Conditional expression and signaling of a specifically designed G_i-coupled receptor in transgenic mice

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Received 18 September 1998; accepted 28 October 1998

To control G protein signaling in vivo, we have modified G protein–coupled receptors to respond exclusively to synthetic small molecule agonists and not to their natural agonist(s). These engineered receptors are designated RASSLs (receptor activated solely by a synthetic ligand). A prototype RASSL (Ro1) based on the G_i-coupled κ opioid receptor was expressed in transgenic mice under the control of the tetracycline transactivator (tet) system. Activation of Ro1 expressed in the heart decreased heart rate by up to 80%, an expected effect of increased G_i signaling. Maximal heart rate changes occurred in less than 1 min, demonstrating the speed of this inducible signaling system. This Ro1-mediated slowing of heart rate was also subject to desensitization, which lasted more than 24 h. Both the initial effect on heart rate and the desensitization occurred, even though Ro1 is derived from a human opioid receptor not normally involved in heart rate control. In addition, the tet system was used to induce Ro1 expression in hepatocytes and salivary gland, where G_i signaling is known to control physiologic events such as proliferation and secretion. These studies demonstrate that a RASSL can be inducibly expressed in several mouse tissues and used in vivo to activate G protein signaling in a controllable fashion.

Keywords: G protein signaling, engineered opioid receptor, synthetic ligand

Control of intracellular signaling pathways in vivo would allow modulation of physiologic events, such as proliferation, chemotaxis, and neurotransmission. Several conditional signaling systems that allow control over transcription and the dimerization of cytoplasmic signaling proteins have been described[1–4]. Although these systems are powerful, their conditional signaling events have relatively slow induction kinetics, and few have been tested in multiple tissues in vivo. Recently, we described a rapid conditional signaling system based on the modification of cell-surface G protein–coupled receptors (GPCRs)[5]. This large family of seven-transmembrane domain receptors transduces extracellular stimuli from hormones, chemokines, and neurotransmitters into intracellular signals. By changing specific amino acids in a GPCR, we greatly decreased receptor activation by endogenous ligands while maintaining activation by small molecule drugs. We call such a modified receptor a RASSL (receptor activated solely by a synthetic ligand)[5].

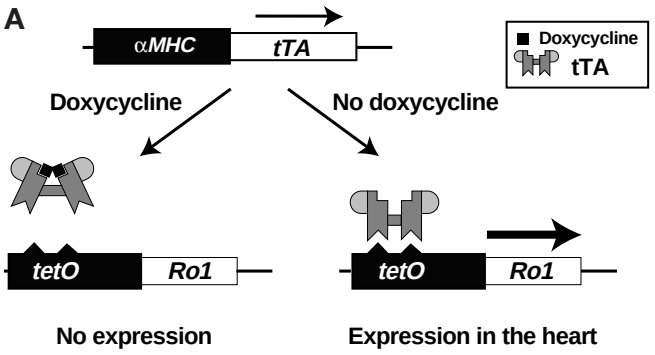
Our modified version of the human κ opioid receptor, Ro1 (RASSL, opioid, no. 1), contains δ opioid receptor sequences in the second extracellular loop and signals through a G_i pathway[5]. These modifications cause a 200-fold reduction in binding by the endogenous agonist (dynorphin) but maintain normal binding and activation by the small molecule drug spiradoline. Ro1 activation increases G_i signaling and subsequent proliferation of Rat-1a cells[5]. Although the tissue culture experiments with Ro1 were highly encouraging, our ultimate goal is to use Ro1 to control physiologic events in mammals.

We chose to study conditional activation of Ro1 in the heart because of the heart's known sensitivity to G_i signaling. Acetylcholine, which signals through the G_i-coupled M_2-muscarinic receptor in the heart, was identified by Otto Loewi in 1920 as the first neurotransmitter ("vagusstoff")[6]. Like Loewi, we are using the heart as a model system to study the physiologic effects of neurotransmitters. Short-term G_i-signaling events in the heart include inhibition of adenylyl cyclase and activation of a membrane potassium channel, resulting in a decreased heart rate (bradycardia)[7]. Recently, aberrant long-term G_i-signaling events have been implicated in human heart failure[8,9].

In the current study, we used the tetracycline transactivator (tet) system to inducibly express Ro1 in mouse heart, liver, and salivary gland. In mice expressing Ro1 in the heart, activation of the receptor by spiradoline caused bradycardia in <1 min that lasted for several hours and was subsequently desensitized for >24 h. These studies demonstrate that a RASSL can be expressed in several mouse tissues and used to control G protein signaling in vivo. Moreover, the speed of that signaling event is unique among conditional signaling systems.

Results

Conditional expression of Ro1. For conditional and tissue-specific expression of Ro1, we used mouse lines that express the tetracycline transactivator (tTA) gene under the control of the α myosin heavy chain promoter (*αMHC-tTA*), the liver-enriched activator protein element (*LAP-tTA*), and the mouse mammary tumor virus long terminal repeat (*MMTV-tTA*) (Fig. 1A)[10–12]. The *tTA* gene encodes a transcriptional activator that binds to and activates transcription from the *tetO* promoter element[1]. The Ro1 transgenic mouse line



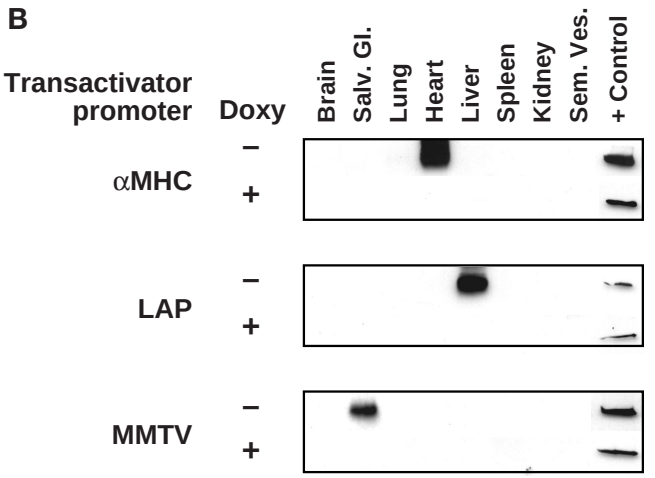


Figure 1. Conditional and tissue-specific expression of Ro1 in heart, liver, and salivary gland. (A) To obtain cardiac-specific doxycycline-regulated transgene expression, a transgenic mouse line containing *αMHC-tTA* was crossed with a second transgenic line containing *tetO-Ro1* to generate *αMHC-tTA/tetO-Ro1* mice. Removal of doxycycline from the drinking water of *αMHC-tTA/tetO-Ro1* mice allowed expression of Ro1 in mouse heart. To obtain liver-specific or salivary gland-specific expression of Ro1, the *LAP-tTA* and *MMTV-tTA* transgenic mouse lines were used, respectively. (B) Representative Western blots of immunoprecipitations from organ extracts of dual heterozygote mice either on (+) or off (–) doxycycline (Doxy). The gel band shown was the predominant 44-kDa band for immunoprecipitated Ro1 (see Fig. 2). In the mice treated with doxycycline, there was no detectable expression of Ro1. Fifty milligrams of tissue from each organ was used for the initial immunoprecipitation. This experiment was repeated in three separate sets of mice. Salv. Gl., salivary gland; Sem. Ves., seminal vesicle; + Control, FLAG-positive control peptide.

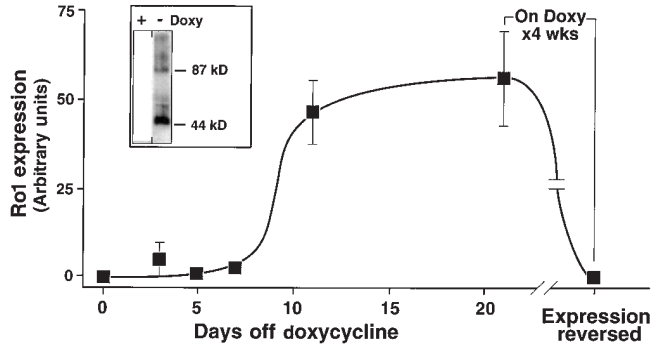


Figure. 2. Timing of Ro1 expression in the heart. Quantitative analysis of Western blots shows Ro1 expression to be near maximal 11 days after doxycycline was removed from the drinking water (day 0) (n = 3 at days 0, 3, 5, and 7; n = 5 at days 11 and 21). Readministration of doxycycline can reverse Ro1 expression ("Expression reversed," n=9). (Inset) Western blots of immunoprecipitation of hearts from two *αMHC-tTA/tetO-Ro1* mice are shown. In the mouse not treated with doxycycline (–Doxy), the entire gel lane represents immunoprecipitated Ro1 (44 kDa) and its glycosylated forms.

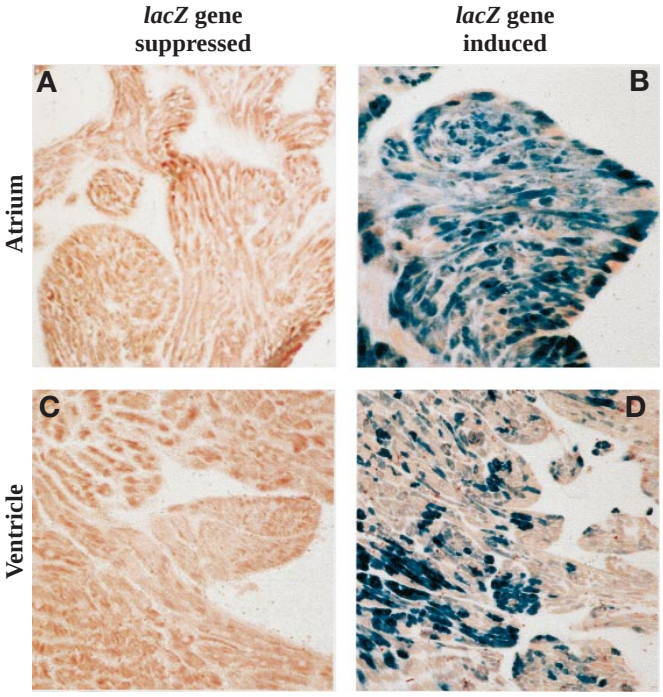


Figure 3. Inducible β-gal activity in the atria and ventricles of *αMHC-tTA/tetO-Ro1/tetO-lacZ* mice. 50× magnification of cross-sections of mouse heart stained for β-gal activity. Cardiac tissue with induction of *lacZ* expression demonstrates heterogeneous but high-level β-gal staining in atria (A and B) and ventricles (C and D). *αMHC-tTA/tetO-Ro1/tetO-lacZ* mice (n=3) were sacrificed 21 days after the removal of doxycycline (B and D). Control mice (n=3) showed no evidence of β-gal activity (A and C).

contains two co-integrated transgenes, *tetO-Ro1* and *tetO-lacZ*. Crossing the tissue-specific-*tTA* mice with the *tetO-Ro1* mice produced dual heterozygote mice in which the tTA protein can bind to *tetO* and induce Ro1 expression. Transcriptional activator binding to DNA is blocked in the presence of the tetracycline analog doxycycline. Therefore, transgene induction is achieved by removing doxycycline from the diet. Dual heterozygotes were conceived and raised in the presence of doxycycline to prevent expression of Ro1 during development. When the mice were 6–8 weeks old, doxycycline was withdrawn. Western blot analysis showed inducible and tissue-specific Ro1 expression in the heart, liver, and salivary gland consistent with the known specificities of these promoters (Fig. 1B).

To determine the timing and level of Ro1 expression that could be induced with the tet system, we performed a time-course experiment (Fig. 2). Western blot analysis of heart tissue showed no expression of Ro1 at day 0 after the removal of doxycycline, minimal expression from days 3 to 7, and maximal expression after day 11. Despite some variability in the absolute amount of receptor expression during the induction period, high-level expression of the G_i-coupled receptor was present in all mice after 11 days.

Because the *tetO-Ro1* mice were also transgenic for *tetO-lacZ*, analysis of β-galactosidase (β-gal) activity was performed as a surrogate for immunolocalization of cardiac Ro1 expression. β-gal activity was found in approximately 90% of atrial myocytes and in about 50% of ventricular myocytes (Fig. 3). Although currently available antibodies are not suitable for immunohistochemistry of

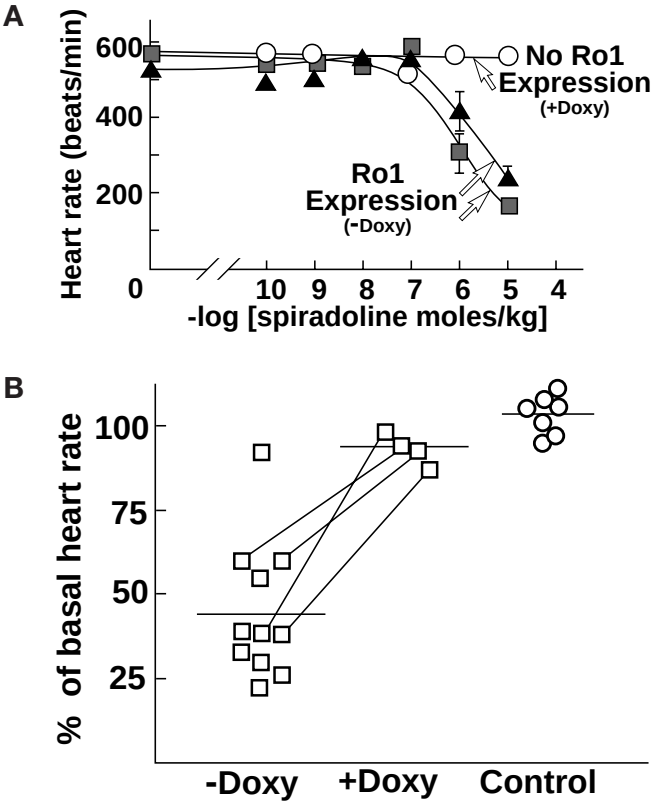


Figure 4. Spiradoline-mediated bradycardia in mice with cardiac Ro1 expression. (A) Dose-response curve of spiradoline-mediated bradycardia in the same mouse. Heart rate at a given dose equals mean heart rate between 3 and 10 min after administration of spiradoline. Dose-response curves with Ro1 expression (■, ▲) were generated 2 weeks apart using the same mouse. Dose-response curve with no Ro1 expression (○) was generated in the same mouse after doxycycline was resumed for 2 weeks. Each dose of spiradoline was separated by ≥24 h. The dose-response curves were repeated in four other mice. Error bars indicate standard error of the mean. (B) Average decrease in heart rate induced by spiradoline. Each point represents the average of two experiments in the same mouse. Heart rate in each experiment equals the mean between 3 and 10 min after spiradoline administration (1×10^{-5} mol/kg). Spiradoline caused significant bradycardia in mice expressing Ro1 (–Doxy). Repression of Ro1 expression (+Doxy, 2 weeks of treatment) in four of these same mice abolished the spiradoline-mediated bradycardia. No spiradoline-mediated effects were seen in seven control mice (○; *αMHC-tTA*, n=4; *tetO-Ro1*, n=2; wild-type, n=1). Horizontal lines indicate the means.

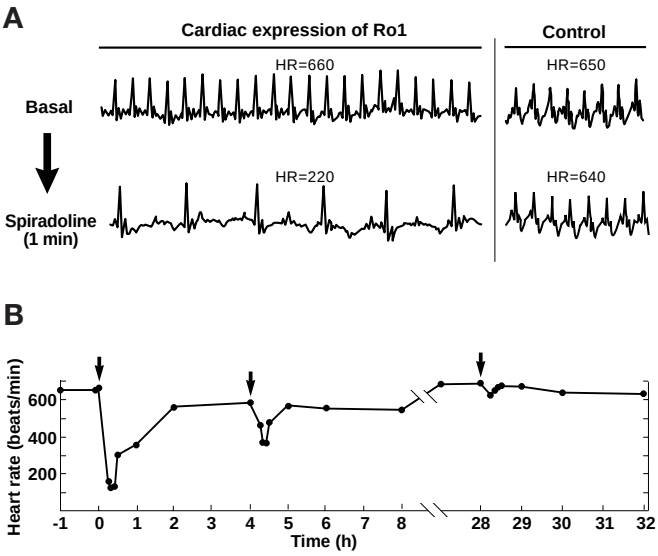


Figure 5. (A) Spiradoline-mediated bradycardia occurs immediately. Less than 1 min after spiradoline injection (1×10^{-5} mol/kg), the mouse expressing Ro1 in the heart has a heart rate one-third of baseline. Electrocardiographic tracings during the bradycardia reveal a relative sinus bradycardia and atrioventricular block. Spiradoline injection (1×10^{-5} mol/kg) had no effect on the control mouse (*αMHC-tTA*). (B) Desensitization of spiradoline-mediated bradycardia. Spiradoline injections (1×10^{-5} mol/kg) indicated by arrows (↓) at 4 and 28 h after the initial spiradoline injection have a diminished effect on heart rate. Each data point represents average heart rate over 5 min.

Ro1 in vivo, β-gal staining should correlate with the number and location of Ro1-expressing cells.

Inducible control of mouse heart rate with Ro1. Because the M_2 muscarinic and A_1 adenosine receptors cause bradycardia via G_i signaling, we hypothesized that activation of Ro1 in mouse heart would also cause bradycardia[7]. To measure heart rate and waveform, we implanted cardiac telemetry units into mice expressing Ro1 and administered the κ opioid receptor agonist spiradoline[5] to activate Ro1. Intraperitoneal administration of spiradoline at doses of 1×10^{-7} to 1×10^{-5} mol/kg (0.05–5 mg/kg) caused a dose-dependent decrease in heart rate (Fig. 4A). In the single mouse depicted here, the dose-response curves were reproducible 2 weeks apart. Doxycycline was then administered to repress Ro1 expression in the same mouse. After 2 weeks of doxycycline, spiradoline had no effect on heart rate.

Spiradoline was injected intraperitoneally at 1×10^{-5} mol/kg in 11 mice with cardiac expression of Ro1. In these mice, heart rates decreased by a mean of 55.5% after spiradoline administration (Fig. 4B). The magnitude of inducible bradycardia varied between mice. This variability may be due to heterogeneous expression of Ro1 in the conduction tissue, as suggested by the heterogeneous β-gal staining in ventricular tissue (Fig. 3). Repression of Ro1 expression in four of these same mice abolished the spiradoline-mediated bradycardia. Injection of spiradoline into seven control mice had no effect on heart rate. Thus, the spiradoline-mediated effects are specific to mice expressing Ro1 in the heart.

To explore the kinetics of the RASSL-induced bradycardia, we measured the time required to reach 50% of baseline heart rate. In 10 of the 11 mice injected with spiradoline, heart rate decreased by more than 50% over an average time of 25.8 s (range, 11–38 s). The mechanism of bradycardia included a decreased rate of atrial depolarization and both prolonged and decreased atrioventricular conduction (Fig. 5A). These Ro1-mediated effects on heart rate and impulse conduction are consistent with the known effects of G_i-coupled receptors in the heart[7].

In a conditional signaling system, the rapidity of onset, the magnitude, and the duration of the induced signal are all important determinants of the observed physiologic changes. The duration of a GPCR-mediated signal can vary from seconds to a few hours and is modulated by well-characterized desensitization mechanisms[13–15]. We tested the effects of repeated spiradoline dosing in mice expressing Ro1. In one mouse, the initial dose of spiradoline caused an 80% decrease (at 25 min) in heart rate from baseline, but a second dose at 4 h (after the heart rate returned to 80% of baseline) caused only a 40% decrease in heart rate, and a third dose at 28 h had only a minimal effect on heart rate (Fig. 5B). The mean response for five mice tested for desensitization was a 61% decrease in heart rate after the initial spiradoline dose, a 27% decrease after a second dose at 4 h, and a 16% decrease after a third dose at 28 h (Fig. 6). Administration of spiradoline to these same mice 1 week later caused a mean decrease in heart rate of 58%.

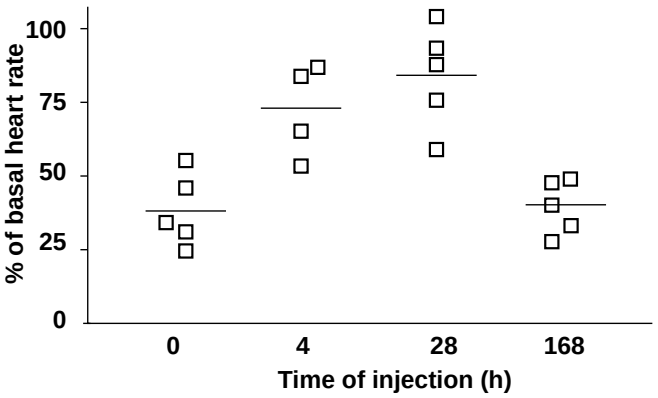


Figure 6. Average decrease in heart rate for five mice (n=4 at 4 h) treated with spiradoline at 4, 28, and 168 h after initial injection (1×10^{-5} mol/kg). The Ro1-mediated bradycardia is desensitized for more than 24 h, but is reproducible 1 week after initial spiradoline injection. Percent decrease in heart rate calculated from basal heart rate at t= 0 h for all time points. Horizontal lines indicate the means.

Thus, activation of Ro1 caused a bradycardia in <1 min that lasted for several hours and was subsequently desensitized for more than 24 h. Moreover, full activation of Ro1 was repeatable within 1 week of previous activation.

Discussion

Recently, several conditional signaling systems have been described that allow control over physiologic events with exogenously administered drugs. One type, based on chimeric transcription factors, can be regulated by tetracycline, mifepristone, tamoxifen or ecdysone[1,3,4,16]. The second type uses drugs, such as FK1012, rapamycin, or coumermycin, to initiate protein-protein dimerization to reconstitute transcription factor activity or to activate intracellular signaling pathways[2,17–19]. We described an additional type of conditional signaling system, based on modification of GPCRs[5]. Here we demonstrate that a RASSL can be expressed in multiple mouse tissues, and activation of that RASSL in mouse heart causes bradycardia. Furthermore, the Ro1-mediated bradycardia occurred in <1 min, lasted several hours, and was subject to desensitization for more than 24 h. Electrocardiographic analysis of adult mice provided a quantitative measure of the range of RASSL-mediated physiologic effects and was reproducible in the same mouse. RASSLs complement, rather than replace the other conditional signaling systems. Each signaling system has unique properties that are best suited for different cellular tasks. For instance, we are using the tet system to control RASSL expression. Once the RASSL is expressed, it can be used to control G protein signaling.

Important characteristics of any conditional signaling system include the speed of activation and the amount, duration, and physiologic relevance of the generated signal. Administration of spiradoline causes a 50% decrease in heart rate in <1 min. Thus, a conditional signaling system based on a modified GPCR can be activated relatively quickly (in seconds) compared with the dimerization of cytosolic proteins, which takes 10–30 min, or the activation of chimeric transcription factors, which can take hours or days. This bradycardia lasted for several hours and was subject to desensitization for >24 h. The duration of the bradycardia is consistent with the duration of effects mediated by the native κ opioid receptor in tissues such as the hippocampus[13]. This duration of Ro1-mediated effects compares favorably with other conditional signaling systems. Desensitization of Ro1 signaling effects, a critical determinant of any receptor's function, suggests that the receptor is recognized similarly to other GPCRs in the heart and may predict the usefulness of Ro1 in other mouse tissues. Moreover, inducible activation of Ro1 in the heart may allow dissection of the relative importance of both short- and long-term desensitization mechanisms in that tissue.

A final feature of a conditional signaling system is the level of basal activity. Although basal or low-level Ro1 activity was not detected in previous tissue culture assays and Ro1 expression alone did not affect heart rate in adult mice, we suspect that basal activity could occur at maximal receptor expression levels. As shown for the β_2 adrenergic receptor (βAR), overexpression (200-fold) of wild-type βAR in mouse heart caused an increase in basal adenylyl cyclase activity, heart rate, and contractility[20]. To assay for basal signaling by Ro1, we plan to test the effects of Ro1 expression during embryogenesis and during long-term (months) expression in adult mice.

Our ability to overexpress and activate a G_i-coupled receptor in mouse heart will allow us to test the role of G_i signaling in cardiomyopathy. Recent studies in mice and humans suggest that increases or dysregulation of G_i signaling may underlie some human dilated cardiomyopathies[9,21,22]. Our control of Ro1 expression and activation will allow us to test both the short- and long-term biochemical, pathological, and gene expression effects induced by G_i signaling in the heart.

In addition to controlling Ro1 expression in the heart, we induced Ro1 expression in mouse liver and salivary gland. These results demonstrate that a conditional signaling system based on a human opioid receptor can be expressed in diverse mouse tissues. Activation of Ro1 in these tissues has both experimental and therapeutic implications. For example, in cells that proliferate in response to a G_i signal, Ro1 activation could cause targeted proliferation of that population. Thus, Ro1 might be used to amplify a population of transfected stem cells for therapeutic purposes. Another possible use of Ro1 is to activate G_i signaling in the nervous system, where G protein signaling is critical for olfaction, taste transduction, weight control, memory, and locomotion[23]. Although naturally occurring κ-opioid receptors are expressed primarily in the brain, the recent knockout of the κ-opioid receptor[24] provides an ideal background to use κ opioid-based RASSLs in the brain. Thus, Ro1 could be used to dissect the neural circuitry involved in complex physiologic processes.

Experimental protocol

Transgenic mouse production. Mice harboring the *αMHC-tTA* (cardiac-specific), *LAP-tTA* (liver-specific), and *MMTV-tTA* (salivary gland–, seminal vesicle–, and lymphocyte-specific) promoter constructs have been described[10–12]. The previously described Ro1 construct[5] (N-terminal FLAG; Sigma, St. Louis, MO) was subcloned into the *tTA*-inducible pUHG 10-3 (*tetO-Ro1*), cut with *Apa*LI and *Nde*I to isolate a 3-kb fragment, and purified for pronuclear injection. The *tetO-Ro1* DNA was injected along with the 4.6-kb *Xho*I-*Bgl*II fragment of pUHG 16-3 (*tetO-lacZ*). Before injection, DNA was purified twice by agarose gel electrophoresis, electroeluted, passed through an Elutip (Schleicher and Schuell, Dassel, Germany), and diluted in injection buffer (10 mM Tris, pH 7.4, and 0.25 mM EDTA) to 5.0 ng/μl. Pronuclear injections into FVB/N oocytes were performed according to standard techniques in the Gladstone Institutes Transgenic Core Facility (San Francisco, CA). Founder mice were identified by PCR analysis of genomic DNA prepared from tail tips. Constructs and detailed protocols are available[25]. All transgenic mouse strains described in this paper (*αMHC-tTA, LAP-tTA, MMTV-tTA,* and *tetO-Ro1*) have been donated to the Jackson Laboratories Induced Mutant Resource[26].

PCR analysis. Genotyping of mice was performed by PCR. The *tTA* transgenes were identified with the following primers: 5′-GCT GCT TAA TGA GGT CGG-3′ and 5′-CTC TGC ACC TTG GTG ATC-3′ resulting in a 507-bp fragment. The *tetO-Ro1* transgene was identified with 5′-CTG CTG TTG GGG GGC AGG-3′ and 5′-GGT TCG TCG CAG AAA GGG TC-3′ resulting in a 178-bp fragment. The *tet*O-*LacZ* transgene was identified with 5′-GGC GTG TAC GGT GGG AGG-3′ and 5′-GTT GGG AAG GGC GAT CGG-3′ resulting in a 421-bp fragment.

Antibiotic treatment. To repress *tTA*-dependent transactivation, the mice were given doxycycline (200 μg/ml) in their drinking water (Sigma, St. Louis, MO).

Western blots. Whole hearts were homogenized in Tris homogenization buffer (50 mM Tris, pH 7.4; Complete Cocktail [Boehringer Mannheim, Indianapolis, IN]; 50 μM dithiothreitol; and 50 μM phenylmethanesulfonyl fluoride) and solubilized in Triton-X 100 (50 mM Tris, pH 7.5, 1% Triton-X 100, and Complete Cocktail). Immunoprecipitation was performed overnight at 4°C with 1 μg/ml monoclonal antibody M1 (M1-Ab; Sigma), Protein A agarose beads, and 2 mM $CaCl_2$. Next, the beads were washed in tris-buffered saline (pH 7.4, 0.1% Tween-20, and 1 mM $CaCl_2$), resuspended in sample buffer (Tris, pH 6.8, 10% glycerol, and 2% SDS), boiled, vortexed, centrifuged, and subjected to SDS-PAGE on 10% gels (Novex, San Diego, CA). The blots were probed with M1-Ab conjugated to horseradish peroxidase (Chromaprobe, Mountain View, CA) and developed by enhanced chemiluminescence detection (Amersham, Cleveland, OH). Western blots were quantitatively analyzed by densitometry (UMAX Data Systems and Jandel Scientific, Fremont, CA).

β-gal staining. Whole mouse hearts were embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and immediately frozen in liquid nitrogen. Cryostat sections (10 μm thick) were mounted on glass slides, fixed (0.2% glutaraldehyde and 2.0% formaldehyde in phosphate-buffered saline [PBS]) for 5 min, incubated in X-gal solution (1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, and PBS) for 24 h, and counterstained with Nuclear Fast Red (Sigma).

Heart rate measurements. Telemetry units were implanted under sterile conditions. Mice were anesthetized with Avertin (10 μl/g) (0.25 g/ml tribromoethanol, 2.5% tert-amylalcohol, and PBS). After opening of the mouse peritoneum, a PhysioTel Implant (Model TA10EA-F20; Data Sciences International, St. Paul, MN) was inserted, and monopolar electrodes were tunneled subcutaneously across the precordium. Heart rate was monitored with AcqKnowledge III software (BIOPAC Systems, Santa Barbara, CA). For all baseline heart rate measurements, mice were injected with H_2O (10 μl/g given intraperitoneally) and monitored for 20 min. After treatment with spiradoline 10^{-3} M, 10 μl/g given intraperitoneally (U-62066E; Research Biochemicals International, Natick, MA, or Pharmacia Upjohn Pharmaceuticals, Kalamazoo, MI), heart rate was monitored for 20 min.

Acknowledgments

We thank Robert W. Mahley and Murielle M. Veniant for valuable discussions and advice; Lara Jensen and Dale Newland for histochemistry assistance; and Gary Howard, Stephen Ordway, and Jane Peredo for editorial assistance. G. Fishman is an established investigator of the American Heart Association. This work was supported by the J. David Gladstone Institutes and the National Institutes of Health Grants CA71779 (C.H.R.) and HL60664-01 (B.R.C.).

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