlation between amygdala plasticity and fear learning, our findings argue against models proposing a direct causative relationship between amygdala depotentiation and fear extinction. Taken together, the behavior and physiology of *Rin1*$^{-/-}$ mice provide new insights into the regulation of memory acquisition and maintenance. In addition, *Rin1*$^{-/-}$ mice should prove useful as a model for pathologies marked by enhanced fear acquisition and retention, such as posttraumatic stress disorder.

Keywords

Amygdala; Fear conditioning; Ras signaling pathways; Tyrosine kinase; Plasticity

The Ras effector Rin1 is expressed in mature telencephalic neurons, most notably in the cerebral cortex, hippocampus, striatum, and amygdala. Rin1 protein localizes to neuronal cell bodies and dendrites, with enrichment in postsynaptic densities (Deininger et al., 2008). Initial studies of *Rin1* null mice revealed elevated long-term potentiation (LTP) at lateral amygdala (LA) synapses onto cells in the basolateral amygdala (BLA), as well as enhanced performance in two amygdala-dependent learning tasks: fear conditioning and conditioned taste aversion (Dhaka et al., 2003). These findings suggested that Rin1 normally acts as an inhibitor of plasticity and learning in the amygdala.

In response to activated Ras, Rin1 signals through two downstream effectors: Abl non-receptor tyrosine kinases that control actin cytoskeleton remodeling (Hu et al., 2005) and Rab5 GTPases

* Correspondence to: David Geffen School of Medicine at UCLA, Los Angeles, California. colicelli@mednet.ucla.edu .

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that control receptor endocytosis and trafficking (Tall et al., 2001). Both Rin1 signaling partners are expressed in forebrain neurons (Koleske et al., 1998; Brown et al., 2005) and have been implicated in plasticity. Abl1 and Abl2, close paralogs that are each directly activated by Rin1 (Cao et al., 2008), localize to neuronal synapses (Moresco et al., 2003) and contribute to short-term synaptic plasticity in the hippocampus. Rin1 may function to modulate the influence of Abl kinases on cytoskeletal remodeling of post-synaptic dendritic spines, a process necessary for synaptic plasticity. In addition, Rin1 contains guanine nucleotide exchange factor (GEF) activity for Rab5, promoting Rab5-mediated endocytosis of cell-surface receptors. Indeed, Rab5 participates in the endocytosis of AMPA receptors during multiple forms of long-term depression (LTD) (Huang et al., 2004; Brown et al., 2005) and Rin1 mediates the endocytosis of Eph4A in amygdala neurons (Deininger et al., 2008). We hypothesize that, following Ras activation, Rin1 coordinates the stimulation of Abl and Rab5 signaling pathways. By integrating actin remodeling with receptor endocytosis and trafficking at the postsynaptic membrane, Rin1 is ideally situated to regulate the mechanisms underlying acquisition, maintenance and/or expression of activitydependent forms of synaptic plasticity in telencephalic neurons.

Here, we show that mice lacking *Rin1* have substantial deficits in fear extinction and latent inhibition, demonstrating that Rin1 is a critical negative regulator of conditioned fear learning. In addition, acute slices from *Rin1*$^{-/-}$ mice showed enhanced depotentiation in LA-BLA synapses. This is consistent with Rin1 normally functioning to dampen synaptic plasticity. Importantly, our results do not support a direct correlation between LA-BLA depotentiation and fear extinction. These data provide insight into the synaptic mechanisms underlying control of conditioned fear, while also validating *Rin1*$^{-/-}$ mice as a potential model for disease states, such as posttraumatic stress disorder, characterized by enhanced retention and recall of fear memories.

MATERIALS AND METHODS

Animals

Rin1$^{-/-}$ mice, in which exons 2-7 of the *Rin1* gene are deleted (Dhaka et al., 2003), were fully backcrossed onto the C57Bl/6 genetic background. Mice were housed in groups of 2-5 with a 12 hr light/dark cycle, and all experiments were conducted during the light period. Behavioral experiments used 3–8 month old 1/1 and −/− littermates (male and female) and were conducted blind to genotype. Electrophysiological experiments used primarily male wild type and *Rin1*$^{-/-}$ mice that were 3-7 months in age. All behavioral experiments were performed in the UCLA Behavioral Testing Core Facility (Dept. of Psychology, UCLA) and were conducted with the approval of the University of California, Los Angeles, Animal Research Committee.

Fear Conditioning Assays

Fear conditioning experiments were run in conditioning chambers (Med Associates, St. Albans, VT) with tones (2.8 kHz, 80–85 dB) and foot shocks (0.75 mA, 2s) controlled by a modular Med Associates system. Tones were produced by a speaker in the conditioning chamber, and foot shocks were delivered through a stainless-steel grid floor. A HEPA air-filtering unit provided both ventilation and background noise (55 dB). Animal movement was recorded with a video camera and was automatically analyzed for freezing using NIH image (Anagnostaras et al., 2000).

Fear extinction consisted of 3 phases (acquisition, extinction, and test) that were spaced 24 hr apart. Acquisition was performed in type A chambers that were square in shape, had a metal grid floor, and were wiped down with diluted acetic acid. Extinction and testing were performed in type C chambers that were hemispherical in shape, had a plastic floor, and were wiped down

with a mint-smelling disinfectant. Each conditioning protocol began 5 min after mice were placed in the conditioning chamber. The individual protocols were as follows: Acquisition − 10 ×[30 sec tone co-terminating with a 2 sec foot shock, 1 min inter-trial interval (ITI)]; Extinction − 30 ×[30 sec tone, 1 min ITI]; Test − 3 ×[30 sec tone, 1 min ITI]. In order to evaluate extinction and latent inhibition, a stronger than usual acquisition protocol (10 tone-shock pairings) was used to maximize fear levels and overcome the enhanced fear seen in *Rin1*$^{-/-}$ mice after weaker training protocols (Dhaka et al., 2003).

Latent inhibition also consisted of 3 phases (pre-exposure, acquisition, and test) that were separated by 24 hr; pre-exposure and testing were in type C chambers and acquisition was in type A chambers. The pre-exposure protocol entailed 20 presentations of a 30 sec tone, 1 min ITI, while acquisition and testing were identical to those for fear extinction. Each protocol began 5 min after the mice were placed in the conditioning chamber. For both fear extinction and latent inhibition, pre-CS freezing was measured during the 5 min before the first test tone, and CS freezing was calculated as the average of time spent freezing during the 3 test tones.

Prepulse Inhibition

Prepulse inhibition was tested in ventilated, sound-attenuating startle chambers (SR-LAB system, San Diego Instruments, San Diego, CA) with an interior speaker that provided both broadband frequency background noise (65 dB) and startle stimuli. To measure the amount of startle, mice were restrained in an acrylic cylinder (3.7 cm in diameter) that rested on a piezoelectric accelerometer. Each mouse was tested in one session of about 35 min in duration. Each session consisted of a 5-min acclimation period, habituation to a 120-dB startle noise, a test of startle intensities to different decibel pulses (Block 1), and a test of prepulse inhibition of startle (Block 2). Block 1 measured the amount of startle to pulses of 90 dB, 105 dB, and 120 dB (6 trials each). Block 2 measured startle to a pulse of 105 dB alone (4 trials) or proceeded by a prepulse of 71 dB (5 trials), and startle to a pulse of 120 dB alone (8 trials) or proceeded by prepulses of 68 dB, 71 dB, or 77 dB (5 trials each). The prepulse duration was 20 ms and the pulse duration was 40 ms. The prepulse and pulse were separated by 100 ms. Within each block, trials were presented in a pseudorandom order and were separated by intertrial intervals that varied from 3–60 sec but most often fell between 5–11 sec. Prepulse inhibition was calculated as [1- (startle to prepulse with pulse/startle to pulse alone)].

Novel Object/Odor Assay

The novel object/odor assay was performed in a white opaque acrylic box measuring 56 cm long ×33 cm wide ×18 cm deep. The box was placed in a well-lit, ventilated room. The objects used were plastic wrapped votive candles of different scents (lemon, cinnamon, fresh linen) under a circular wire frame (~10 cm tall ×7 cm in diameter). After placing a mouse in the box, the experimenter left the room and monitored behavior via a video camera mounted above the box. Following 1 hour of acclimation to an empty box, two objects (lemon and cinnamon) were placed in the center of the box, approximately 20 cm apart, and the mouse was allowed to explore the objects for 15 min. Immediately afterward, oneobject (alternating between lemon and cinnamon) was replaced with a novel object (fresh linen), and the mouse was allowed to explore for 10 min. Exploration (time with object) was defined as contact between the object and the nose or front paws of the mouse. The box and the wire frames were disinfected and deodorized with 5% bleach in between mice. There was no apparent preference for any object/odor used. We noted that unscented objects differing in size, shape, color and texture failed to elicit sufficient sustained investigation by subject mice for use in this assay (such objects work well with rat subjects).

Spontaneous Alternation

Spontaneous alternation was performed in a dimly lit, well-ventilated room on a T-shaped maze made of white opaque acrylic. Each arm of the maze was 71 cm long with walls that were 20 cm tall. The three arms intersected in a hemispherical center area (36 cm in diameter) with one arm (start arm) forming the bottom of the "T" and two other arms (goal arms) forming the cross of the "T". Each arm could be blocked with a transparent acrylic door that was raised and lowered from behind a concealing curtain. There were no rewards associated with arm choice. Behavior was monitored from behind the curtain via a video camera mounted above the maze. Each mouse was tested in 14 consecutive choice trials, which each began with confinement in the start arm for 5 sec, followed by opening of all arm doors. Once a mouse had entered a goal arm, the door to the other goal arm was lowered, and the mouse was allowed to return to the start arm, completing the trial. Percent alternation was calculated as the percent of trials (out of 14) where the mouse alternated its choice of goal arms compared to the previous trial.

Electrophysiology

Acute coronal brain slices (400 μm thick) were prepared using standard techniques from halothane-anesthetized mice. Slices were maintained and recordings performed at 30°C in interface-type chambers (Fine Science Tools, Foster City, CA) with continuous perfusion of oxygenated (95% O_2, 5% CO_2) artificial cerebral spinal fluid (ACSF) at a flow rate of 2–3 mL/min. ACSF was prepared fresh each day and contained 124 mM NaCl, 4.4 mM KCl, 25 mM $NaHCO_3$, 1.0 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, and 10 mM glucose. After ≥ 1.5 hr of recovery, slices were transferred to a recording chamber. Slices were stimulated with a bipolar stimulating electrode made from Formvar-insulated 38 μm diameter nichrome wire (A-M Systems Inc., Carlsborg, WA) and extracellular responses were recorded with low-resistance (5-10 MΩ) glass microelectrodes filled with ACSF. For experiments examining Schaffer collateral plasticity, both electrodes were placed in the stratum radiatum of the CA1 region to record CA1 field excitatory postsynaptic potentials (fEPSPs) from activation of CA3 axonal fibers. For amygdaloid plasticity, basolateral population responses (fPSPs) were recorded after stimulation of the lateral amygdala (LA-BLA). Stimulation intensity was set to a level that evoked 50% of the maximal response and presynaptic pulses were given once every 20 sec (amygdala experiments) or 50 sec (hippocampal experiments).

LTP was induced with 2 ×100 Hz (2 trains of 100 Hz pulses; 1 sec train duration; 10 sec inter-train interval (ITI)), 5 ×100 Hz (5 trains of 100 Hz pulses; 1 sec train duration; 3 min ITI), or 3 trains of theta-burst stimulation (TBS) [each train of TBS consisted of 5 × 100Hz (5 trains of 100 Hz pulses; 50 ms train duration; 200 ms ITI), with an ITI of 20 sec]. LTD or depotentiation was induced with 15 min of 1 Hz stimulation (900 pulses). For experiments using imatinib (STI571 or Gleevec), recordings were performed from sub-merged slices, and slices were perfused with ACSF containing 5 μm imatinib for at least 60 min prior to induction of LTP. Stock preparation of STI571 (Novartis) has been previously described (Hu et al., 2005).

Data Analysis

Results are presented as mean values ± the standard error. Two-way ANOVA (analysis of variance) was used to analyze differences between genotype (WT vs. *Rin1*$^{-/-}$) and group (control vs. extinction/pre-exposed) in fear extinction and latent inhibition. For all other experiments, unpaired, two-tailed *t* tests were used to compare WT and *Rin1*$^{-/-}$ for a given measurement. Differences were considered significant for p values less than 0.05.

For electrophysiological experiments, the average response during the last 10 min of recording was compared across WT and *Rin1*$^{-/-}$ slices, with the average response values presented as

the mean ± the standard error. Statistical significance was assessed with unpaired, two-tailed *t* tests, and p values less than 0.05 were considered significant.

RESULTS

Rin1$^{-/-}$ Mice are Defective in Conditioned Fear Extinction

Mice lacking Rin1 show enhanced acquisition of short-term and long-term cued fear memories (Dhaka et al., 2003). To test whether the biochemical changes underlying this increased associative memory formation occurs through a mechanism that also controls learned disassociation, we performed fear extinction experiments. Wild type and *Rin1*$^{-/-}$ mice were first trained with multiple pairings of a tone conditioned stimulus (CS) and a mild foot shock unconditioned stimulus (US). We employed training conditions that resulted in strong and equivalent fear conditioning in both genotypes. Half of each group was then exposed to an extinction protocol with repeated presentations of the CS alone. Twenty-four hr later the mice were examined for their level of CS-associated fear, as assessed by freezing during tone presentation (Figure 1A). Both *Rin1*$^{-/-}$ and wild-type (WT) mice showed low amounts of pre-CS freezing, a measurement of baseline fear. During presentation of the tone (CS), wild-type mice that had undergone extinction training froze significantly less than their corresponding controls (Fig. 1B, WT ctrl. 57.6 ± 5.8%, WT extn. 32.5 ± 6.7%, $p < 0.05$). In contrast, *Rin1*$^{-/-}$ mice displayed similar levels of freezing between control and extinction groups (Fig. 1C, *Rin1*$^{-/-}$ ctrl. 45.4±4.5%, *Rin1*$^{-/-}$ extn. 43.7±6.0%), indicating a substantial deficit in extinction (ANOVA $p < 0.05$, $F = 4.08$).

Rin1$^{-/-}$ Mice Show Reduced Latent Inhibition

We next asked whether acquisition of cued fear memories by *Rin1*$^{-/-}$ mice could be influenced by pre-exposure to tone (CS). This training paradigm (Fig. 2A) is referred to as latent inhibition (LI) because prior exposure to CS alone inhibits learning during subsequent training trials in which the CS is paired with a US. This was the case for wild-type mice in our experiments (Fig. 2B). Pre-exposure to tone decreased the effectiveness of fear conditioning, as measured by freezing behavior (WT non-pre-exposed 82.1 ± 3.1%, WT pre-exposed 63.2 ± 6.2%, $p < 0.05$), consistent with inhibited formation of a CS-US association. In direct contrast to the result from wild-type mice, *Rin1*$^{-/-}$ mice showed no inhibition of freezing in the pre-exposed group (*Rin1*$^{-/-}$ non-pre-exposed 71.6 ± 6.1%, *Rin1*$^{-/-}$ pre-exposed 78.9 ± 6.0%) (Fig. 2C). Both sets of *Rin1*$^{-/-}$ mice froze to the CS at levels comparable to control wild-type mice and above the pre-exposed wild-type mice (ANOVA $p < 0.05$, $F = 5.72$). The measured pre-CS freezing in control *Rin1*$^{-/-}$ mice appeared lower than in the pre-exposed *Rin1*$^{-/-}$ mice, although this difference in baseline freezing failed to reach significance and was not seen in other fear conditioning experiments performed with *Rin1*$^{-/-}$ mice. We cannot, however, rule out a more complex effect of the *Rin1*$^{-/-}$ mutation in this particular training protocol.

Attentional Processes are Normal in *Rin1*$^{-/-}$ Mice

The deficits of *Rin1* null mice in fear extinction and latent inhibition reveal that these mice lack a key mechanism for appropriate negative regulation of fear associations. While fear extinction and latent inhibition both pair a classical acquisition phase with a separate interference training phase, some have argued that the two assays inhibit fear via different mechanisms. Fear extinction is generally accepted to result from overriding the original fear-based association by learning a new “CS - no US” association (reviewed in Bouton, 2004). It has been postulated that during latent inhibition mice form a “CS-nothing” association that inhibits the acquisition or expression of a subsequent association between the same CS and a US. An alternative theory posits that latent inhibition works through attentional processes (Lubow, 2005). In this model, pre-exposure familiarizes the mice with the CS to a degree that they give it less attention during fear training, resulting in weaker CS-US associations. If a

deficit in attentional processing underlies the diminished latent inhibition seen in *Rin1*$^{-/-}$ mice, these mice would also be expected to show a phenotype in behavioral assays of attention.

Prepulse inhibition, also known as inhibition of startle, is an attentional assay commonly linked to latent inhibition, and deficits in both are observed in schizophrenics (reviewed in Meyer et al., 2005). Prepulse inhibition assesses the diminished startle response that results when a loud noise (the pulse) is immediately preceded by a less intense noise (the prepulse). Inhibition of startle response is an indication of innate attentional processing. Wild type and *Rin1*$^{-/-}$ mice were tested for their levels of startle to a pulse of 105 or 120 decibels (dB) alone or preceded by prepulses of 68, 71, or 77 dB. Both strains of mice showed similar levels of startle inhibition (Fig. 3). For prepulses of 71 or 77 dB before a pulse of 120 dB, *Rin1*$^{-/-}$ mice showed slightly less inhibition but the differences were not significant, suggesting that Rin1 does not regulate innate attention processes.

To confirm that the *Rin1*$^{-/-}$ mice were unaltered in attention, we used two other assays. Spontaneous alternation evaluates the animal's systematic exploration of its environment and is a model for working memory (Maurice et al., 1994; Ukai et al., 1998) and aspects of attention (Wall et al., 2003; Mishima et al., 2004). The recognition of novel objects (or odors) test similarly assesses a mixture of working memory and attention, as well as perception and motivation (Carey et al., 2009; Kim et al., 2009). In both of these assays, the performance of *Rin1*$^{-/-}$ mice was indistinguishable from that of wild-type mice (data not shown), leading to the conclusion that Rin1 does not have a significant role in working memory or attentional processing. Instead, our data suggest that reduced latent inhibition in *Rin1*$^{-/-}$ mice reflects a deficit in an inhibitory pathway normally invoked by CS pre-exposure.

Rin1$^{-/-}$ Mice Have Enhanced Amygdaloid Depotentiation but Normal Long-term Depression

The amygdala is the central location for fear-based learning and is directly implicated in fear extinction (reviewed in Sigurdsson et al., 2007). In addition, Rin1 is normally expressed in mature neurons of this and other forebrain structures (Dhaka et al., 2003; Deininger et al., 2008). Further, *Rin1*$^{-/-}$ mice show elevated amygdaloid long-term potentiation (LTP), which likely contributes to their enhanced fear conditioning pheno-type (Dhaka et al., 2003). We therefore examined *Rin1*$^{-/-}$ mice for changes in amygdaloid depotentiation, which has been proposed as a mechanism for fear extinction. In control experiments, we first examined the induction of LTP at LA to BLA synapses in slices from wild type and *Rin1*$^{-/-}$ mice using a strong high frequency stimulation (HFS) protocol consisting of 5 trains of 100 Hz stimulation (1 sec in duration) delivered with an inter-train interval of 3 min. As shown in Figure 4A, this pattern of synaptic stimulation induced robust LTP with no significant difference between slices from wild type and *Rin1*$^{-/-}$ mice (PSPs were potentiated to 134 ± 6% of baseline in wild type slices, and potentiated to 126 ± 5% of baseline in slices from *Rin1*$^{-/-}$ mice). The previously reported LTP enhancement in *Rin1*$^{-/-}$ mice was not observed in these experiments due, in part, to a modified induction protocol intended to maximize LTP levels.

Another difference between the studies is the genetic background of the mice. The previous study used C57Bl × 129 F2 crosses while the present study employed >98% C57Bl/6 backcrossed animals. The C57Bl/6 background *Rin1*$^{-/-}$ mice still show a statistically significant enhancement in amygdala LTP (Deininger et al., 2008), but the difference from wild type is less than the original C57Bl × 129 F2 animals (Dhaka et al., 2003) or F2 crosses newly generated from backcrossed C57Bl/6 *Rin1*$^{-/-}$ and wild type 129/SvJ (Supp. Info. Fig. 1). These observations suggest the presence of a modifier locus in the 129/SvJ background, but do not otherwise alter conclusions from the present analysis. 129/SvJ strain loci that modify electrophysiological and behavioral measurements have been suggested before (Nguyen et al., 2000; Rodgers et al., 2002; Schimanski et al., 2007) but have rarely been isolated. A late onset elevation in LTP was noted in the *Rin1*$^{-/-}$ C57Bl × 129 F2 animals (Fig. 1B). The basis for

this is not clear, although weaker late onset effects have been seen in TBS-induced LTP done by ourselves (unpublished) and others (Frey et al., 2009).

To investigate whether Rin1 has a role in depotentiation, we examined the effects of low-frequency stimulation (LFS) delivered 20 min after the last train of HFS used to induce LTP in slices from wild type and *Rin1*$^{-/-}$ mutant mice. As shown in Figure 4B and 4C, LFS induced little, if any, depotentiation in slices from wild-type mice (responses elicited 90 min post-HFS were still potentiated to 126 ± 5% of baseline, n = 8 slices from 4 mice) and PSPs were still significantly potentiated ($p < 0.05$, paired t-test comparison to pre-HFS baseline). Surprisingly, although LFS had little effect on LTP in slices from wild type animals, it induced strong depotentiation in slices from Rin1 mutants (responses evoked 90 min. post-HFS were 104 ± 9% of baseline, n = 7 slices from 4 mice, $p < 0.05$ compared to wild-type littermates) and evoked responses were not potentiated significantly compared to pre-HFS baseline. Thus, depotentiation at LA-BLA synapses is strongly *enhanced* in *Rin1*$^{-/-}$ mutants, even though the mutant exhibited *deficits* in extinction.

The LFS protocol used in our experiments can not only induce depotentiation at potentiated synapses but can also induce LTD of basal synaptic transmission. Therefore, we next examined the effect of LFS on basal synaptic transmission at LA to BLA synapses in slices from *Rin1*$^{-/-}$ and wild-type mice. WT and *Rin1*$^{-/-}$ slices showed initial depression of synaptic responses, but both groups returned to baseline by 75 min post-LFS (PSPs in slices from wild-type mice were 105 ± 7% of baseline, n = 18 slices from 7 mice and were 92 ± 4% of baseline in slices from *Rin1*$^{-/-}$ mice, n = 20 slices from 7 mice; Figure 4D). These results suggest that while Rin1 is required for negative regulation of amygdaloid depotentiation of LTP, it is not a negative regulator of LTD induction at LA to BLA synapses. The data also support the hypothesis that LTD and depotentiation are separate processes occurring through distinct mechanisms.

Inhibition of Abl Tyrosine Kinases Impairs Amygdaloid Depotentiation

We next examined the role of Abl tyrosine kinases, well-established Rin1 signaling transduction effectors (Hu et al., 2005; Cao et al., 2008), in depotentiation. Abl kinases are expressed in forebrain neurons (Koleske et al., 1998; Moresco et al., 2003), and forebrain tissue from *Rin1*$^{-/-}$ animals shows reduced levels of tyrosine phosphorylated Crkl, an Abl substrate (Hu et al., 2005). Thus, to explore the possible role of Abl kinases in depotentiation we examined the effects of the Abl inhibitor imatinib mesylate (a.k.a. STI571 or Gleevec) on depotentiation in slices from wild-type mice. Consistent with Abl tyrosine kinase inhibition, immunoblot analysis of extracts from imatinib-treated forebrain slices showed that the level of tyrosine phosphorylated Crkl was more than 2-fold lower compared to extracts from vehicle treated slices (Fig. 5A). As shown in Figure 5B, inhibiting Abl kinases with imatinib had no detectable effect on HFS-induced LTP at LA-BLA synapses (90 min post-HFS PSPs were potentiated to 127 ± 3% of baseline in control slices, n = 5, and were potentiated 118 ± 7% of baseline in imatinib-treated slices, n = 6). To examine the effect of inhibiting Abl on depotentiation we next compared the effects of LFS delivered after HFS on LTP in untreated and imatinib-treated slices. Because our results with LFS delivered 20 min post-HFS showed that LFS delivered at this time point post-HFS induces little depotentiation in slices from wild-type mice (Fig. 4B), we delivered LFS at an earlier time point post-HFS (15 min) in these experiments to facilitate the induction of depotentiation (Fujii et al., 1991; O'Dell and Kandel 1994; Huang et al., 1999). As shown in Figure 5C, although LFS delivered 15 min post-HFS induced strong depotentiation in untreated control slices (PSPs were 96 ± 3% of baseline, n = 7), significantly less depotentiation was observed in imatinib-treated slices (PSPs were 118 ± 12% of baseline, n = 4, $p < 0.05$ compared to control). While keeping in mind that imatinb has some cross-kinase inhibitory effects (Heinrich et al., 2000), these results suggested that Abl

tyrosine kinases might play a positive role in amygdaloid depotentiation and perhaps fear extinction. The decreased amygdala depotentiation following inhibition of Abl activity appeared to conflict, however, with the increased amygdala depotentiation associated with a *Rin1* gene disruption that reduces Abl kinase activity. Therefore, while the net impact of Rin1 signal transduction is to inhibit amygdaloid depotentiation, this appears to be either independent of Abl kinase or to strictly require Abl activation in the context of other Rin1 downstream pathways.

Hippocampal Depotentiation Remains Normal in *Rin1*$^{-/-}$ Mice

Although Rin1 is expressed throughout the telencephalic region of the mature forebrain, including the hippocampus, *Rin1*$^{-/-}$ mice show no change in hippocampal LTP (Schaffer collateral) or in the acquisition of hippocampal-dependent forms of learning (Morris water maze) (Dhaka et al., 2003). Our data showing elevated depotentiation in the amygdala of *Rin1*$^{-/-}$ mice prompted us to re-examine synaptic plasticity in the CA1 region of the hippocampus of these mutant animals. As shown in Figure 6A, HFS-induced LTP was not altered in slices from *Rin1*$^{-/-}$ mice (60 min post-HFS fEPSPs were potentiated to 157 ± 6% of baseline in slices from *Rin1*$^{-/-}$ mice, n = 8 slices from 8 mice, compared to 153 ± 4% of baseline in slices from wild-type mice, n = 8 slices from 7 mice). Similarly, both genotypes showed comparable levels of LFS-induced depotentiation (Fig. 6B) and LTD (Fig. 6C). Taken with our previous results (Dhaka et al., 2003), we conclude that NMDA receptor-dependent forms of synaptic plasticity at excitatory synapses onto hippocampal CA1 pyramidal cells are normal in *Rin1*$^{-/-}$ mice. This finding adds to the evidence of unique characteristics in amygdala and hippocampus LTP (Chapman et al., 2003; Huang and Kandel 2007a, 2007b).

DISCUSSION

The formation and maintenance of associative fear memories is the subject of intense investigation using behavior, electrophysiology and biochemical techniques. Indeed, much has been learned using these experimental approaches, although a unified working model has remained elusive. The unique phenotype of *Rin1*$^{-/-}$ mutant mice (enhanced fear conditioning with reduced fear extinction and deficient latent inhibition) and relatively well-defined signaling properties of the Rin1 protein (activation of Abl kinases and Rab5 GTPases) offer a useful tool to address this problem.

The fear extinction deficit of *Rin1*$^{-/-}$ mice was accompanied by enhanced LA-BLA depotentiation, and is the first example of a specific genetic alteration connecting decreased fear extinction with enhanced amygdala plasticity. The results, together with previous characterization of this mutant, strongly implicate Rin1 as a negative regulator of fear learning and a required component for experience-mediated extinction. At the same time, our data argue against a direct correlation between fear extinction and depotentiation. There are, of course, important caveats to this conclusion. As with other comparisons between behavioral and electrophysiological data, the time course of the extinction training (several days) is quite a bit longer than the time course of the depotentiation protocol (about an hour) and may therefore be measuring distinct processes. In addition, it remains possible that other amygdaloid circuits in the *Rin1*$^{-/-}$ mutant might show an opposite effect (i.e. reduced depotentiation) to that reported here.

One of the circuits most strongly implicated in fear extinction is that connecting the infralimbic region of the prefrontal cortex (PFC) and the lateral amygdala, perhaps through intermediary “intercalated” neurons (reviewed in Quirk and Mueller, 2008; Sotres-Bayon et al., 2008). Rin1 is expressed in mature neurons throughout the telencephalon, but its contribution to extinction through PFC neurons is difficult to predict. Rin1 may be restricted to a subset of PFC neurons (unpublished data) and distinct PFC circuits appear to have opposing effects on extinction

(Vidal-Gonzalez et al., 2006). Thalamic to lateral amygdala (T-LA) circuits may also contribute to fear conditioning and extinction (Kim et al., 2007). Future studies using *Rin1*$^{-/-}$ mutant animals should help define more clearly an electrophysiological signature of fear extinction.

The best-characterized downstream signaling pathways from Rin1 are activation of Abl tyrosine kinases that regulate actin remodeling (Hu et al., 2005; Cao et al., 2008) and activation of Rab5 GTPases to promote receptor endocytosis (Tall et al., 2001; Barbieri et al., 2003; Hu et al., 2008). Our results suggest that Abl kinase activation alone cannot account for the contribution of Rin1 to LA-BLA plasticity. Indeed, inhibition of Abl kinase activity reduced depotentiation levels while a *Rin1*$^{-/-}$ mutation enhanced them. Several caveats apply to this interpretation, however. Importantly, the Abl inhibitor, imatinib, is known to cross-inhibit a few other tyrosine kinases including Kit and Pdgfr (Heinrich et al., 2000). In addition, this compound has been shown to reduce hippocampal paired pulse facilitation (Moresco et al., 2003), an indicator of presynaptic function. It seems likely that the contribution of Rin1, which localizes to the postsynaptic density, requires the coordinated activation of Abl tyrosine kinases and Rab5 GTPases. This could occur through the directed trafficking of endosomes, which may require actin remodeling in the wake of receptor internalization. We are currently investigating the possibility that RIN1-mediated effects on plasticity are NMDA or AMPA receptor dependent.

Our results provide new evidence that Rin1 normally functions as a negative regulator of fear learning and memory (*Rin1*$^{-/-}$ mice have enhanced fear conditioning and diminished fear extinction). Rin1 also appears to reduce the contribution of established memories to new fear conditioning (*Rin1*$^{-/-}$ mice have reduced latent inhibition). Importantly, the *Rin1*$^{-/-}$ mutant mice show no deficits in baseline behaviors, including assays of attention to environmental cues. Together, these characteristics should make the *Rin1*$^{-/-}$ mutant a useful model for studying aspects of neuropsychiatric conditions, such as post-traumatic stress disorder (PTSD), that are rooted in enhanced formation and/or retention of fearful memories. In particular, a better understanding of Rin1 signaling could provide much needed insights on individual variation in PTSD susceptibility and exposure therapy responsiveness.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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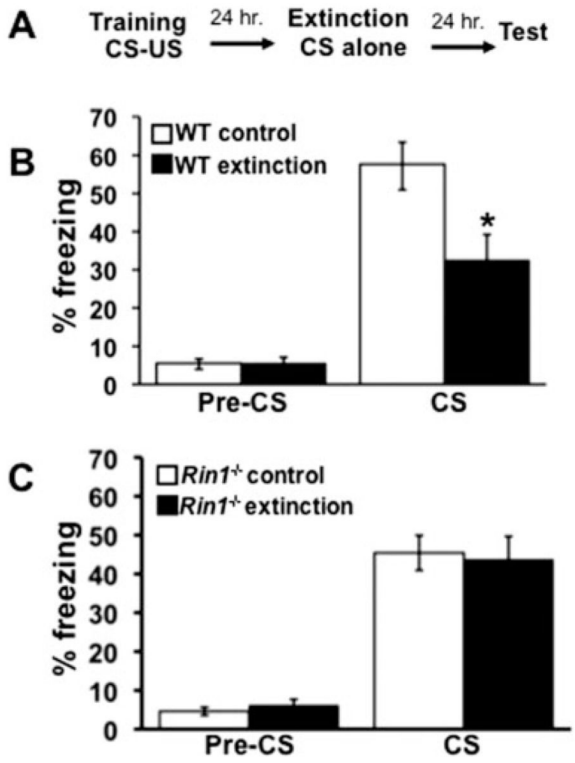

Fig. 1.

Fear Extinction is reduced in *Rin1*$^{-/-}$ mice. **A**. Outline of extinction protocol used. **B**. WT mice that have undergone extinction freeze less than controls during a tone test (WT ctrl., n = 17 mice; WT extn., n = 17 mice) *$p < 0.05$. **C**. *Rin1*$^{-/-}$ mice show no extinction with equal freezing in control and extinguished mice (*Rin1*$^{-/-}$ ctrl., n = 17 mice; *Rin1*$^{-/-}$ extn., n = 17 mice). PRE-CS = baseline freezing before CS presentation; CS = freezing during CS presentation.

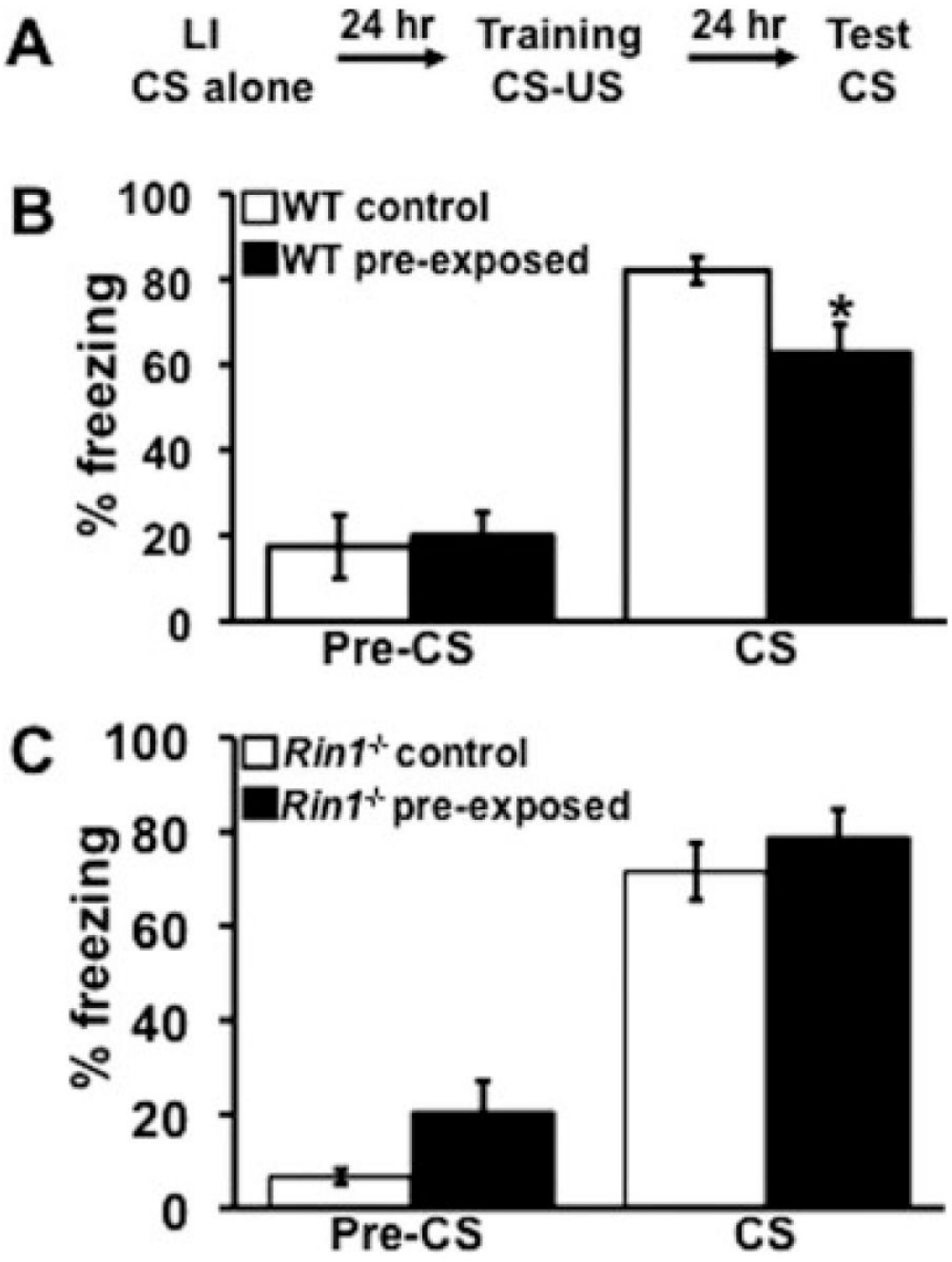

Fig. 2.

Rin1$^{-/-}$ mice are deficient in latent inhibition. **A**. Outline of latent inhibition protocol used. **B**. WT mice show latent inhibition of fear, with less freezing in preexposed (prx) mice compared to non-preexposed controls (WT non-prx, n = 10 mice; WT prx, n = 10 mice) *$p < 0.05$. **C**. *Rin1*$^{-/-}$ mice display little to no latent inhibition (*Rin1*$^{-/-}$ non-prx, n = 10 mice; *Rin1*$^{-/-}$ prx, n = 10 mice). PRE-CS = baseline freezing before CS presentation; CS = freezing during CS presentation; LI = latent inhibition.

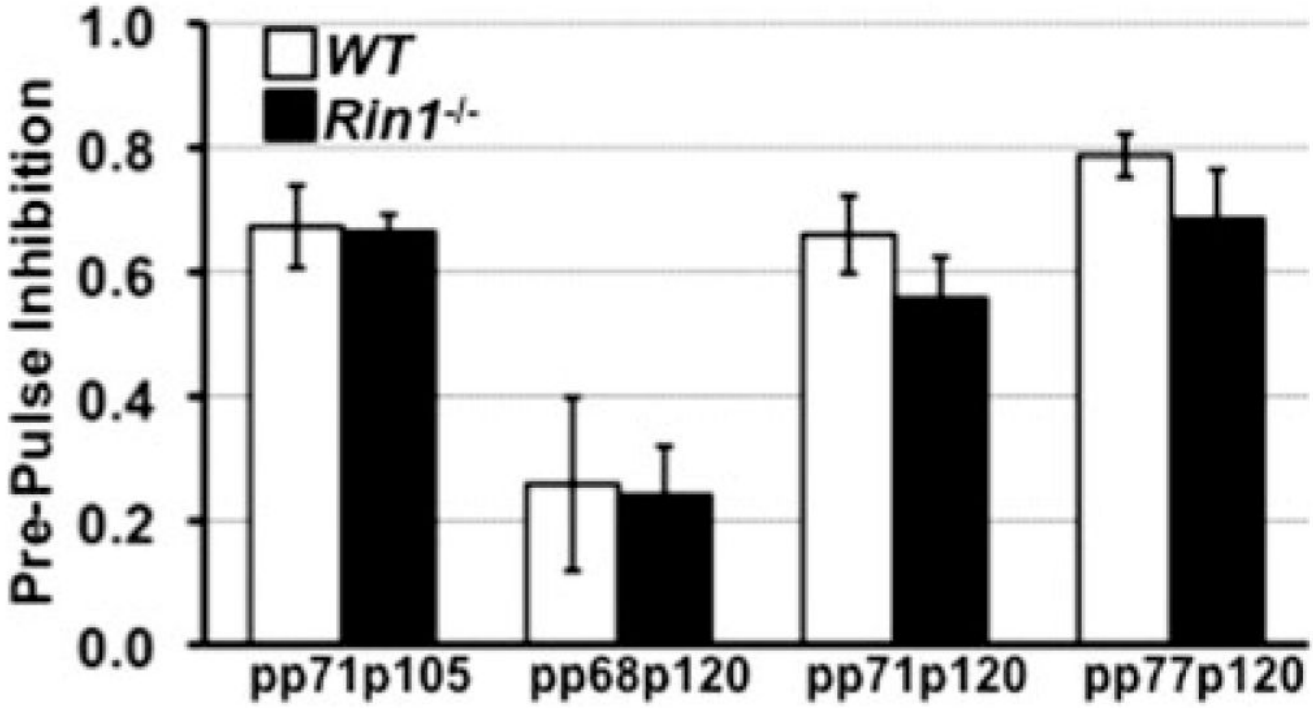

Fig. 3.

Rin1$^{-/-}$ mice show normal levels of prepulse inhibition. Levels of startle were measured for pulses (p) of 105 or 120 dB with or without prepulses (pp) of 68, 71, or 77 dB. Prepulse inhibition (PPI) is calculated as [1-(startle to prepulse with pulse/startle to pulse alone)], i.e. a decrease in startle after the prepulse translates to greater PPI. *Rin1*$^{-/-}$ mice were not significantly altered from WT mice at any of the prepulse or pulse levels. (WT, n = 13 mice; *Rin1*$^{-/-}$, n = 18 mice).

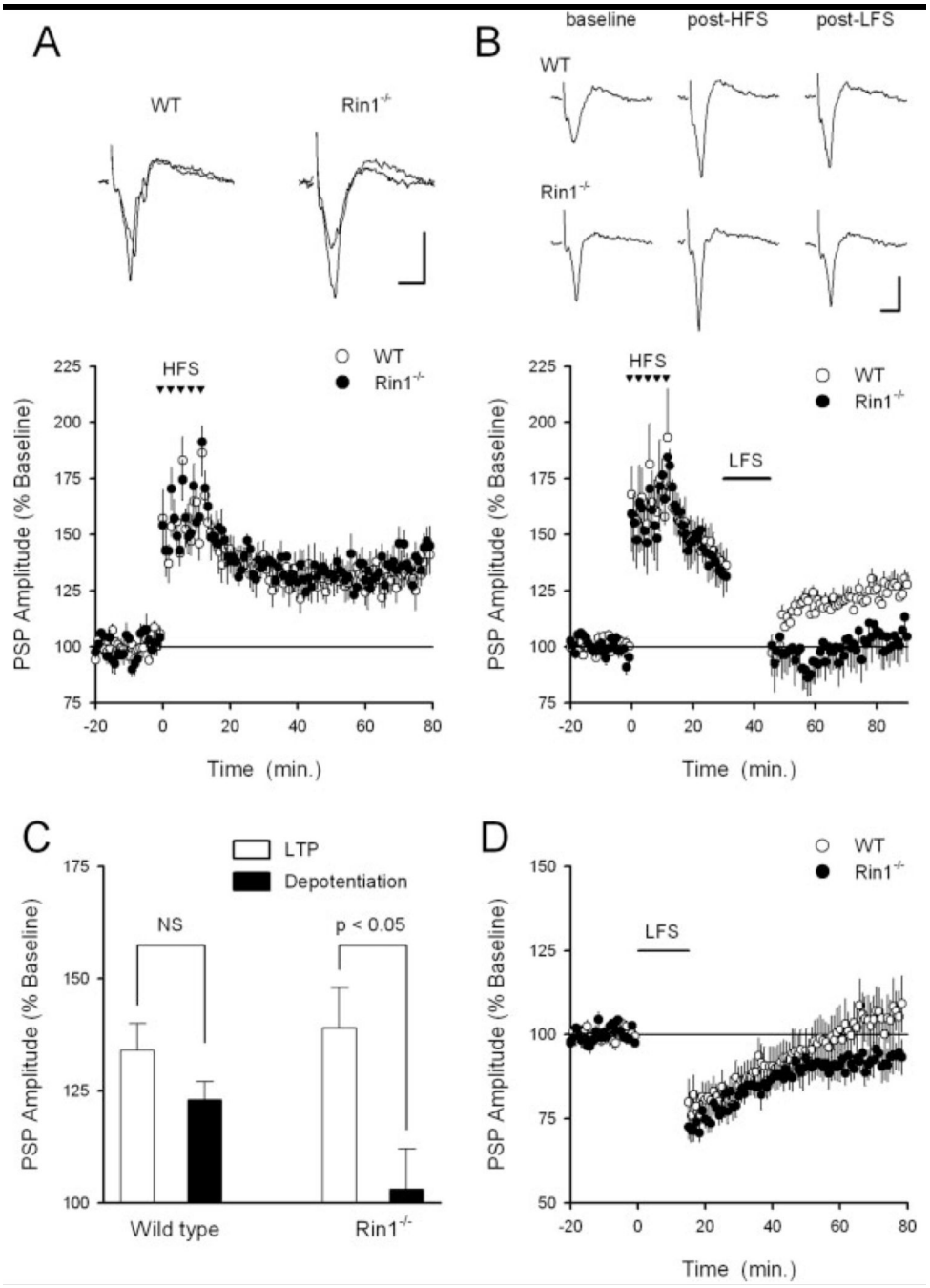

Fig. 4.

Depotentiation at LA-BLA synapses is enhanced in *Rin1*$^{-/-}$ mice. **A**. High-frequency presynaptic fiber stimulation (HFS; 5 trains of 100 Hz stimulation, 1 sec in duration) induced similar levels of LTP in slices from wild type (open symbols, n = 5 slices from 5 mice) and *Rin1*$^{-/-}$ mice (filled symbols, n = 5 slices from 5 mice). The inset shows superimposed postsynaptic responses recorded during baseline and 60 min post-HFS in slices from wild type (left) and *Rin1*$^{-/-}$ mice. Calibration bars are 5.0 milliseconds and 0.5 mV. **B**. Low-frequency stimulation (LFS, 1 Hz for 15 min.) delivered starting 20 min after the last train of HFS induced stronger depotentiation in slices from *Rin1*$^{-/-}$ mice (filled symbols, n = 7 slices from 4 mice) compared to wild-type mice (open symbols, n = 8 slices from 4 mice). Over the last 10 min of

the experiment PSPs in wild type slices were significantly larger that those seen in slices from *Rin1*$^{-/-}$ mice ($p < 0.05$). The inset shows postsynaptic responses recorded during baseline (left), 20 min post-HFS (middle), and 45 min post-LFS (right) in slices from wild type (top) and *Rin1*$^{-/-}$ mice (bottom). Calibration bars are 5.0 milliseconds and 0.5 mV. **C**. Summary of the effects of HFS and HFS followed by LFS in slices from wild type and *Rin1*$^{-/-}$ mice. Values correspond to normalized PSP amplitudes recorded 80 min post-HFS in control LTP experiments (open bars) and in experiments where LFS was delivered post-HFS (filled bars) shown in panels A and B. Note that while LFS had no significant effect on LTP in wild type slices it induced significant depotentiation in *Rin1*$^{-/-}$ slices. **D**. Fifteen min of 1 Hz stimulation had little lasting effect on LA-BLA synaptic transmission in slices from wild type (open symbols, n = 14 slices from 7 mice) and *Rin1*$^{-/-}$ mice (filled symbols, n = 20 slices from 7 mice). Although LFS appears to induce larger LTD in slices from *Rin1*$^{-/-}$ mice, this difference was not statistically significant.

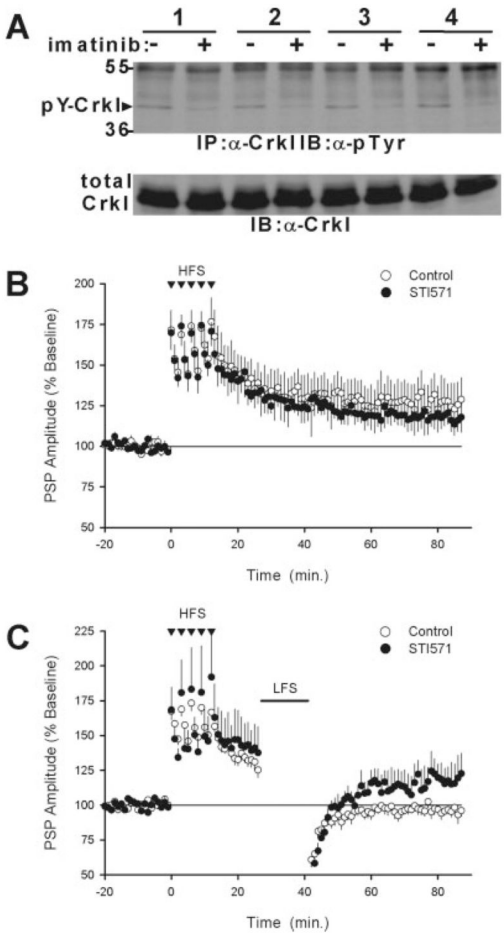

Fig. 5.

The ABL tyrosine kinase inhibitor imatinib inhibits depotentiation at LA-BLA synaspes. **A**. Forebrain slices from four mice were pre-incubated with 5 μM imatinib or drug free ACSF for one hr before electrophysiology. Subsequently, slice extracts were analyzed by immunoprecipitation and immunoblot for changes in tyrosine phosphorylation of endogenous Crkl, an Abl substrate. MW markers (kDa) are shown at left. The constant 55 kDa band corresponds to antibody heavy chain. **B**. HFS induced similar levels of LTP in imatinib-treated (filled symbols, n = 6 slices from 6 mice) and control untreated (open symbols, n = 5 slices from 5 mice) samples. **C**. LFS delivered 15 min post-HFS induced strong depotentiation in control experiments (open symbols, n = 7 slices from 7 mice) but induced significantly less depotentiation in slices bathed in ACSF containing 5 μM imatinib (filled symbols, n = 4 slices from 4 mice, $p < 0.05$ compared to untreated controls).

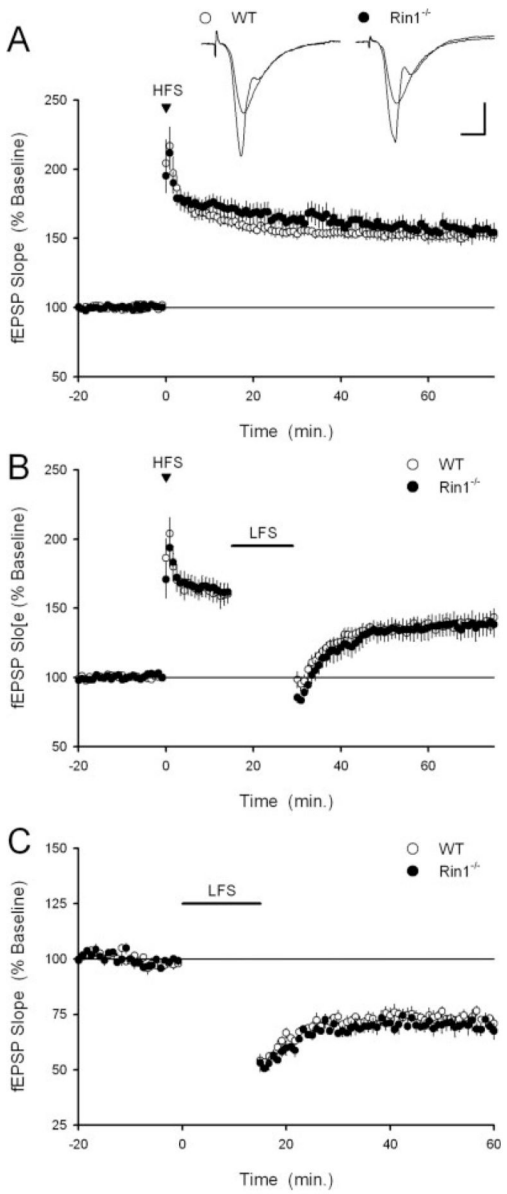

Fig. 6.

LTP, depotentiation and LTD are normal in the hippocampal CA1 region of *Rin1*$^{-/-}$ mice. **A**. Two trains of 100 Hz stimulation (HFS) delivered at time = 0 induced similar levels of LTP in slices from wild-type mice (open symbols, n = 8 slices from 7 mice) and *Rin1*$^{-/-}$ mice (filled symbols, n = 8 slices from 8 mice). The inset shows fEPSPs recorded during baseline and 60 min post-HFS in slices from wild type (left) and *Rin1*$^{-/-}$ mice (right). Calibration bars are 5.0 milliseconds and 2.0 mV. **B**. LFS (1 Hz/15min.) delivered 15 min post-HFS induced similar levels of depotentiation in slices from wild-type mice (open symbols, n = 8 slices from 7 mice) and *Rin1*$^{-/-}$ mutant mice (filled symbols, n = 8 slices from 8 mice). **C**. The induction of LTD in the hippocampal CA1 region is not enhanced in *Rin1*$^{-/-}$ mice. Sixty minute post-LFS fEPSPs were depressed to 73 ± 2% of baseline in slices from wild-type mice (open symbols, n = 10 slices from 5 mice) and were depressed to 70 ± 3% of baseline in slices from *Rin1*$^{-/-}$ mice (filled symbols, n = 8 slices from 4 mice).