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R-RAS CONTRIBUTES TO LTP AND CONTEXTUAL DISCRIMINATION

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Abstract—The ability to discriminate between closely related contexts is a specific form of hippocampal-dependent learning that may be impaired in certain neurodegenerative disorders such as Alzheimer's and Down Syndrome. However, signaling pathways regulating this form of learning are poorly understood. Previous studies have shown that the calcium-dependent exchange factor Ras-GRF1, an activator of Rac, Ras and R-Ras GTPases, is important for this form of learning and memory. Moreover, the ability to discriminate contexts was linked to the ability of Ras-GRF1 to promote high-frequency stimulation long-term potentiation (HFS-LTP) via the activation of p38 Map kinase. Here, we show that R-Ras is involved in this form of learning by using virally-delivered miRNAs targeting R-Ras into the CA1 region of the dorsal hippocampus and observing impaired contextual discrimination. Like the loss of GRF1, knockdown of R-Ras in the CA1 also impairs the induction of HFS-LTP and p38 Map kinase. Nevertheless, experiments indicate that this involvement of R-Ras in HFS-LTP that is required for contextual discrimination is independent of Ras-GRF1. Thus, R-Ras is a novel regulator of a form of hippocampal-dependent LTP as well as learning and memory that is affected in certain forms of neurodegenerative diseases.

Key words: Ras-GRF1, synaptic plasticity, hippocampus.

BACKGROUND

The hippocampus is a critical processor of contextual information from the outside world and essential contributor to the formation of long-term memories. One specific form of hippocampus-dependent memory is contextual discrimination, the specialized ability to distinguish between closely related contexts (Frankland et al., 1998; Giese et al., 2001; Jeffery et al., 2004). Aging results in progressive weakening of the ability to process contextual information for the purpose of new memories. This behavior can be mimicked in mouse models of neurodegenerative disorders such as Down syndrome(Hyde and Crnic, 2001) and Alzheimer's disease and thus may be useful as a biomarker for these hallmark disorders (Tronche et al., 2010). However, the mechanisms underlying this form of learning are poorly understood.

In mice, contextual discrimination ability requires Ras-GRF1(Giese et al., 2001), which is expressed in the post-synaptic space of CNS neurons (Shou et al., 1992; Sturani et al., 1997; Zippel et al., 1997). Along with Ras-GRF2 (Fam et al., 1997), they form a family of calcium-activated exchange factors for Ras and Rac GTPases (Feig, 2011). Both Ras-GRF1 and Ras-GRF2 have Dbl homology exchange factor domains for Rac and CDC25 exchange factor domains for Ras. Interestingly, the CDC25 domain of RasGRF1, but not Ras-GRF2, also has the capacity to activate the Ras-related R-Ras GTPase (Gotoh et al., 1997, 2001; Tian and Feig, 2001).

R-Ras is similar to Ras proteins, but clear differences exist in that they share only a subset of both downstream targets and upstream regulators and have distinct negative regulators. While GTP-bound Ras proteins can activate the Raf/Erk Map kinase, Ral GTPase, and PI3 kinase signaling cascades, GTP-bound R-Ras only activates PI3 kinase. Moreover, only R-Ras activates inside-out integrin signaling (Reuther and Der, 2000). While both Ras and R-Ras can be activated by GRF1, only R-Ras is activated by the exchange factor C3G (Overbeck et al., 1995). In contrast, negative regulators of these proteins are distinct in that Ras-GAPs only promote hydrolysis of GTP to GDP bound to Ras proteins (Iwashita and Song, 2008) and R-Ras GAPs such as plexin-B1 only suppress R-Ras proteins (Negishi et al., 2005).

R-Ras has been implicated in cell adhesion and neurite outgrowth associated with developing hippocampal or cortical neurons (Ivins et al., 2000; Negishi et al., 2005). R-Ras localizes to active zones to regulate axonal morphogenesis and branching (Oinuma et al., 2007; Iwasawa et al., 2012). This involves downregulation of R-Ras by Plexin-B1 receptor activation in response to its ligand Semaphorin 4D to control growth cone collapse (Oinuma et al., 2004a,b, 2010). While these studies identified a role for R-Ras in development of the presynaptic nerve terminal, a role for R-Ras in mature neurons has not been explored.

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Abbreviations: AAV, adeno-associated viruses; ACSF, artificial cerebrospinal fluid; HFS-LTP, high-frequency stimulation long-term potentiation; LTD, long-term depression; PB, phosphate buffer; PBS, phosphate-buffered saline; NGS, normal goat serum; PFA, paraformaldehyde; SDS, sodium dodecyl sulfate; TBS, theta-burst stimulation.

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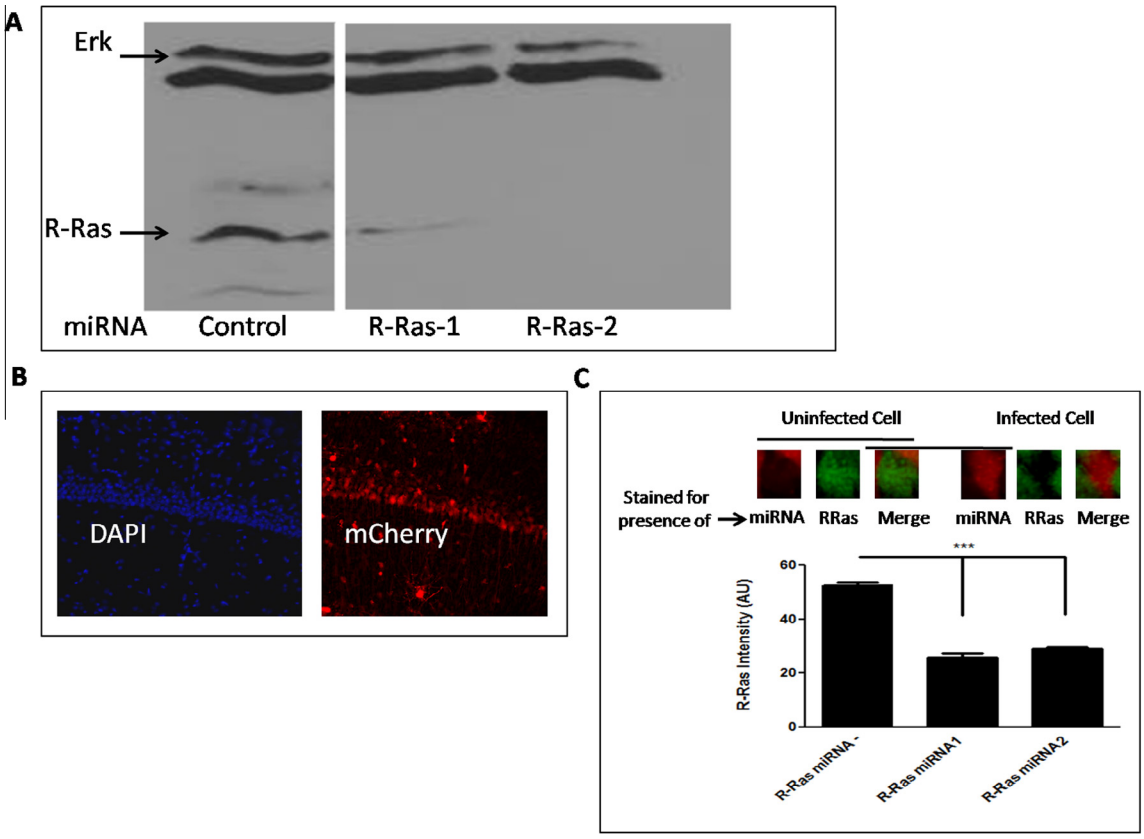

Fig. 1. miRNA knockdown of R-Ras in cells and in animals. (A) Selection of R-Ras miRNAs for *in vivo* use. Lysates of NIH 3T3 cells transfected with two R-Ras-specific miRNAs or a control-unrelated miRNA sequence were immunoblotted to detect endogenous R-Ras expression. (B) Expression of AAV encoding R-Ras miRNA and mCherry in the CA1 hippocampus. Hippocampal brain slices from mice infected with AAV encoding R-Ras miRNA were stained with DAPI to detect cell nuclei and mCherry fluorescence to detect expression of virus. (C) R-Ras miRNAs reduce endogenous R-Ras expression in area CA1 hippocampus. Top: Representative images of cells uninfected or infected with R-Ras miRNAs and immunostained for endogenous R-Ras. Bottom: Cells infected with either R-Ras miRNA showed significantly lower staining intensity compared to neighboring cells that were uninfected (One-way ANOVA, $F_{2,512} = 112.6$, $p < 0.0001$; Bonferroni post hoc, R-Ras miRNA – vs. R-Ras miRNA1, $t = 8.40$, $p < 0.05$, R-Ras miRNA – vs. R-Ras miRNA2, $t = 13.73$, $p < 0.05$, R-Ras miRNA1 vs. R-Ras miRNA2, $t = 0.98$, $p > 0.05$).

We previously reported that RasGRF1 contributes to contextual discrimination by regulating a specific form of postsynaptic plasticity called high-frequency long-term potentiation (HFS-LTP, (Jin et al., 2013) in the CA1 region of the hippocampus. Additionally, this signaling was dependent on p38 activation. Because RasGRF1 can potentially activate R-Ras through the same CDC25 exchange domain that activates R-Ras, in this paper we tested the possibility that R-Ras contributes to HFS-LTP and/or contextual discrimination. Using viral-delivered miRNAs specific for knockdown of R-Ras *in vitro* and *in vivo*, we found that R-Ras is necessary for HFS-LTP and contextual discrimination, but it is not functioning as an effector of Ras-GRF1 in this context.

EXPERIMENTAL PROCEDURES

Animals

Two- to three-month-old male C57Bl/6J animals were used for all experiments. All procedures were carried out in accordance with the Institutional Animal Care and Use Committee guidelines of the Tufts University.

Generation of R-Ras miRNA constructs

R-Ras miRNA sequences were purchased from Open Biosystems as an shRNAmir set (TRCN0000077618, TRCN0000077619, TRCN0000077620, TRCN0000077622) cloned into the pGIPZ expression vector. Unrelated miRNA sequence was used as a control. Each clone was individually transfected into 3T3 cells to test for endogenous knockdown efficacy of R-Ras. 1.5 mL Opti-MEM media containing DNA for the pGIPZ expression vector (3 μg), VSVG (3 μg), and delta 8.91 plasmids (6 μg) were combined with 36 μl Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) and 1.5 mL Opti-MEM, allowed to mix for 20 min and then added to 3T3 cells. After overnight incubation, the cells were washed with phosphate-buffered saline (PBS) and lysed in ice-cold RIPA buffer containing protease inhibitors. Cell lysates were spun at 13,000 g for 15 min, sodium dodecyl sulfate (SDS)-loading buffer was added to the supernatant, and SDS–polyacrylamide gel electrophoresis (PAGE) was run to assay for total R-Ras using anti-R-ras antibody (sc-523, Santa Cruz, Dallas, TX, USA) and anti-ERK antibody (sc-94, Santa Cruz) for loading control. Positive sequences (miRNA #1: