Neuroscience Letters 489 (2011) 182–186

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Mice lacking rhes show altered morphine analgesia, tolerance, and dependence

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ARTICLE INFO

Article history:
Received 18 August 2010
Received in revised form 12 October 2010
Accepted 7 December 2010

Keywords:
RASD2
Opioid
Tolerance
Analgesia
Dependence
GTP-binding protein

ABSTRACT

Rhes, the Ras Homolog Enriched in Striatum, is an intermediate-size GTP binding protein. Although its full functions are not yet known, it has been shown to affect signaling and behaviors mediated by G protein-coupled receptors. Here we have tested whether Rhes affects behaviors mediated by opioid receptors. Wild type and rhes-deficient mice were administered morphine and tested for analgesia in formalin and tail flick tests. Rhes$^{-/-}$ mice showed significantly enhanced analgesia in both tests relative to rhes$^{+/+}$ mice. Furthermore, rhes$^{-/-}$ mice did not display tolerance to repeated morphine administration and displayed significantly less withdrawal than rhes$^{+/+}$ mice. These findings indicate that Rhes is involved in behaviors mediated by mu opioid receptors and in the adaptive response to repeated morphine administration.

Rhes (Ras Homolog Enriched in Striatum, RASD2) is a GTP-binding protein that is highly expressed in the striatum of rodents and has a high degree of homology with the Ras superfamily of small GTPase proteins [7]. It was originally characterized by using subtractive hybridization for genetic sequences differentially expressed in striatum [7,26]. The rhes gene codes for a 266 amino acid protein of intermediate size between Ras-like GTPases and α subunits of heterotrimeric G proteins. Rhes is most closely related to AGS1/Dexras1, showing 62% identity [7]. Both proteins contain an elongated C-terminus, defining them as the founding members of a novel subfamily of the Ras superfamily [7]. Furthermore, both genes are regulated by hormones, rhes by thyroid hormone and AGS1/Dexras1 by dexamethasone [11,27].

In vitro investigations of Rhes have shown its ability to attenuate Gs and, more recently, Gi/o signaling after agonist stimulation of G protein coupled receptors (GPCR). In PC12 cells, Rhes was shown to attenuate reporter gene activation by the Gs-coupled thyroid stimulating hormone receptor, but not by the M_2 muscarinic receptor [28]. However, in HEK293 cells expressing Rhes and M_2, attenuation by Rhes of agonist-initiated inhibition of $Ca_V2.2$ was demonstrated. This effect is mediated by Gβγ subunits liberated from Gi/o and demonstrates that Rhes, like AGS1/Dexras1, can affect Gi/o-mediated signaling [25]. In addition, the efficacy of Gi/o activation through D2 receptors is lower in rhes$^{-/-}$ mice compared with rhes$^{+/+}$ counterparts, suggesting that the presence of Rhes protein is important in normal signal transduction by this dopamine receptor [5].

Rhes has been shown to be preferentially expressed in neurons of the striatum [5,7,9,28], and in vivo studies point to its role in dopaminergic signaling and behavior. Lesion of the nigrostriatal pathway results in a decrease in rhes mRNA and protein expression in the striatum [9,10]. Rhes$^{-/-}$ mice show increased D1 agonist-initiated locomotor activation, increased D2 antagonist-initiated catalepsy, and increased D1 signaling through adenylyl cyclase (AC) relative to rhes$^{+/+}$ mice [5]. These studies suggest that absence of Rhes leads to an increase in dopamine receptor activation of AC, or similarly a decrease in inhibitory tone through AC. However, rhes$^{-/-}$ mice also show a decrease in dopamine D1 receptor-mediated grooming behavior relative to rhes$^{+/+}$ mice [17], a behavior thought to be mediated by activation of the phospholipase C pathway [4,16], thus suggesting that Rhes may differentially affect signaling pathways.

Although dopamine systems have been emphasized in early behavioral and cellular studies of Rhes due to the enrichment of both dopamine receptors and Rhes in striatum, there is no a priori reason to assume that only dopamine receptors are modulated by Rhes. Indeed, opioid receptors are also highly expressed in striatum [13], and striatum has been shown to play a role in analgesia [1,12]. Here we have tested whether these GPCRs are also modulated by Rhes. We hypothesized that because there is increased excitatory tone through AC in rhes$^{-/-}$ mice and a decrease in the ability to activate Gi/o [5], these mice would show decreased morphine anal-

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doi:10.1016/j.neulet.2010.12.012

gesia relative to wild type mice. To test this hypothesis, we tested analgesia in two different models of pain and found, surprisingly, that rhes$^{-/-}$ showed enhanced analgesia relative to rhes$^{+/+}$ mice. In addition, we tested whether Rhes affects the development of tolerance and dependence upon repeated morphine administration.

Rhes$^{+/+}$ and rhes$^{-/-}$ mice were generated as previously described [22] and backcrossed for ten generations onto the C57/BL6 background. Male mice from heterozygous and homozygous matings were used for all experiments. Genotypes were verified by using PCR of tail biopsies, and mice were used at 2–4 months of age. Mice were group housed (n = 4/cage) in a climate-controlled vivarium on a 12-h light/dark cycle (lights on at 06:00). Food and water were provided ad libitum with the exception of testing times. All animal experiments were performed in accordance with the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals, and experimental protocols were approved and supervised by the University of New Orleans Institutional Animal Care and Use Committee. Every effort was made to minimize the suffering of the animals and to reduce the number of animals used.

Morphine sulfate (Paddock Laboratories; Minneapolis, MN) was injected at a dose of either 0.3, 1, 3, or 10 mg/kg, and naloxone hydrochloride (Sigma; St. Louis, MO) was injected at a dose of 3 mg/kg. Drugs were dissolved in 0.9% saline and administered IP at an injection volume of 10 ml/kg.

Formalin test: Thirty minutes after morphine administration, animals received a 40 μl SC injection of 5% formalin into the plantar surface of the left hind paw and were immediately placed in an elevated 11 cm × 23 cm Plexiglas® enclosure with a clear glass floor (IITC, Woodland Hills, California, USA). A mirror positioned at 45° under the floor allowed for un-obscured observation of the animals' behavior. Time spent licking the injected hind paw was recorded over 5 min intervals for 1 h.

Tail flick: Analgesia was tested with the tail flick paradigm using the IITC tail flick testing apparatus. Mice were loosely restrained in a towel, and a 1 cm^2 beam of light was focused on the distal 1/3 of the tail. Latencies to withdraw were measured at baseline, at 5 and 15 min post-injection, and at 15 min intervals thereafter until 2 h post drug administration. A maximum withdrawal latency of 12 s was imposed to prevent tissue damage.

Opioid tolerance was measured in animals receiving single daily injections of 3 or 10 mg/kg morphine for 5 consecutive days. The percent maximum possible effect in the tail flick test was calculated 30 min after morphine on days 1 and 5 of drug administration by using the following formula: Percent Maximum Possible Effect (%MPE) = 100 × [(test latency − basal latency)/(cut-off time − basal latency)]. Opioid withdrawal was measured using a naloxone challenge. On day 5 of repeated morphine administration, animals were given IP injections of naloxone hydrochloride (3 mg/kg) 30 min post-administration of morphine and immediately placed in clear Plexiglas® cylinders approximately 15 cm in diameter and 30 cm high. A variable for global withdrawal score was computed by weighting number of jumps by 1, wet dog shakes by 5, and fecal boli by 5.

For the formalin test, results are presented as time spent licking group means ± SEM for each dose by time. For statistical analysis, data were divided into early (1st 5 min) and late (remaining 55 min) phases, and two-factor (genotype × phase) repeated measures ANOVAs were performed for each dose. Tail flick results are presented as time courses for each genotype, which were analyzed by two-factor (dose × time) repeated measures ANOVA for each genotype. Nonlinear regression was used to fit dose–response data from the 45 min time point and to calculate ED_{50} values and 95% confidence limits. Tolerance is presented as tail flick latency %MPE group mean ± SEM for acute and repeated administration of morphine sulfate. These data were analyzed by two-factor (genotype × day) repeated measures ANOVA for each dose. Results of withdrawal are presented as global withdrawal score group means ± SEM, which were compared by genotype using independent samples t-test. Results were considered significant at $p < 0.05$, and follow-up planned multiple comparisons were made subject to a modified Bonferroni correction.

In the formalin test, rhes$^{-/-}$ and rhes$^{+/+}$ mice did not show any differences in licking in the early or late phase when administered vehicle [$F(1,18) = 0.424$, $p > 0.05$] or morphine at a dose of 3 mg/kg [$F(1,17) = 0.155$, $p > 0.05$] (Fig. 1a and b). However,

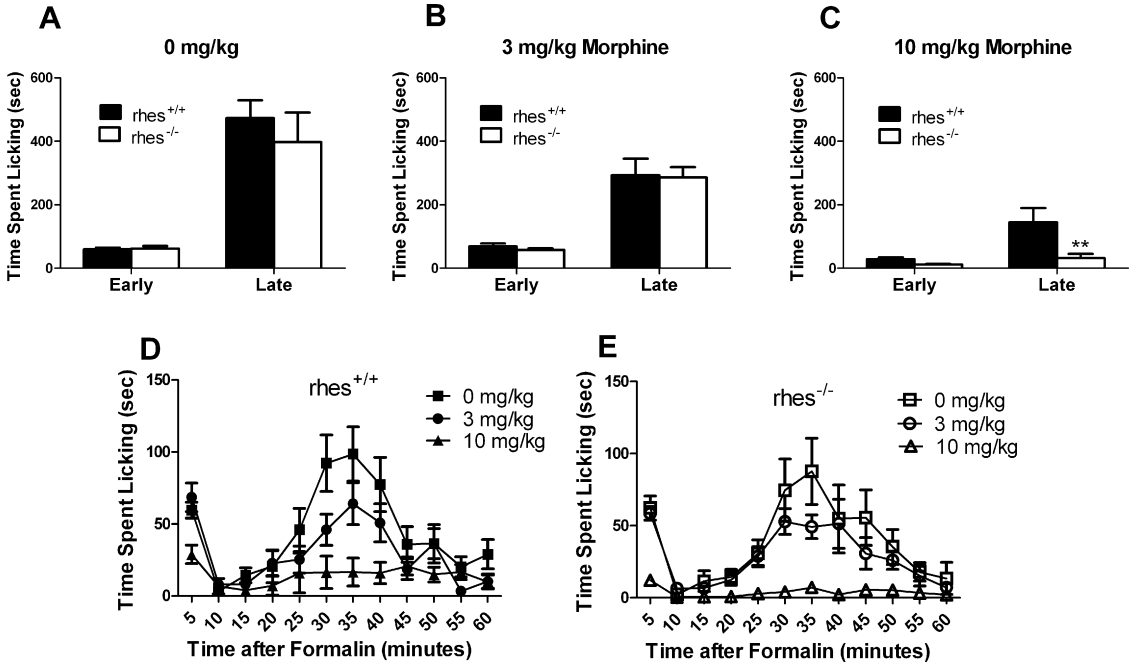

Fig. 1. Analgesia assessed with the formalin test. (A–C) Time spent licking (group mean ± SEM) in the early (1st 5 min) or late (remaining 55 min) phases after 0 mg/kg (A), 3 mg/kg (B), or 10 mg/kg (C) morphine. (D and E) The data are shown as a time course over 60 min with group mean ± SEM for each 5 min interval for rhes$^{+/+}$ (D) and rhes$^{-/-}$ (E) mice. $^{**}p < 0.01$ compared with rhes$^{+/+}$ mice at the same phase. n = 9–10.