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The Effects of Rhes on Opioid Analgesia

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The Effects of Rhes on Opioid Analgesia

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Terms

AC – Adenylate Cyclase

CAAX- C-cysteine, AA-Aliphatic, X-amino acid

cAMP – Cyclic adenosine-monophosphate

CPP – Conditioned place preference

ERK – Extracellular signal-regulated kinases

GDP – Guanosine diphosphate

GFP- Green fluorescent protein

GPCR – G protein-coupled receptor

GTP – Guanosine triphosphate

MAPK – Mitogen-activated protein kinase

MOR – mu-opioid receptor

MSN – Medium spiny neurons

PAG – Periaqueductal grey

PCR- Polymerase chain reaction

PI3K – Phosphoinositide 3-kinase

PKA – Protein Kinase A

PKC – Protein Kinase C

PLC – Phopholipase C

Rhes – Ras homolog enriched in striatum

RVM – Rostral ventromedial medulla

Abstract

Rhes (Ras homolog enriched in striatum) has been identified as a novel monomeric G-protein involved in dopaminergic and other signaling in the striatum. Given the many effects of opioids that involve striatal circuitry, genetically engineered mice that are incapable of making Rhes ($rhes^{-/-}$) and their control littermates ($rhes^{+/+}$) were subjected to behavioral tests to determine if any differences existed in opioid analgesia, tolerance, withdrawal, reward, and locomotion. $Rhes^{-/-}$ mice showed an increased opioid mediated analgesia, along with an absence of tolerance and decrease in withdrawal when compared with $rhes^{+/+}$ littermates. However, no significant changes were seen in opioid induced locomotor activation or conditioned place preference. These results provide strong evidence for the implication of Rhes in opioid signaling.

Rhes, analgesia, opioid, tolerance, dependence, GTP-binding protein

Introduction

Exogenous opioids are currently the best treatment for severe intractable pain. These substances show profound analgesic effect, but at the cost of being highly addictive. Clinicians are tasked with weighing the benefits of these potent analgesics against the costs of side effects such as respiratory depression, euphoria, and addiction. Opioid addiction is a major public health concern, especially considering the high likelihood of overdose due to its effects on respiration. Better understanding of the mechanisms that lead to both analgesia and addiction can be beneficial in the development of therapies that maximize the analgesic effects of opioids while simultaneously decreasing the liability of side effects.

Given the concerns of using opioids in the treatment of pain, it is important to understand the mechanism by which opioids exert their actions in the body. When properly understood in the context of pain and addiction, the mechanisms will help scientists to develop therapies that increase the therapeutic window of opioid analgesics thereby increasing analgesia and reducing abuse potential. The mechanisms by which opioids exert their actions, despite the large amount of research dedicated to the field, are still not well understood. Basic scientific research continually adds to an increasingly complex picture of the action of opioids at both the cellular and systems levels. In the current study, we have attempted to classify the effects of a novel protein Rhes (Ras Homolog Enriched in Striatum) in regard to opioid analgesia. The approach focuses on the study of behavior to draw inferences regarding both cellular mechanisms in context of the current literature.

Rhes, a monomeric G-protein, is known to function as a special type of G-protein that does not signal in the same manner as the traditional heterotrimeric G-proteins. Rather, Rhes is part of the Ras superfamily of small GTPases that seem to function as modulators of traditional G-protein functions (Campbell, Khosravi-Far, Rossman, Clark, & Der, 1998; Falk et al., 1999). Current research suggests that Rhes interferes with the intracellular messaging of G protein-coupled receptors (GPCRs), specifically dopamine, β-adrenergic, muscarinic, and thyroid-hormone receptors. Our aim was to determine if this interference could lead to behavioral changes in opioid induced analgesia, tolerance, withdrawal, reward, and locomotion, all of which involve GPCRs in the striatum, a place where Rhes has been shown to be localized (Errico et al., 2008; Falk et al., 1999; Harrison & LaHoste, 2006).

G Protein-Coupled Receptors

GPCRs, also known as metabotropic receptors, are found throughout the central nervous system (CNS), and provide an important means of transduction of signals from neurotransmitters to cellular activity. Every major neurotransmitter, as well as a host of hormones, neuropeptides, and exogenous compounds, activates this class of receptors. Stimulation of these receptor types plays a role in every type of cellular function from altering membrane potential to transcription of genes. GPCRs can be implicated in many illnesses from cancer to schizophrenia. Understanding the role of GPCR signaling is important to understanding the role of such pathologies, and behavior in general, at the level of the cell.

GPCRs share a homologous structure containing seven membrane spanning domains that are connected by extracellular loops that form a ligand binding site (Kandel, Schwartz, & Jessell, 2000). The third intracellular loop is typically attached to a heterotrimer, a structure composed of 3 individual proteins. These G-proteins, alpha (α), beta (β), and gamma (γ), are responsible for transmitting extracellular signals via GPCRs to intracellular responses of the cell. When a ligand binds this receptor type, the receptor goes through a conformational change that allows the α subunit of the heterotrimeric G-protein complex to become activated, uncoupling from a constitutively bound guanosine diphosphate (GDP) molecule which is then replaced by a guanosine triphosphate (GTP) molecule. This activates the α subunit and allows it to move away from the βγ complex and the receptor. The subunit then moves along the inner membrane surface to effector proteins which it can then activate setting in motion any number of possible outcomes via activation and inactivation of various intracellular signaling pathways. When this happens, the βγ proteins, which are thought to exist mostly in a dimerized state, are allowed to move away from the receptor and can activate second messenger cascades as well (Hamm, 1998; Kandel et al., 2000).

Dopamine Receptors

Dopamine signaling has been widely studied, and it has been shown that dopamine acts through two metabotropic receptor types including the D1 (D1, D5) family receptors which operate via coupling to the stimulatory G_s α subunit, and the D2 (D2,D3,D4) family receptors that couple to the inhibitory $G_{i/o}$ α subunits. Both D1 and D2 family receptors have been shown to be expressed abundantly in the striatum,

nucleus accumbens (NAc), and limbic structures (Missale, Nash, Robinson, Jaber, & Caron, 1998).

$G_{\alpha s}$ α subunits are so named because of their stimulatory effect on adenylyl cyclase (AC). These subunits, when activated by an agonist bound receptor, have been shown to upregulate cyclic adenosine monophosphate (cAMP) through stimulation of AC (Kebabian & Calne, 1979; Missale et al., 1998). $G_{\alpha i/o}$ are so named because, in the presence of an agonist bound receptor, they inhibit the production of cAMP via effects on AC (Kandel et al., 2000). Both excitatory $G_{\alpha s}$ and inhibitory $G_{\alpha i/o}$ coupled receptors are bound to $G_{\beta\gamma\alpha}$ heterotrimers in their resting state (Kandel et al., 2000). At rest the G_{α} subunit is bound to the $G_{\beta\gamma}$ complex due to its constitutive binding of a GDP molecule. When an agonist is presented, GDP is replaced by GTP which causes a conformational change in G_{α} that disassociates it from the βγ complex, allowing it to move along the plasma membrane to a nearby effector protein, AC (Missale et al., 1998). G_{α} subunits have an intrinsic ability to dephosphorylate GTP into GDP, thus allowing the subunit to re-associate with the βγ again forming the heterotrimer that is associated with the receptor (Missale et al., 1998).

AC's major function is the production of cAMP. cAMP is a soluble intracellular second messenger that binds several effector proteins and activates their various functions. Most isoforms of AC are activated by the $G_{\alpha s/olf,}$ and inactivated by $G_{\alpha i/o,}$ but some forms can also be stimulated by protein kinase C, Ca2+/Calmodulin, and βγ signaling (Neve, Seamans, & Trantham-Davidson, 2004). The main target of cAMP is the protein kinase A (PKA). PKA has been implicated in many cell functions, including Ca2+ concentration via regulation of extracellular calcium influx/efflux and intracellular

calcium stores, regulation of ion channels, protein inactivation through activation of phosphodiesterases and phosphatases, activation of the phopholipase C (PLC) pathway, cell proliferation, and gene transcription. Many regulatory effects of dopamine receptors are also a consequence of the signaling of $G_{\beta\gamma,}$ which has been shown to regulate AC, and directly interact with the PLC/DAG pathway (Missale et al., 1998; Neve et al., 2004).

Rhes

Rhes is an intermediate size GTP binding protein that is heavily expressed in the striatum of rodents and has a high degree of nucleotide conservation with the Ras superfamily of small GTPase proteins (Errico et al., 2008; Falk et al., 1999; Harrison & LaHoste, 2006). Ras GTPases act as cellular switching mechanisms that interact with other effector proteins to change specific cellular messaging systems within the intracellular cytoplasmic domain (Campbell et al., 1998). They have been studied extensively for their role in the activation of the mitogen-activated protein kinase/extracellular signal-rated kinase (MAPK/ERK) signaling pathway which is intimately involved in cell proliferation and implicated in the mechanics of many cancers (Bos, 1989; Campbell et al., 1998; Rodriquez-Viciana, Sabatier, & McCormick, 2004). However, despite the large body of research conducted on the relationship between the MAPK/ERK signaling pathways and small GTPases Rhes has not been shown to affect MAPK/ERK signaling (Vargiu et al., 2004)(Laura Harrison Unpublished Results).

Rhes was originally characterized by Falk and colleagues after the discovery of its closely related family member Dexras1/AGS1 (1999). In bacterial cells expressing

Rhes, these researchers determined that Rhes did bind GTP, likely giving it intrinsic GTPase activity, and that it was induced by thyroid hormone unlike its close relative Dexras1, which is induced by dexamethasone (Falk et al., 1999; Kemppainen & Behrend, 1998). This group also made the observation that like Dexras1, Rhes also contained an unusually long c-terminus, the part of the protein that is known to associate with other proteins, making it likely that these proteins posses unique but similar signaling characteristics (Falk et al., 1999).

Vargiu and colleagues further characterized Rhes (2004). *In situ* hybridization was performed, and it was determined that the Rhes mRNA was heavily expressed in the striatum, but to a lesser extent in the nucleus accumbens, olfactory bulb, olfactory tubercle, piriform cortex, hippocampus, anterior thalamic nuclei, inferior colliculus, cerebellum, cortex, and outside of the CNS in the thyroid. A later study provided evidence that expression of Rhes is determined by dopamine innervation. By experimentally lesioning dopaminergic projections, a decrease in rhes mRNA was measured in the caudate putamen and shell of NAc suggesting that rhes expression is regulated by dopaminergic tone and also that supersentivity of dopamine receptors correlates with rhes mRNA downregulation (Harrison & LaHoste, 2006).

By attaching a GFP (green fluorescent protein) to the CAAX terminal, an area of proteins susceptible to membrane binding, researchers were able to determine that Rhes is targeted to the plasma membrane in vitro. Through a series of pharmacological interventions it was also determined that farnesylation is the most likely candidate for its post translational targeting to the membrane (Vargiu et al., 2004).

In vitro immuno-precipitation showed evidence that Rhes did not stimulate the MAPK/ERK pathway, but that it did stimulate the phosphoinositide 3-kinase (PI3K) pathway (Vargiu et al., 2004). In vitro GTP binding showed that 30% of Rhes is constitutively bound by GTP, meaning its effects remain active at basal conditions. Evidence also suggests an inability of several well known guanidine exchange factors (GEFs), small proteins that assist in exchanging GTP and GDP molecules on other proteins, to alter GTP binding. Most importantly, Rhes was found to inhibit reporter gene activation, a cAMP dependent mechanism, in cells expressing the metabotropic, $G_{\alpha s}$ coupled, thyroid hormone receptor, suggesting that Rhes interferes with signaling of $G_{\alpha s}$ coupled receptors (Vargiu et al., 2004). This was supported by Errico et al (2008), who found that PKA dependent phosphorylation of the GluR1 subunit of the AMPA receptor in medium spiny neurons, a direct result of $G_{\alpha s/olf}$ stimulation of AC/cAMP, was increased in $rhes^{-/-}$ membrane preparations (2008). Since this phosphorylation is a direct result of $G_{\alpha s/olf}$ stimulation of AC/cAMP, it suggests that Rhes provides negative modulation of the $G_{\alpha s/olf}$ stimulation of AC.

To determine if Rhes modifies $G_{\alpha i/o}$ signaling like its closest relative Dexras1, researchers using PC12 cells transfected with Rhes and the M2-muscarinic receptor found no change in reporter gene activity suggesting no alteration of $G_{\alpha i/o}$ (Vargiu et al., 2004). Errico et al. (2008) suggested that Rhes may have an effect on $G_{\alpha i/o}$ signaling after finding that in $rhes^{-/-}$ membrane preparations of striatal medium spiny neurons, lack of Rhes slightly lowered the ability of D2 agonists to stimulate GTP binding (2008). A later study found that Rhes does influence signaling through $G_{\alpha i/o}$. Cells stably expressing the M2-muscarinic receptor were shown to produce maximal inhibition of

$Ca_{v2.2}$ channels under agonist stimulation when transfected with mutant Rhes; however agonist stimulation produced less inhibition in the presence of fully functioning Rhes, suggesting that Rhes attenuated $G_{\alpha i/o}$ inhibitory signaling (Thapliyal, Bannister, Christopher, & Brett, 2008).

Another important finding of the previously mentioned study was the possible role of Rhes' effect on $G_{\beta\gamma}$ signaling. This group transfected M2-muscarinic receptor expressing cells with Rhes and showed tonic inhibition of $Ca_{v2.2}$ channels, a $G_{\beta\gamma}$ mediated effect, versus cells transfected with a Rhes CAAX mutated control (Thapliyal et al., 2008). In a recent study by Hill, Goddard, Ladds, and Davey (2009), cells expressing both Rhes and either $G_{\beta1}$, $G_{\beta2}$, $G_{\beta3}$, $G_{\beta4}$, or $G_{\beta5}$ were compared, and it was found that Rhes showed much more binding affinity for $G_{\beta1\text{-}3}$, but not $G_{\beta4,5}$. These results suggest that Rhes could possibly interfere with $G_{\alpha s}$ binding due to its affinity to interact with $G_{\beta1\text{-}3}$, the units that preferentially bind $G_{\alpha s}$, and not $G_{\beta4,5}$, units that preferentially bind $G_{\alpha i/o}$. Hill et al. suggest that this might be possible due to the fact that $G_{\alpha s}$ interaction with the plasma membrane is much weaker than that of $G_{\alpha i/o}$, and therefore $G_{\alpha i/o}$ subunits are able to overcome the interference of Rhes in binding G_{β} subunits (2009).

This would lend support to the assertion of Thalapiyal et al. (2008) that Rhes may interfere with $G_{\alpha i/o}$ signaling by allowing the $G_{\alpha i/o}$ subunit to disassociate from the heterotrimeric complex and signal but as this happens allowing Rhes to bind the $G_{\beta\gamma}$ subunits disrupting re-association of the $G_{\alpha i/o}$ subunit with the $G_{\beta\gamma}$ complex. They suggest that this may be due to the ability of Rhes to function as a guanidine exchange factor for $G_{\alpha i/o}$ (Thapliyal et al., 2008).

These findings, although unclear almost to the point of contradiction, suggest that Rhes may play a differential role depending on what type of receptor complex it targets as a function of cell type, receptor type, and expression of the rhes.

Analgesia

The classical centrally mediated analgesia pathway has been shown to originate in the periacquaductal gray matter (PAG) where mu-opioid binding inhibits GABAergic inhibition of serotonergic projection neurons of the rostral ventromedial medulla (RVM). These neurons in turn project to the spinal cord where they act to block the incoming pain signals of afferent nociceptors (Kandel et al., 2000). However, mu-opioid receptor (MOR) activation is also well known to disinhibit the mesolimbic dopaminergic projections of the ventral tegmental area (Altier & Stewart, 1998). These dopaminergic projections send their axons to the limbic structures and most notably to the NAc which has been implicated as the reward center of the brain (Kandel et al., 2000). Through activation of the NAc, the mu opioid agonists show great reward and motivation to seek these substances. However the NAc has also been shown to make connections to the PAG suggesting that there is a direct modulation of the classical analgesic pathway (Ikemoto & Panksepp, 1999).

A small body of research however has focused on the idea that structures in the striatum can themselves be shown to affect analgesia, in particular the NAc. A study by Magnussen and Martin (2002) has shown that direct injection of bupivacaine, an anesthetic, into the core of the NAc causes enhanced formalin-induced nociception while no effects were shown when the anesthetic was injected into the shell . Another

study found that injection of quinpirole, a potent D2 agonist, into the NAc showed direct inhibition of nociception in phase II of the formalin test, and this effect was reversible using raclopride, a D2 antagonist (Taylor, Joshi, & Uppal, 2003). Another pharmacological study finds similar effects using D1 agonist and antagonist co-administered with DAMGO. A potent MOR agonist DAMGO was co-administered with haloperidol, a D2 antagonist, or apomorphine, a D1/D2 agonist. Results show that when administered with haloperidol, DAMGO caused a dose-dependent decrease in antinociception in the tail flick assay, and conversely, when administered with apomorphine, it showed a dose-dependent increase in antinociception (Capasso, 2008). Another study finds that noxious capsaicin administration significantly increases mechanical antinociception, but that this effect can be blocked by intra-accumbens injection of flupixentol, a non-specific dopamine antagonist (Gear, Aley, & Levine, 1999). In light of these studies, dopamine signaling in the striatum becomes an important target of centrally mediated analgesic effect.

Because Rhes is highly expressed in the shell of the NAc, and to a lesser extent in the core, and has been shown to affect dopaminergic signaling in the striatum, its involvement in signaling may be of importance to NAc mediated analgesia (Errico et al., 2008; Harrison & LaHoste, 2006; Quintero, Spano, LaHoste, & Harrison, 2008). Lack of rhes in the NAc could cause analgesia by allowing un-modulated activation of the $G_{\alpha s}$ /cAMP pathway in response to stimulation by dopamine projections from the VTA and inability for Gαi/o to inhibit this response. Specifically we hypothesize that $rhes^{-/-}$ mice will have increased centrally mediated analgesia.

Reward

The NAc is most notably involved in the rewarding aspects of stimuli and is often referred to as the pleasure center of the brain. The NAc, being part of the basal ganglia, is primarily composed of GABAergic medium spiny neurons. The NAc receives input from the VTA, and forms efferent connections with the thalamus, striatum, cortex, PAG, and a number of other structures (Ikemoto & Panksepp, 1999). The NAc also forms a feedback loop with the VTA, allowing further upregulation of dopaminergic tone when salience has been identified thus contributing to instrumental conditioning (Hagelberg et al., 2004). Numerous studies have shown that drugs of abuse are linked with dopaminergic upregulation in the NAc, especially in the shell (Di Chiara et al., 2004; Marinelli, Rudick, Hu, & White, 2006). One study shows that both D1 and D2 receptor stimulation in the NAc is required to elicit self administration (Nakjima, Xinhe, & Lau Loong, 1993).

Rhes could play a vital role in regulating the ability of dopaminergic inputs to affect novel stimulation and continued assignment of salience to exogenous cues. Rhes' ability to modulate both D1 and D2 receptor signaling allows a certain amount of error signal to the system. In mice lacking the Rhes protein, signaling for D1 receptors should increase and D2 should decrease. It is not clear how this will affect reward, as the circuitry within the NAc is not clearly defined. If in fact activation of both receptor subtypes is crucial to reward learning, then lack of Rhes may cause an attenuation of learning due to overactivation of D1 receptors and underactivation of D2 receptors.

In the current study we sought to determine if Rhes has effects on either the analgesic pathways of the periaqueductal gray and if there is a possible effect on mesolimbic dopaminergic pathways involved in analgesia, reward, and locomotion. The Rhes protein has been shown both through behavioral and molecular studies to modulate the effects of GPCRs. Rhes has been shown to differentially effect G_s and $G_{i/o}$ dopamine signaling as well, and we investigated the behavioral effects of this signaling on the mesolimbic reward and analgesia pathways.

Locomotion

Rhes is expressed in the medium spiny neurons (MSNs) of the striatum (Harrison & LaHoste, 2006; Spano et al., 2004). MSNs of the striatum are inhibitory GABAergic cells of two types, those expressing D1 receptors, Substance P, and dynorphins, and those expressing D2 receptors and enkephalins. The cells expressing D1 receptors make up the direct circuit of the striatal behavioral activation system, and the cells expressing D2 receptors compose the indirect pathways of the striatal behavioral activation system. In the absence of dopamine, the direct and indirect pathways act to suppress behavior, but when presented with stimuli that are salient, dopaminergic projections from the substantia nigra activate the pathways allowing the direct and indirect pathways to disinhibit the thalamus, thus allowing behavioral responses to proceed (Kandel et al., 2000). However, one review suggests that circuitry in the striatum is not so straightforward, as there are medium spiny cells that coexpress D1 family and D2 family receptors, as well as cholinergic interneurons expressing D1/D2 receptors (Nicola, Surmeier, & Malenka, 2000).

In vivo studies have shown that Rhes is normally modulatory of the effects of the activation of the striatal system. In rhes$^{-/-}$ mice, Spano et. al. (2004) showed a slight decrease in locomotor activation. In the same study it was also shown that rhes$^{-/-}$ mice showed marked motor coordination deficits as measured by falling in the rotarod test (Spano et al., 2004). One study found that Rhes normally provides negative modulation of behaviors that are specific to D1/D2 costimulation and D2 stimulation whereas certain D1 behaviors are facilitated (Quintero et al., 2008). Errico and colleagues, using pharmacological ligands specific to D1 and D2 receptors, show that D1 receptor-mediated behaviors are increased in rhes$^{-/-}$. They also showed an increase in D2-antagonist mediated cataleptic response (Spano et al., 2004). These studies all show support for the involvement of Rhes in motor circuitry. However, it is not clear as to exactly how Rhes works at the receptor level to modulate behavior. In the current study, we intend to measure locomotor activity in the context of MOR stimulation of the ventral tegmental area, as this behavior has been well characterized. Specifically we hypothesize rhes$^{-/-}$ mice will show increased locomotion, replicating previous studies.

Design and Methods

Genotyping

All procedures carried out in this study were done under the approval and supervision of the University of New Orleans Institutional Animal Care and Use Committee (Approval #08-001 & #08-002).

All mice used in this study have been bred locally from a colony generously donated to Dr. Gerald LaHoste by Dr. Daniela Spano. Mice were created of a CD1 and then backcrossed 10 generations onto a C57BL/6 background. Using a site specific homologous recombination, a null mutation was created at the Rhes locus resulting in a strain of mice null for the Rhes gene. An EGFP cassette was inserted into the locus of the Rhes gene allowing access to a reporter gene for genotyping. To determine the genotype of the mice, tail biopsies were performed to provide a DNA sample of each animal. DNA was amplified using two separate polymerase chain reactions (PCR), one using primers for the wild type allele and one using primers for the EGFP allele which only recognizes the null mutation. Products were separated and imaged using gel electrophoresis. Only male mice between 2-5 months of age were used for behavioral testing.

Tail Biopsy

Tail biopsies were performed on mice to render a sample of DNA for genotyping. Mice were anesthetized using 100-150 mg/kg of a ketamine/xylazine solution. Depth of anesthesia was checked using toe pinch and corneal reflex. Once the animal displayed no reflex, the distal 2-5 mm of its tail was clipped using a straight razor, and the site was

cauterized using a razor blade heated over a spirit lamp. The tail clipping was placed in a solution containing 300µl of DirectPCR™ lysis reagents and 11.6µl of proteinase K. Samples were placed on rotation in an incubator at 55˚c overnight to allow for complete cell lysis. Animals were monitored until they had fully recovered from anesthesia.

Polymerase Chain Reaction

After tail samples had incubated at 55˚C overnight, they were placed in an oven at 85˚c for approximately 1 hour to deactivate the proteinase K, an enzyme that can inactivate the PCR reaction enzymes. Reactions were prepared consisting 12.5µl GoTaq Green Master Mix™ (400 µM dNTPs, 3mM $MgCl_2$, Taq Polymerase, & proprietary buffer), 3µl of sense and antisense primers, 11.5µl of nuclease free water, and 0.5µl of DNA sample. Primers for the wild type gene include upstream 5'-TCCTAGCTCAGCGAGAGGAA-3', and downstream 5'-CTAGACAGGGCCCACAGAGA-3'. Primers for the EGFP reporter include 5'-CCTACGGCGTGCAGTGCTTCAGC-3', and 5'-GCGAGCTGCACGCTGCGTCCTC-3'. Wild type reactions were allowed to anneal at 60˚C for 30 seconds with a 1 minute extension time, whereas EGFP reactions were allowed to anneal at 55˚C for 30 seconds with a 1 minute extension time. Both reactions were allowed to complete 35 cycles and then held at 4˚C to stop the reaction.

Gel Electrophoresis

Gel electrophoresis was carried out using a 3% agarose gel to allow proper separation of PCR products. 5µl of each PCR reaction sample was placed in each well. A 100bp ladder was used at the distal wells of the gel providing a standard to judge the

length of the alleles. Samples were run through the gel for 5 minutes at 35v to allow the DNA to move into the gel evenly. After the initial 5 minutes the voltage was increased to 95v, and the DNA was allowed to run until the marker dyes approached the edge of the gel. The gel was post-stained using a 0.5µg/ml solution of ethidium bromide. The gel was then imaged using a Biorad™ gel imager. Expected size of the wild type gene was 400bp, while expected size of the EGFP reporter was 345bp.

Supraspinal Analgesia

To assess the role of Rhes in centrally mediated analgesia we used the formalin test paradigm in mice (Tjolsen, Berge, Hunskaar, Rosland, & Hole, 1993). In the formalin test, subjects are injected with a 5% formalin solution in the plantar surface of the hindpaw. Pain was quantified as the amount of time the animals spend licking the site of injury. Formalin initially causes painful injury to the tissue at the site of injection leading to immediate nociception due to activation of c-fibers (Tjolsen et al., 1993). However, five minutes post injection the original injury is no longer painful as displayed by lack of licking and flinching behavior in the animals due to the fixative ability of the formalin. The animals will then begin to lick approximately ten minutes post injection leading to the second phase of the formalin test. In this phase, centrally mediated input in the dorsal horn has been sensitized due to the initial c-fiber activation, and now allows random firing of the nociceptive pathway despite lack of painful injury (Tjolsen et al., 1993). This second phase of the formalin test is thus a model of chronic inflammatory pain that is the result of the failure of efferent pathways to modulate the activation of c-fibers in the dorsal horn (Tjolsen et al., 1993).

To measure the effects of Rhes on centrally mediated analgesia, separate groups of $rhes^{+/+}$ and $rhes^{-/-}$ mice were tested in the formalin test after receiving i.p. injections of 0.0, 3.0 or 10.0 mg/kg morphine. Thirty minutes after drug administration, animals received a 40µl s.c. injection of 5% formalin into the plantar surface of one hind paw and were placed in an elevated 6"x 6" Plexiglas enclosure with a glass floor. A mirror positioned at 45º under the floor allows for un-obscured observation of the animal's behavior. Time spent licking the injected hindpaw was recorded at five minute intervals for one hour.

Spinal Analgesia

To assess the role of Rhes in spinally mediated analgesia we used the tail flick test (Lewis, Sherman, & Liebeskind, 1981; Paronis & Holtzman, 1992). In the tail flick test, a halogen heat source is directed at the distal tip of the tail, and the amount of time to withdraw the tail from the source is recorded. In this test the withdrawal of the tail is a reflex mediated by local interneurons in the spinal cord. As c-fibers are activated, they cause activation of a local interneuron that synapses on a motor fiber. In this way, as the nociceptors are turned on, so is the reflex to withdraw. This reflex requires no input from the CNS (although it can be modified by descending input), and therefore allows a test of efficacy of analgesics in spinally mediated nociception by their ability to block nociceptors from activating the withdrawal reflex. We hypothesize that presence or absence of Rhes will have a minimal affect on this reflexive nocifencisive response due to its localization. The presence of Rhes has not been tested in the spinal cord and at present we have no a priori evidence of it being expressed there, therefore it should show minimal involvement in a spinal reflex.

To determine the effects of Rhes on spinally mediated analgesia, separate groups of rhes$^{+/+}$ and rhes$^{-/-}$ mice were tested in the tail flick test after receiving i.p. injections of 0.0, 1.0, 3.0, or 10.0 mg/kg morphine. For tail flick testing, animals were held loosely in a towel, and latency to withdraw from a 1 cm^2 beam of concentrated halogen light shone on the distal 1/3 of their tail was recorded. Latency to withdraw was recorded prior to injection, at 5 minutes post-injection, 15 minutes post injection, and at 15 minute intervals thereafter for 90 minutes.

Tolerance

Tolerance is an overall term applied to a set of mechanisms that are responsible for the decreased ability of a drug to produce an effect. In the case of exogenous opioids, much research is dedicated to determining what these mechanisms are. Current research trends lean toward the idea that continued activation of MORs leads to a conformational change in those receptors making them desensitized to the exogenous opioid, and consequently upregulating the cAMP system. Compensatory mechanisms attempt to correct this system and lead to development of tolerance. Given that Rhes has been shown to couple to several types of GPCR, it is possible that it may play a role in MOR signaling. This part of the experiment was an exploratory analysis of Rhes' effects on opioid tolerance.

To test the development of tolerance, rhes$^{+/+}$ and rhes$^{-/-}$ mice were given repeated daily doses of morphine sulfate at a dosage of 3mg/kg. Before this dosing regimen was started, mice were baseline tested for tail withdrawal latency using the tail

flick test as described above. Mice were tested again after the fifth day of repeated morphine injections, and latency to withdraw was recorded.

Withdrawal

Opiate withdrawal has been well characterized in the literature, and is a function of the supersentivity of the compensatory mechanism of tolerance as explained above. To test withdrawal we used a naloxone precipitated withdrawal test. $Rhes^{+/+}$ and $rhes^{-/-}$ mice were administered repeated injections of morphine over a period of five days at a dose of 3mg/kg. Morphine was allowed to reach peak efficacy by allowing 30 minutes to pass. Animals were then injected I.P. with a 3mg/kg dose of naloxone, a competitive mu opioid antagonist. Animals were then placed in a 6 inch diameter tube that is 1 foot tall. Number of jumps in which the animal's 4 paws cleared the floor were counted. Also, "wet dog shakes" in which the animals entire body shakes much like that of a wet dog were counted. After testing was complete number of fecal boli left in the chamber were counted as well. These scores were combined into a global withdrawal score by assigning a weight of 1 to jumps, 5 to wet dog shakes, and 5 to fecal boli, and summed for each animal (Christie, 2008).

Conditioned Place Preference

To assess the role of Rhes in reward we used the conditioned place preference paradigm (Carr, Fibiger, & Phillips, 1989; Tzshentke, 1998). The conditioned place preference (CPP) paradigm is a test designed to assess the rewarding properties of a stimulus based on the animal's preference for cues paired with that rewarding stimulus. Rewarding stimuli such as drugs of abuse cause activation of the NAc, the pleasure

center of the brain. The NAc receives input from the cortex, amygdala, and VTA (Kandel et al., 2000). When a stimulus is rewarding, the animal is conditioned to seek the context which the stimuli have been previously paired, thus showing a place preference. The animals in this study were placed in two equally salient contexts, one paired with morphine and the other with saline, and the ability of the drugs to establish a preference for a side were measured by the amount of time spent in each side. This allowed us to determine if Rhes affects place preference, and reward.

To assess the effects of Rhes deletion on the rewarding properties of morphine, separate groups of $rhes^{+/+}$ and $rhes^{-/-}$ mice were tested in the CPP paradigm. To do this, a 6" x 12" Plexiglas chamber was constructed so that the walls and flooring were interchangeable. During habituation and testing trials, the boxes were in a divided configuration, split equally into 6" x 6" areas, one with white walls and a wood-chip floor and another with black walls and a wire mesh floor. On drug-pairing trials, the box was in a whole configuration, where the entire 6"x12" chamber was arranged to have either white walls/wood chip floor or black walls/wire mesh floor. A camera positioned above the clear Plexiglas roof of the chamber recorded all behavior during habituation, the first morphine trial and the test trial.

On day one, animals were placed in the divided chamber for a 10-minute habituation trial to detect any inherent preference for one side or the other. The design was unbiased, and equal number of animals showed a preference for each side during habituation (data not shown). For the next 3 days, with the boxes in the whole configuration, i.p. injections of saline (morning) or 10 mg/kg morphine (afternoon) were paired with the environments that were preferred and not preferred during habituation,

respectively. Injections preceded placement in the CPP box by 30 minutes to allow the drug to take effect, and animals were left in the box for 30 minutes to allow for the formation of associations between the drug effects and environmental cues. On the final day, drug-free animals were placed in the divided box, and time spent on each side was recorded. Difference scores were calculated such that time spent in the conditioned side was subtracted from the time spent in that side on day 1.

Locomotion

To detect the relationship between Rhes and the locomotor effects of morphine, video recordings of the animals' response to a 3mg/kg acute dose of morphine were analyzed using AnyMaze ® software. Mice were placed into a 6" x 12" box and video recorded for 10 minutes at 30 minutes post morphine administration. Distance traveled was recorded for 5 minutes beginning after the animal had been in the chamber for 2 minutes to allow for habituation and compared for the $rhes^{+/+}$ and $rhes^{-/-}$ mice.

Statistical Analysis

For the formalin test of supraspinal analgesia, results are presented as time spent licking group means ± SEM for each dose by time. For statistical analysis, data were divided into early (1^{st} 5 minutes) and late (remaining 55 minutes) phases, and 2-factor (genotype x phase) repeated measures ANOVAs were performed for each dose. Tail flick results are presented as a time course for each genotype, which was analyzed by 3-factor (dose x time x genotype) repeated measures ANOVA. These data are also presented as latency to withdraw group mean ± SEM for each dose by genotype at the 30 minute time point. Tolerance is presented as tail flick latency %MPE [(Tested

Latency-Baseline Latency)/(Cutoff Latency-Baseline Latency) X 100] group mean ± SEM for acute and repeated administration of morphine sulfate. These data were analyzed by a two factor (genotype x phase) repeated measures ANOVA. Results of withdrawal are presented as global withdrawal score group mean ± SEM. Global withdrawal score means were compared by genotype using independent samples t-test. Results of the CPP test are presented as group mean of difference score ± SEM. These results were analyzed using independent samples t-test. Locomotion data are presented as group mean ± SEM. The data were analyzed using independent samples t-test. Follow up planned comparisons were made as necessary, and multiple comparisons were subject to a modified Bonferroni correction beginning with an α of $p < 0.05$.

Results

Supraspinal Analgesia

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 [$F(1,17)=0.155$, $p >0.05$] (Figure 1a and b). However, when administered a dose of 10 mg/kg of morphine, rhes$^{-/-}$ mice showed significantly less licking in the late phase [$F(1,18)=6.587$, $p <.05$] compared with rhes$^{+/+}$ mice (figure 1c). This finding suggests that at higher doses of morphine, rhes$^{-/-}$ mice show increased supraspinal analgesia. Figue 1d shows the time course of formalin induced licking for each genotype by dose.

Supraspinal Analgesia

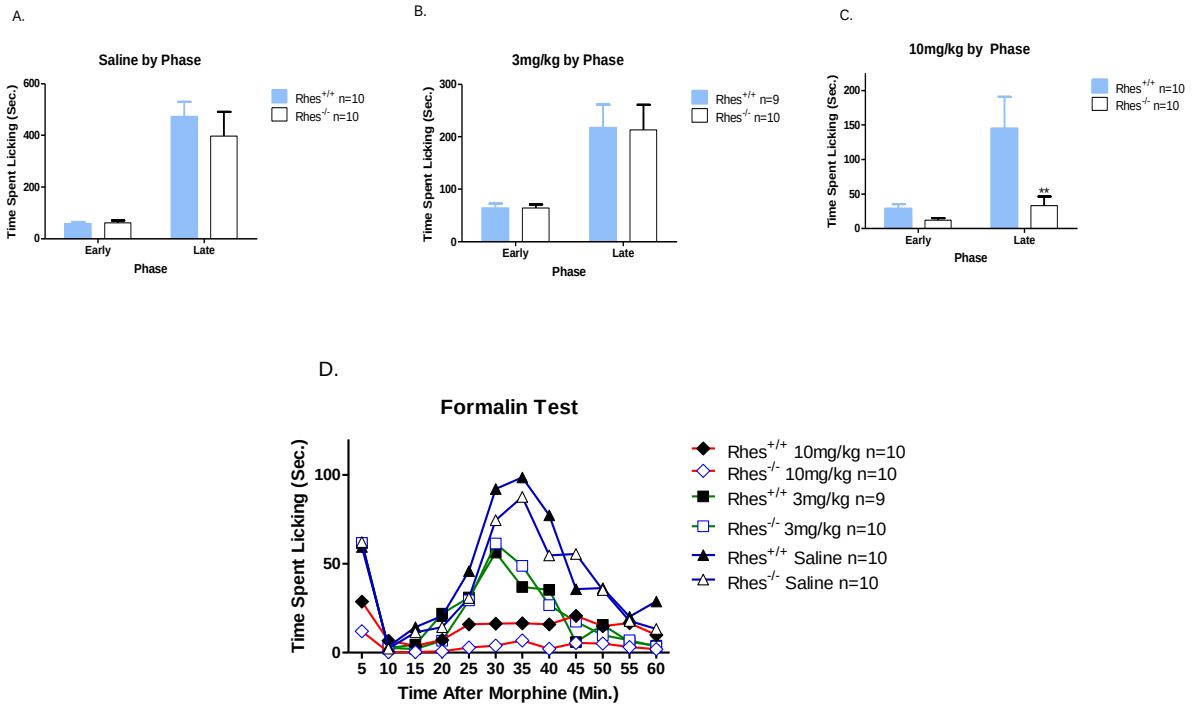

Figure 1. No differences in supraspinally mediated analgesia were observed between rhes$^{-/-}$ and rhes$^{+/+}$ mice when administered (A) 0mg/kg or (B) 3 mg/kg morphine. However, at (C) 10mg/kg of morphine, rhes$^{-/-}$ displayed significantly less licking than rhes$^{+/+}$ mice. (D) Time course of formalin nociception displayed as time spent licking at 5 minute intervals beginning with injection of formalin.

Spinal Analgesia

Results for the tail flick test yielded an expected interaction effect for time x dose [$F(27)=5.195$, $p < 0.001$], as well as an interaction effect of time x genotype [$F(9)=2.516$, $p <0.01$] (figure 2a and b). Follow up comparisons of time points by genotype indicate that $rhes^{-/-}$ mice showed a significant shift in time to onset of analgesia such that the maximum efficacy was realized at 30 minutes [$t(29)=3.303$, $p <0.01$], whereas $rhes^{+/+}$ mice did not reach maximum efficacy until the 45 minute time point [$t(29)=2.879$, $p <0.01$]. Analgesia was attenuated in $rhes^{+/+}$ beginning at the 90 minute time point [$t(29)=-2.532$, $p <.01$], whereas $rhes^{-/-}$ mice remained analgesic [$t(29)=-1.557$, $p >0.05$]. An unexpected main effect of genotype was also present [$F(1,52)=6.168$, $p < 0.05$]. A planned comparison of the 30 minute time point for each dose reveals that at 1mg/kg [$t(8)=3.598$, $p < 0.01$] and 3mg/kg [$t(17)=3.577$, $p < 0.01$], $rhes^{-/-}$ mice are more sensitive to the effects of morphine than $rhes^{+/+}$ (figure 2 c). It should be noted that a cutoff of 12 seconds was imposed in this test to prevent tissue damage. It is very possible that $rhes^{-/-}$ would have shown much more analgesia when administered 10mg/kg of morphine, however, at every time point past 15 the $rhes^{-/-}$ mice reached the cutoff, whereas the $rhes^{+/+}$ mice did not achieve a ceiling effect. Thus $rhes^{-/-}$ mice display enhanced spinal analgesia relative to $rhes^{+/+}$ littermates.

Spinal Analgesia

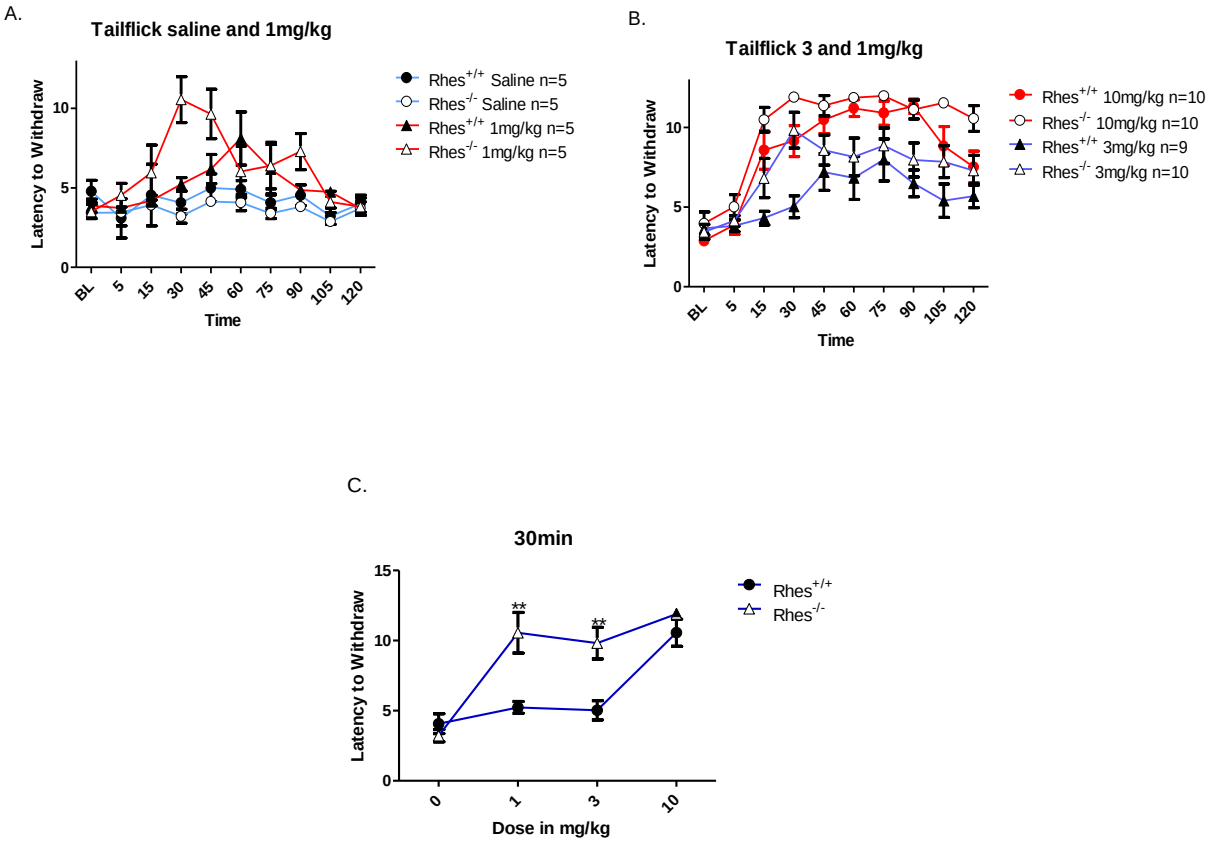

Figure 2. Time course of tail flick test of spinally mediated nociception presented as time to withdraw for (A) 0mg/kg and 1mg/kg morphine, as well as (B) 3mg/kg and 10mg/kg of morphine. (C) Planned comparison of 30 minute time point for each genotype by dose indicating rhes$^{-/-}$ show greater sensitivity to 1mg/kg and 3mg/kg of morphine when compared with rhes$^{+/+}$ mice.

Tolerance & Withdrawal

To test for effects of Rhes on adaptation to repeated morphine treatment, mice were given repeated injections (over 5 days) of morphine and tested for tolerance, using the tail flick test, to the analgesic effects of 3 mg/kg morphine at 30 minutes post-drug administration. Results of the tolerance test indicate significant main effects for genotype [$F(1,15)=33.10$, $p < 0.01$] and time [$F(1,19)=6.354$, $p <0.05$] (figure 3), and no significant interaction [$F(9, 153)=1.649$, $p >0.05$]. $Rhes^{+/+}$ mice showed significantly reduced %MPE after both the acute [$t(16)=-4.395$, $p <0.001$] and repeated morphine administration [$t(16)=-6.191$, $p <0.001$] compared with $rhes^{-/-}$ mice. This result is expected given that $rhes^{+/+}$ show a later onset of morphine induced analgesia compared with $rhes^{-/-}$ as presented in the tail flick test. $Rhes^{+/+}$ mice also showed significantly reduced %MPE between the acute and chronic administrations [$t(9)=-2.303$, $p <0.05$], whereas $rhes^{-/-}$ showed no significant difference between administrations [$t(9)=-.362$, $p >0.05$] indicating induction of tolerance in $rhes^{+/+}$ but not $rhes^{-/-}$ mice (Figure 3a). Global withdrawal scores for $rhes^{-/-}$ mice were significantly decreased [$t(19)=-4.241$, $p<0.001$] as compared with $rhes^{+/+}$ mice (figure 4).

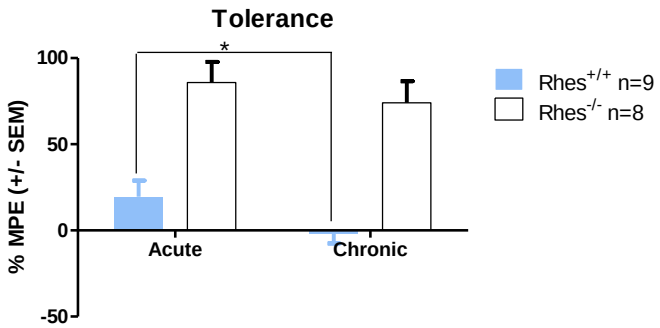

Figure 3. Tolerance to the effects of morphine represented as acute (day one) percent maximum possible effect versus repeated (day 5) percent maximum possible effect. These results indicate that rhes$^{+/+}$ mice show a significantly decreased maximal possible analgesic effect of morphine after repeated administrations, whereas rhes$^{-/-}$ showed no difference between acute and chronic administrations.

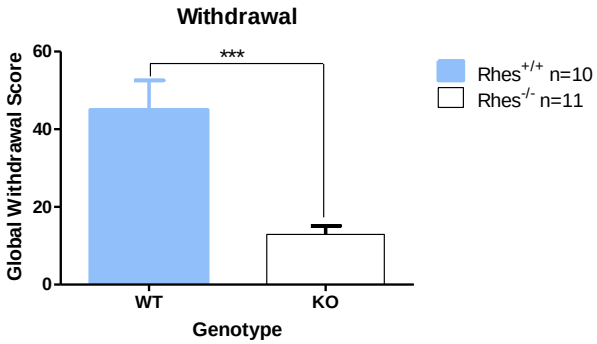

Figure 4. Withdrawal displayed as global withdrawal scores by genotype. Results show a significantly lower amount of withdrawal behaviors measured in rhes$^{-/-}$ mice when compared with rhes$^{+/+}$ mice.

Conditioned Place Preference and Locomotion

Rhes$^{-/-}$ mice did not show any difference in conditioned place preference when compared to rhes$^{+/+}$ [$t(6)=0.702$, $p > .05$] (figure 5). Similarly, rhes$^{-/-}$ mice did not show any significant difference in locomotor activity when compared to rhes$^{+/+}$ mice [$t(7)=0.092$, $p > 0.05$] (figure 6). It should be noted that the sample size for these groups was very low.

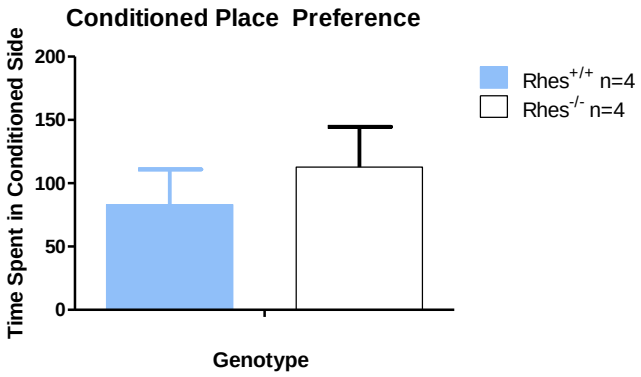

Figure 5. Place preference displayed as time spent in the conditioned chamber by genotype. $Rhes^{-/-}$ mice did not significantly differ in morphine induced preference when compared to $rhes^{+/+}$ mice.

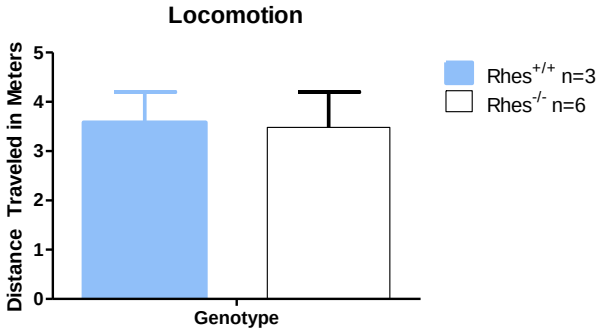

Figure 6. $Rhes^{-/-}$ mice did not travel significantly farther than $rhes^{+/+}$ mice when administered 3mg/kg or morphine.

Discussion

Results of this study indicate that we have supported our hypothesis that rhes$^{-/-}$ mice show increased supraspinal analgesia when compared to rhes$^{+/+}$ littermates. We found evidence to the contrary of our hypothesis that rhes$^{-/-}$ mice would show no differences in spinally mediated analgesia when compared with rhes$^{+/+}$ mice. Further exploratory experiments regarding opioid induced tolerance and withdrawal have shown that lack of Rhes does modify opioid tolerance and withdrawal. We also failed to find any differences between rhes$^{-/-}$ mice and rhes$^{+/+}$ littermates in opioid induced locomotor activity or conditioned place preference, however these hypothesis were not adequately tested.

Supraspinal Analgesia

Rhes$^{-/-}$ mice show an increase in supraspinal analgesia. However, interpreting the effects of Rhes' action in this centrally mediated response becomes important. As demonstrated (Capasso, 2008), D1/D2 agonists in conjunction with a potent mu opioid agonist can cause an increase in spinally mediated analgesia, however, a D2 agonist (Taylor et al., 2003) can similarly effect antinociception in the NAc. As hypothesized, lack of Rhes may be able to cause an upregulation of activation of $G_{\alpha s}$ as a result of increased dopaminergic afferent activation from the VTA into the NAc, however this hypothesis is inadequately supported by the current literature. As discussed below, analgesia in the striatum is a D2, not a D1 effect. It cannot be ruled out that lack of Rhes' effect is a result of the attenuation of signaling through $G_{\alpha i/o}$, in which case any agonist stimulation would result in a net increase of cAMP as a result of inability to inhibit AC. These mechanisms are not mutually exclusive, and it is also equally

plausible that lack of Rhes results in the overactivation of $G_{\alpha s}$ and understimulation of $G_{\alpha i/o}$. Antinociception in the nucleus accumbens has been shown to be an effect of stimulation of D2 receptors with or without the involvement of D1 receptors (Magnusson & FIsher, 2000; Morgan & Franklin, 1991; Taylor et al., 2003). In our experiments, $Rhes^{-/-}$ mice do not show a change in baseline nociception in either the formalin or tail flick test, suggesting that Rhes is not contributing to pronociceptive processes. This does not support the hypothesis that lack of Rhes results solely in an upregulation of cAMP, be it through increases in AC activation via $G_{\alpha s}$ or attenuation of signaling through $G_{\alpha i/o}$. In fact, given this hypothesis, lack of Rhes should provide for pronociception and at least some attenuation of morphine induced analgesia.

Spinal Analgesia

Further complication in interpreting the results of the supra-spinally mediated analgesia results from the test of spinally mediated analgesia. $Rhes^{-/-}$ mice show greater sensitivity to morphine induced analgesia as compared with $rhes^{+/+}$ mice. No prior attempt has been made to asses Rhes expression in the spinal cord. In this study we were operating under the assumption that any involvement of Rhes in Supraspinal analgesia would be the result of descending modulation to the spinal cord. Indeed, morphine has been shown to cause a supraspinal activation of neurons responsible for inhibiting the tail flick reflex (Hanaoka et al., 1978; Sinclair, Main, & Lo, 1988), but this is complicated by the fact that much of morphine's effect can be attributed to local action at the site of the dorsal horn neurons (Soja & Sinclair, 1983). In the current study animals were injected with morphine systemically, suggesting that at least much of the anti-nociception in the tail flick reflex is a result of morphine acting at μ-opiate receptors

in the dorsal horn. However, involvement of central dopaminergic systems in the control of the tail-flick reflex has been demonstrated (Capasso, 2008; Hnasko, Sotak, & Palmiter, 2005; King, Bradshaw, Chang, Pintar, & Pasternak, 2001; Kiritsy-Roy et al., 1994). The methodologies employed in these studies do little to pinpoint the exact locus of dopamine's control on antinociception at the spinal level. Dopamine's actions on spinal antinociception have been seen not only in the striatum but in the PAG (Meyer, Morgan, Kozell, & Ingram, 2009), and through projections to the spinal cord itself (Levant & McCarson, 2001; Millan, 2002). A western blot provided by Dr. Laura Harrison reveals no detection of Rhes in the spinal cord; however this may be an aberration of the antibody, which may not be sensitive enough to detect low levels of protein. Preliminary RT-PCR does suggest that rhes mRNA is present in the spinal cord. Future evaluation of the presence or absence of Rhes expression in the spinal cord could help to explain these results and guide future work.

One explanation in this case would be that Rhes, being striatally enriched, exerts its antinociceptive effects via dopamine receptors in the striatum. This is an incomplete explanation given previous findings regarding the involvement of Rhes in dopamine signaling. Future work using site specific delivery of specific agonists and antagonists in $rhes^{-/-}$ and $rhes^{+/+}$ mice would provide much information regarding the contribution of Rhes to dopamine signaling in analgesia. Given the result of both the supraspinal and spinal test of analgesia, alternative explanations of Rhes' effect on analgesia are warranted.

Tolerance and Withdrawal

Rhes$^{-/-}$ mice showed no effect of tolerance and very little withdrawal to morphine compared to rhes$^{+/+}$ mice. These results are intriguing. Tolerance is the phenomenon by which increasing doses of a drug are needed to yield the same behavioral effect as a function of repeated use of that drug. The cellular response of repeated administration of the drug leads the cell to undergo compensatory mechanisms for prolonged exposure. It is widely accepted that morphine shows a very high affinity and efficacy at the MOR although it is considered a partial agonist. The MOR is a GPCR much like D1 and D2 family receptors, couples to $G_{\alpha i/o}$, and when activated causes the inhibition of cAMP (Harrison, Kastin, & Zadina, 1998). A major effect of the activation of the MOR is the activation of the inwardly rectifying potassium channel which acts to shunt excitatory transmission, thereby inhibiting the cell (Christie, 2008; Harrison et al., 1998).

Administration of morphine causes rapid onset of tolerance, leading to the need for increasing amounts of drug to evoke this same cellular response. The mechanisms that underlie tolerance are not so clear. Many studies have shown that tolerance is affected by a multitude of cellular and synaptic events. The overarching effects of these events seem to involve the ability of the receptor to couple to its effector (desensitization), ability of the receptor to internalize, and the sensitization of AC (Christie 2008). Each one of these mechanisms is reliant upon the activation of the GPCR and the subsequent activation of the signaling cascades that regulate them.

The current literature suggests that the locus of Rhes actions in dopamine signaling lies within the pathway between receptor and the first effector (AC). This

mechanism is more or less analogous in opiate receptor signaling. Given in vitro evidence, Rhes has been shown to affect the signaling of other GPCRs besides the D1 and D2 family receptors. Rhes is heavily expressed in the striatum in neurons that are known to coexpress both dopamine and MORs. In light of these findings, it is not out of the question that Rhes may affect signaling through opiate receptors, and in particular the MOR. Unpublished data from Laura Harrison's lab provide compelling evidence that this may be the case. Assays of GTP binding in striatal membrane preperations using Endomorphin-1, a potent endogenous opioid, have found that Rhes promotes binding to $G_{\alpha i/o}$. Given that Rhes has been shown to promote signaling through $G_{\alpha i/o}$, loss of Rhes should hypothetically cause an inability of MOR to inhibit production of cAMP through AC, if Rhes does indeed act at the MOR the same as what has been seen in the D2R and M_2-muscarinic receptor. This does not fit very well with what has been observed behaviorally in the tests of supraspinal and spinal analgesia. Lack of Rhes in vivo has been shown here to increase morphine induced analgesia, while also decreasing tolerance and withdrawal.

One signaling pathway can explain both of these phenomena. Rhes has been shown to possibly interfere with the binding of GPCR to the heterotrimeric subunits of the G-protein motif. Both Thapliyal et al. (2008) and Hill et. al (2009) hinted at the idea that Rhes may interfere with the association of the α subunits to either the receptor or the βγ subunit. Rhes could promote signaling through βγ, thereby activating the phospholipase C - inositol 1,4,5-triphosphate/diaglycerol - protein kinase C pathway (PLC-PKC). PKC has been shown to be important in opioid analgesia, tolerance, and withdrawal. Numerous studies using pharmacological and genetic approaches have

shown that blockade of the PKC can cause an attenuation of MOR desensitization and tolerance, to some degree based on the agonist (Bailey et al., 2009; Xie et al., 1999). This mechanism seems to be uniquely efficacious in the induction of morphine induced tolerance whereas tolerance to higher efficacy MOR agonists (i.e. DAMGO) operates through a GRK-arrestin mediated mechanism that promotes receptor internalization and recycling, a phenomenon that does not happen in agonist stimulation of MORs using morphine (Hull et al., 2010). Studies have also shown that blocking PKC can attenuate features of opioid dependence and withdrawal (Cerezo, Laorden, & Milanes, 2002; Kogan & Aghajanian, 1995; Maldonado, Valverde, Garbay, & Roques, 1995; Valverde, Tzavara, Hanoune, Roques, & Maldonado, 1996).

Behavioral studies have also indicated that blockade of the activation of the PLC-PKC pathway results in an increase in antinociception. One study found that PLCβ3 knockout mice had a 10 fold increase in morphine induced antinociception (Xie et al., 1999). Another study using an agent selected to bind and inactivate signaling through βγ subunits found a similar outcome with an increase in morphine induced antinociception (Mathews, Smrcka, & Bidlack, 2008). The phenotype of $rhes^{-/-}$ mice closely resembles that of the animals in which βγ signaling, and subsequently PLC-PKC activation, was blocked. Rhes has been shown to increase the GTP binding of $G_{\alpha i/o}$ subunits when presented with a potent MOR agonist. This would cause an increase in the disassociation of the heterotrimeric complex, allowing an increase in the activation of βγ subunits. This in turn could lead to an increase in PKC mediated phosphorylation of MOR, and receptor desensitization.

Another possibility is that Rhes activates PLC-PKC through $G_{\alpha q}$ signaling. $G_{\alpha q}$ has been shown to promote activation of PLC-PKC (Wu, Lee, Rhee, & Simon, 1992). Quintero et al. (2008) showed a decrease in grooming in $rhes^{-/-}$ mice, a behavior which is mediated by $G_{\alpha q}$ signaling through D1 receptors. Promotion of βγ signaling through MORs, and promotion of $G_{\alpha q}$ through D1 colocalized with MORs receptors could both contribute to the phenotype seen in this study.

Conditioned Place Preference and Locomotion

No significant differences in locomotion or conditioned place preference were found when comparing $rhes^{-/-}$ and $rhes^{+/+}$ mice. Previous work has shown that $rhes^{-/-}$ mice show an increase in D1 receptor mediated locomotion. Failure to reproduce this result as hypothesized may be due to interactions in signaling between opioid and dopamine systems. Treatment with a D1 agonist in conjunction with morphine increases locomotor activation (Funada, Suzuki, & Misawa, 1994). Deletion of Rhes would theoretically provide for an increase in signaling through $G_{\alpha s}$ causing an increase in locomotion, however no differences were found. One possible explanation is that, in the absence of Rhes, modification of MOR signaling in the same cells activates a compensatory mechanism that attenuates the effects of upregulation in D1 signaling.

No modification of place preference was observed in $rhes^{-/-}$ mice. This result can be complicated to interpret given that dopaminergic involvement and MOR signaling can affect reward differentially (Petit, Ettenberg, Bloom, & Koob, 1984). Studies show that opioid induced upregulation of cFos and ΔFosB, regulators of gene expression for acquiring and maintaining place preference, can be attenuated when given a D1

antagonist (Liu, Nickolenko, & Sharp, 1994; Muller & Unterwald, 2005; Nestler & Kelz, 2000). Given that deletion of rhes should promote increased signaling through D1 receptors, deletion of rhes should therefore induce greater place preference. As with locomotion, the unknown variable is the role of Rhes in MOR signaling. Deletion of Rhes may alter the crosstalk between dopaminergic and MOR signaling pathways.

Interpreting the results of these two investigations is difficult due to methodological issues. Neither experiment had a very large sample, therefore, statistically speaking, the results are not robust enough to draw conclusions from. In both tests, only one dose of morphine was administered. Differences in groups may have been overshadowed by either floor or ceiling effects based on dosage. For example, in the test for locomotion, 3mg/kg of morphine is not enough to produce morphine hyperlocomotion as seen with 10mg/kg of morphine. This test would best be repeated using a logarithmic dosing scheme to compare groups such that measures were taken at 0, 1, 3, and 10mg/kg. This issue similarly affects the conditioned place preference test performed here. The dose of morphine used, 10mg/kg, is relatively high and caused robust place preference in both genotypes. Subtle differences between groups may not have been distinguishable at such a high dose. The study would also best be repeated using logarithmic dosing of morphine.

Conclusion

The results presented in this study provide strong evidence for the involvment of Rhes in opioid signaling. Opiate medications are the most widely used and efficacious pharmacological treatment for moderate to severe pain. Opiate medications, however, carry a very high liability including tolerance, withdrawal, abuse, and respiratory

depression. We have shown here a phenotype displayed by mice lacking the Rhes protein reduces behaviors associated with opiate drug treatment while increasing the efficacy of the desired effect of analgesia. Clinically, this finding is very important. Information regarding Rhes and MOR signaling can lead to the development of pharmacological interventions that maximize the efficacy of opiate treatments while minimizing the liabilities.

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