Enhanced Hypotensive, Bradycardic, and Hypnotic Responses to α_2-Adrenergic Agonists in Spinophilin-Null Mice Are Accompanied by Increased G Protein Coupling to the α_{2A}-Adrenergic Receptor

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ABSTRACT

We previously identified spinophilin as a regulator of α_2 adrenergic receptor (α_2AR) trafficking and signaling in vitro and in vivo (Science 304:1940–1944, 2004). To assess the generalized role of spinophilin in regulating α_2AR functions in vivo, the present study examined the impact of eliminating spinophilin on α_2AR-evoked cardiovascular and hypnotic responses, previously demonstrated to be mediated by the α_{2A}AR subtype, after systemic administration of the α_2-agonists 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK14,304) and clonidine in spinophilin-null mice. Mice lacking spinophilin expression display dramatically enhanced and prolonged hypotensive, bradycardic, and sedative-hypnotic responses to α_2AR stimulation. Whereas these changes in sensitivity to α_2AR agonists occur independent of any changes in α_{2A}AR density or intrinsic affinity for agonist in the brains of spinophilin-null mice compared with wild-type control mice, the coupling of the α_{2A}AR to cognate G proteins is enhanced in spinophilin-null mice. Thus, brain preparations from spinophilin-null mice demonstrate enhanced guanine nucleotide regulation of UK14,304 binding and evidence of a larger fraction of α_{2A}AR in the guanine-nucleotide-sensitive higher affinity state compared with those from wild-type mice. These findings suggest that eliminating spinophilin expression in native tissues leads to an enhanced receptor/G protein coupling efficiency that contributes to sensitization of receptor mediated responses in vivo.

The α_2-adrenergic receptor (AR) is a prototypical G protein-coupled receptor (GPCR) that couples to the $G_{i/o}$ subfamily of G proteins (Wang and Limbird, 2007). In native cells, stimulation of the α_2AR leads to inhibition of adenylyl cyclase and voltage-gated Ca^{2+} currents and to activation of receptor-operated K^+ currents and mitogen activated protein kinase (Limbird, 1988; Kobilka, 1992; Richman and Regan, 1998; Wang et al., 2004; Lu et al., 2009). Among the three different subtypes of α_2AR, α_{2A}-, α_{2B}-, and α_{2C}AR, the α_{2A}AR subtype is the major mediator of the therapeutic effects of α_2-adrenergic agents on blood pressure, pain perception, volatile anesthetic sparing, analgesia, and working memory enhancement, as revealed by genetic studies exploiting mice made null or mutant for this receptor subtype (MacMillan et al., 1996; MacDonald et al., 1997; Hein et al., 1999; Kable et al., 2000). Activation of the α_{2A}AR in the nucleus tractus solitarius represents an important central mechanism to lower blood pressure (Sved et al., 1992; MacMillan et al., 1996). In addition, the α_{2A}AR inhibits synaptic firing and induces sedative and hypnotic effects via the locus ceruleus (Lakhlani et al., 1997).

Receptor interacting partners other than heterotrimeric G proteins play pivotal roles in modulating nearly every aspect of GPCR activity, including pharmacological recognition, signaling activation and desensitization, and receptor traffick-

ABBREVIATIONS: AR, α_2-adrenergic receptor; GPCR, G protein-coupled receptor; UK14,304, 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine; WT, wild type; LORR, loss of righting reflex; Gpp(NH)p, 5′-guanylimidodiphosphate.

ing among cellular compartments (Bockaert et al., 2004; Gainetdinov et al., 2004; Kenakin, 2004; Rashid et al., 2004; Tilakaratne and Sexton, 2005; Sato et al., 2006). G protein-coupled receptor kinase (GRK) 2 binds to and phosphorylates the agonist-activated α_2AR (Jewell-Motz and Liggett, 1996; Pao and Benovic, 2005), which subsequently interacts with arrestin (Wu et al., 1997; DeGraff et al., 2002; Wang and Limbird, 2002). GRK phosphorylation represents one major mechanism for α_2AR desensitization after agonist stimulation (Eason et al., 1995; Jewell-Motz and Liggett, 1996; Desai et al., 2006). Whether or not heterologous desensitization pathways (Jones et al., 1990; Jewell-Motz et al., 1998; Liang et al., 1998) and sensitization pathways (Jones et al., 1987; Jones and Bylund, 1988, 1990) reported in vitro also contribute to desensitization of this receptor in vivo is not yet known.

Arrestin is a multifaceted regulator of GPCRs that terminates G protein coupling, mediates receptor internalization, and scaffolds cellular signaling cascades (Reiter and Lefkowitz, 2006; DeWire et al., 2007). Our previous studies identified spinophilin (Allen et al., 1997; Satoh et al., 1998) as an α_2AR-interacting partner that regulates multiple aspects of α_2AR trafficking and signaling by antagonizing GRK2 interaction with the receptor and subsequent arrestin binding and functions (Wang et al., 2004). Interaction of spinophilin with the α_2AR lessens arrestin-dependent internalization of the α_2AR (Brady et al., 2003; Wang et al., 2004), and slows the rate of both activation and resensitization of receptor-mediated signaling, presumably through decelerating the receptor internalization/recycling cycle (Wang et al., 2004). In vivo, the reciprocal regulation of the α_2AR by spinophilin and arrestin is manifest by the fact that agonist sensitivity of α_{2A}AR-evoked sedative response (as assessed by Rotarod latency) is suppressed in arrestin 3-deficient [Arr3(−/−)] mice but enhanced in spinophilin-null [Sp(−/−)] mice.

Given the diversity of α_{2A}AR functions in vivo, it is not clear whether other α_{2A}AR-mediated responses are also regulated by spinophilin in a similar manner. In the present study, we investigated hypotensive, bradycardic, and hypnotic responses elicited by the α_{2A}AR in mice lacking spinophilin expression. Our data revealed an enhanced and prolonged hypotensive effect and bradycardia in response to the α_2-agonists 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine (UK14,304) and clonidine in these mice, as well as enhanced sedative and hypnotic effects. Complementary in vitro studies revealed an enhanced G protein coupling efficiency of the α_{2A}AR in Sp(−/−) mice without changes in receptor density or its intrinsic affinity for the α_2-adrenergic agonist compared with wild-type (WT) mice. These findings not only suggest a molecular mechanism that could contribute to the enhanced in vivo responsiveness to activation of the α_{2A}AR in Sp(−/−) mice but also provide additional compelling evidence that α_{2A}AR-G protein interactions, and their signaling consequences, are in constitutive regulation by spinophilin in vivo.

Materials and Methods

Animals

Sp(−/−) and corresponding WT mice were obtained and maintained as described previously (Feng et al., 2000). Mice were housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited Animal Resources Program at the University of Alabama at Birmingham in accordance with procedures of the Animal Welfare Act and the 1989 amendments to this Act. All studies with these animals followed protocols approved by University of Alabama Institutional Animal Care and Use Committee.

Coimmunoisolation of Spinophilin and α_{2A}AR from Mouse Brain Lysates

Adult male mice 10 to 12 weeks of age were injected with saline or UK14,304 at 1 mg/kg, the EC_{50} dose for inducing the sedative response in WT mice (Wang et al., 2004). Thirty minutes after injection, mice were sacrificed, and the whole brain was removed by dissection. The brain was homogenized in a Dounce homogenizer with 10 to 15 strokes on ice in lysis and biological detergent extraction buffer (10 mM Tris Cl, pH 8.0, 1% Nonidet P-40, 10% glycerol, 5 mM EDTA, 5 mM EGTA supplemented with 1 μg/ml soybean trypsin inhibitor, aprotinin, leupeptin, and 0.1 μM phenylmethylsulfonyl fluoride as protease inhibitors). The detergent extract was further homogenized by trituration through a 25-gauge needle 10 times and incubation on ice for 20 min. After centrifugation of the detergent extract at 100,000g at 4°C for 30 min, the supernatant, defined as the detergent-solubilized preparation, was transferred into a microfuge tube and subjected to immunoprecipitation assays with an anti-HA antibody (HA.11; Covance Research Products (Princeton, NJ) as described previously (Wang and Limbird, 2002).

Measurement of Cardiovascular Responses

Cardiovascular responses were measured as described previously (MacMillan et al., 1996; Tan et al., 2002). In brief, male mice (10–12 weeks of age) were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg). The left femoral artery and right jugular vein were catheterized to measure arterial pressure and to administer anesthetic, respectively. Twenty-four hours after this surgery, the systolic, diastolic, and mean arterial blood pressure and heart rate were recorded continually in conscious, freely moving animals by connecting the tubing implanted through left femoral artery to a pressure transducer that is linked to a computerized system, BIOPAC's AcqKnowledge 3.8.2 (BioPac, Goleta, CA). Baseline was established during infusions of saline through the right jugular vein. Twenty minutes later, responses to a bolus injection of 0.1 mg/kg UK14,304 into the right jugular vein were recorded. Previous studies have established that this dose of UK14,304 is insufficient for provoking a sedative response in mice (Tan et al., 2002). Indeed, in this study, no sedative response was observed in the mice as a result of the bolus injection of 0.1 mg/kg UK14,304.

Measurement of Sedative-Hypnotic Responses

Rotarod Latency. Male mice (10–12 weeks of age) were injected intraperitoneally with saline or different doses of clonidine and then tested for time (seconds) staying on a rotating Rotarod (10 rpm), as described previously (Lakhlani et al., 1997; Tan et al., 2002).

Loss of Righting Reflex. Male mice (10–12 weeks) were injected intraperitoneally with saline or 5 mg/kg UK14,304, and LORR was evaluated by sleep time as described previously (Lakhlani et al., 1997; Tan et al., 2002).

Radioligand Binding

Saturation binding was performed to assess α_{2A}AR receptor density in mouse brains isolated from WT and Sp(−/−) mice as described previously (MacMillan et al., 1996; Lu et al., 2009). Prazosin (1 μM) was added to block binding of the radiolabeled α_2-antagonist, [^{3}H]rauwolscine, to the α_{2B} and α_{2C}AR subtypes in this preparation (MacMillan et al., 1996; Lu et al., 2009).

Competition binding was performed using preparations derived from mouse brains isolated from WT and Sp(−/−) mice to evaluate agonist affinity in the absence or presence of a hydrolysis-resistant GTP analog, 5′-guanylimidodiphosphate (Gpp(NH)p) (MacMillan et

al., 1996; Lu et al., 2009). Computer-assisted analyses of the data obtained in these experiments provide a means to indirectly assess G protein coupling to the receptor. G protein interactions with the receptor increase the apparent affinity of the receptor for agonist agents, whereas addition of the hydrolysis-resistant analog of GTP, Gpp(NH)p, reverses these interactions and allows the assessment of the *intrinsic* affinity of the receptor for agonist in the absence of interactions with G proteins. In the absence of Gpp(NH)p, receptors coupled to G proteins have a higher affinity for agonists and data fit a two-site model, whereas in the presence of Gpp(NH)p, effects of G protein regulation of receptor affinity for agonist are eliminated, apparent receptor affinity is reduced, and data fit a one-site model (De Lean et al., 1980; Samama et al., 1993; Weiss et al., 1996).

All data were analyzed using Prism software (GraphPad Software, San Diego, CA), and the K_d values and percentage of receptors in the higher versus lower affinity states were estimated using nonlinear regression curve fitting.

Results

Spinophilin Preferentially Interacts with the Agonist-Stimulated $\alpha_{2a}AR$ in Mouse Brain. Taking advantage of HA-α_{2A}AR knock-in mice (Lu et al., 2009), we confirmed an endogenous interaction between spinophilin and the α_{2A}AR in mouse brain. As shown in Fig. 1, spinophilin was present in a complex with the HA-α_{2A}AR and could be isolated from the detergent extracts of the mouse brain using an anti-HA antibody. Systemic administration of an α_2-agonist UK14,304 at the EC_{50} dose for inducing the sedative response in mice (1 mg/ml) (Wang et al., 2004) dramatically enhanced the amount of spinophilin coisolated with the HA-α_{2A}AR in brain. These data demonstrate both that an endogenous interaction exists between spinophilin and the α_{2A}AR in mouse brain and that spinophilin preferentially interacts with the agonist-activated form of the α_{2A}AR, consistent with our previous findings examining the α_{2A}AR-spinophilin interaction in in vitro systems (Richman et al., 2001; Wang and Limbird, 2002; Wang et al., 2004).

The Hypotensive and Bradycardia Response to α_2AR-Agonists Was Enhanced and Prolonged in Spinophilin-Null Mice. Clinical use of α_2AR agonists to lower blood pressure relies on their central actions at the α_{2A}AR subtype (MacMillan et al., 1996; Tan et al., 2002). In con-

scious, freely moving animals, basal mean arterial pressure and heart rate did not significantly differ between WT and Sp(−/−) mice (Table 1). To avoid inducing sedative responses to mice, 0.1 mg/kg UK14,304 was employed to examine the cardiovascular response to this α_2AR agonist (Tan et al., 2002). As shown in Fig. 2, administration of UK14,304 led to a drop in arterial pressure in both WT and Sp(−/−) mice. However, compared with WT mice, Sp(−/−) mice responded to UK14,304 treatment with a steeper and longer lasting decrease in arterial pressure (Fig. 2A). In Sp(−/−) mice, the decrease in blood pressure in response to UK14,304 administration was significantly greater than that in WT mice at 10, 35, and 95 min after bolus UK14,304 administration (Fig. 2B). In addition, although blood pressure in WT mice returned to baseline by 95 min after the UK14,304 injection, in Sp(−/−) mice, the maximum hypotensive response endured for at least 95 min after injection of the α_2AR agonist (Fig. 2A). We also analyzed cardiovascular responses to 0.1 mg/kg clonidine administration. Clonidine was able to induce a greater and longer reduction of blood pressure (Fig. 2C) in Sp(−/−) mice compared with WT mice.

Changes in blood pressure in response to α_2AR agonists are accompanied by reflexive changes in heart rate. Thus, we also observed that for both UK14,304 and clonidine, injection of the α_2AR agonist induced a more dramatic and prolonged decrease in heart rate in Sp(−/−) mice than in WT mice (Fig. 3, A–C).

Sedative-Hypnotic Effects in Response to α_2-Agonists Were Enhanced in Mice Lacking Spinophilin. We have shown that Sp(−/−) mice are more sensitive to UK14,304-evoked sedation (Wang et al., 2004) than WT mice as measured by Rotarod latency. Here, we extended our study by testing clonidine, a partial α_2AR-agonist, for its ability to induce sedation in WT, Sp(+/−), and Sp(−/−) mice. Because sedative effects in mice require high receptor occupancy (Tan et al., 2002), the partial agonist effects of clonidine are evident in examining sedation, whereas they are not when examining hypotensive responses. As shown in Fig. 4A, the same dose of clonidine induced a significantly stronger sedation (reflected by shorter Rotarod latency) in Sp(−/−) mice than in WT and Sp± mice. We failed to detect any difference in the ability of clonidine in inducing sedation in WT versus Sp± mice (Fig. 4A). Further analysis of the protein level of spinophilin in mice with different genotypes revealed no significant difference between WT and Sp± mice (Fig. 4B).

We also examined the hypnotic response evoked by an α_2-agonist in WT and Sp(−/−) mice by measuring the sleep time, or LORR, which is known to be mediated by the α_{2A}AR subtype (Lakhlani et al., 1997). After systemic administration of UK14,304 at 5 mg/kg, a dose evoking maximal sedation in WT animals (Wang et al., 2004), both WT and Sp(−/−) mice developed LORR. However, the average sleep time for
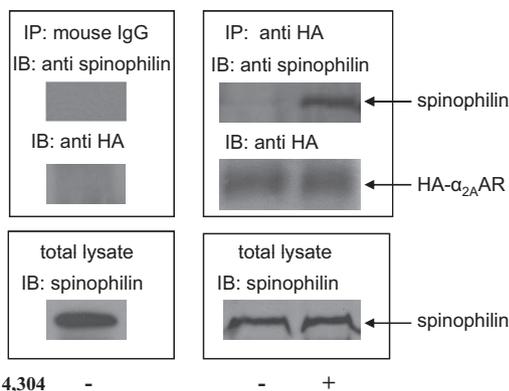

Fig. 1. Endogenous interaction of spinophilin with the α_{2A}AR in mouse brain. HA-α_{2A}AR knock-in mice were intraperitoneally injected with saline (control) or UK14,304 (1 mg/kg). Thirty minutes after injection, mouse brains were isolated, homogenized, and coimmunoisolation assays of detergent-solubilized preparations were performed as described under *Materials and Methods*. Shown are representative blots from three or more independent experiments.

TABLE 1
Basal mean arterial pressure and heart rate in WT and Sp(−/−) mice

Mean arterial pressure (MAP) and heart rate (HR) was measured in awake, freely moving WT and Sp(−/−) mice as described under *Materials and Methods*. Values represent mean ± S.E.M.; $n = 5$ for each genotype.

Genotype	MAP (mm Hg)	HR (beat/min)
WT	103 ± 1.6	598 ± 32
Sp(−/−)	101 ± 2.5	632 ± 12

WT mice was 29 min, whereas the sleep time for Sp($-/-$) mice was 179 min, more than 6 times longer (Fig. 4C). These data demonstrate that the absence of the ability of $\alpha_{2A}AR$ to interact with spinophilin leads to a much more prolonged hypnotic response evoked by the α_2AR agonists.
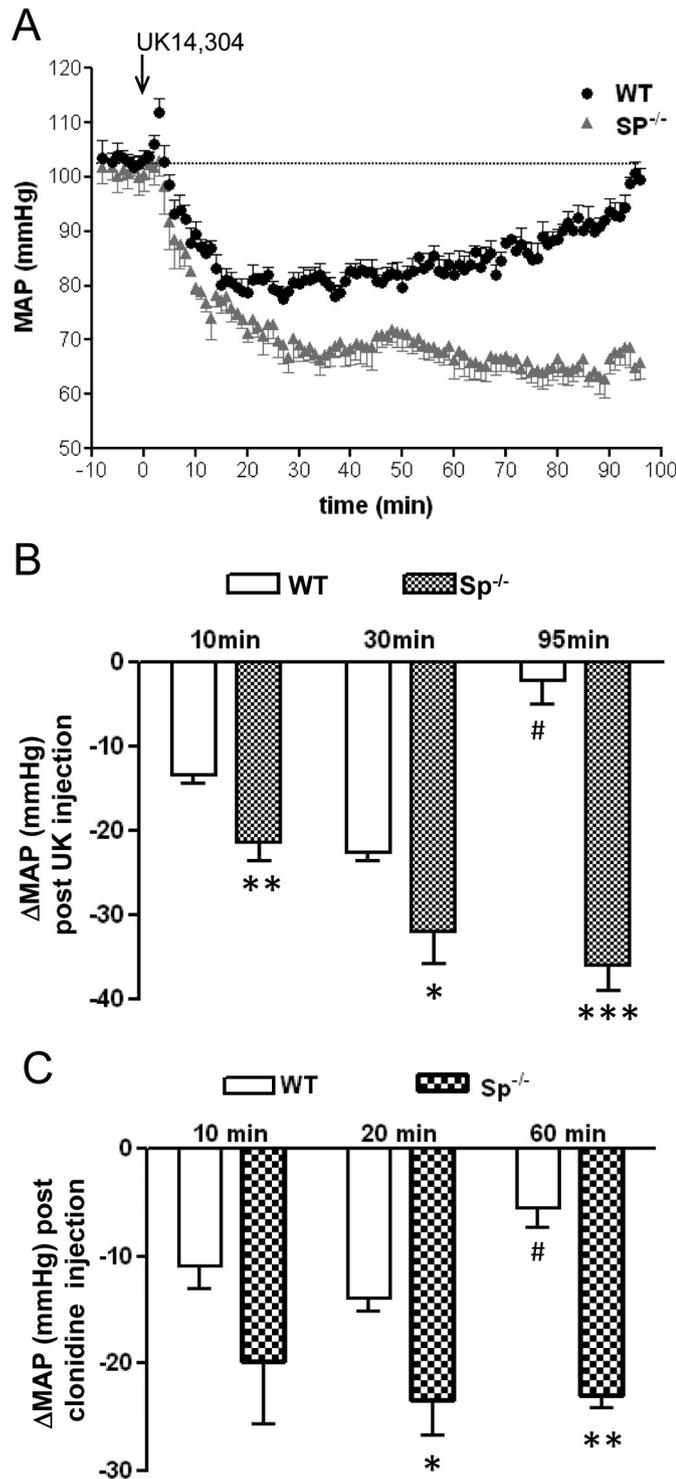

Fig. 2. Enhanced and prolonged hypotensive effects are observed in response to α_2-agonists in Sp($-/-$) mice. A, mean arterial pressure (MAP) was measured in WT and Sp($-/-$) mice after injection of UK14,304 (0.1 mg/kg i.v.). B, change in MAP after bolus injection of UK14,304 at the indicated times in WT and Sp($-/-$) mice. Values represent mean ± S.E.M. *, $p < 0.05$; ***, $p < 0.001$, comparing Sp($-/-$) with WT mice at the same time point. #, $p < 0.01$, comparing WT at 90 min with WT at 35 min. $n = 5$ for each genotype. C, change in MAP after bolus injection of clonidine (0.1 mg/kg) at the indicated times in WT and Sp($-/-$) mice. Values represent mean ± S.E.M. *, $p < 0.05$; **, $p < 0.01$, comparing Sp($-/-$) with WT mice at the same time point. #, $p < 0.05$, comparing WT at 60 min with WT at 20 min. $n = 4$ for WT and $n = 3$ for Sp($-/-$).
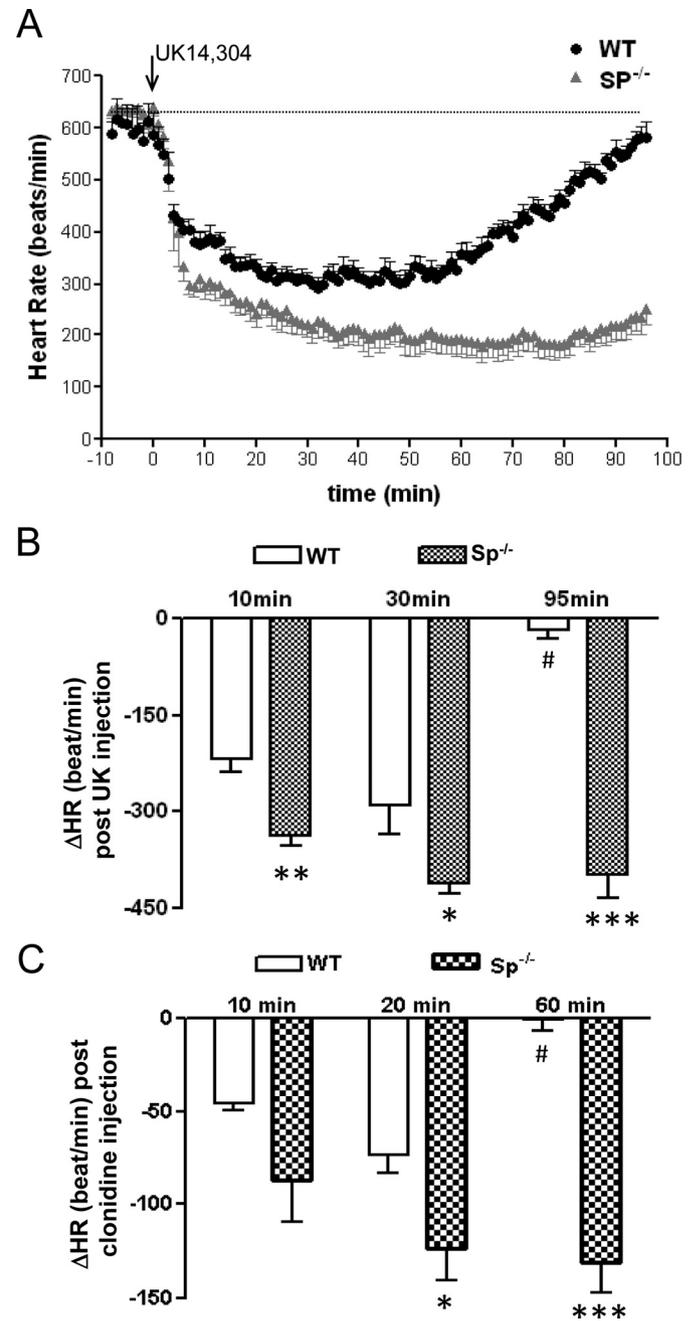
Fig. 3. Increased and prolonged bradycardia are observed in response to the α_2-agonist UK14,304 in Sp($-/-$) mice. A, heart rate (HR) was evaluated in WT and Sp($-/-$) mice after injection of UK14,304 (0.1 mg/kg i.v.) as described under *Materials and Methods*. B, change in HR after bolus injection of 0.1 mg/kg UK14,304 at the indicated times in WT and Sp($-/-$) mice. Values represent mean ± S.E.M. *, $p < 0.05$; ***, $p < 0.001$, comparing Sp($-/-$) with WT mice at the same time point. #, $p < 0.01$, comparing WT at 90 min with WT at 35 min. $n = 5$ for each genotype. C, change in HR after bolus injection of clonidine (0.1 mg/kg) at the indicated times in WT and Sp($-/-$) mice. Values represent mean ± S.E.M. *, $p < 0.05$; ***, $p < 0.001$, comparing Sp($-/-$) with WT mice at the same time point. #, $p < 0.01$, comparing WT at 60 min with WT at 20 min. $n = 4$ for WT and $n = 3$ for Sp($-/-$).

The α_{2A}AR Had a Comparable Density in WT and Sp($-/-$) Mice. A potential explanation for changes in receptor sensitivity in WT versus Sp($-/-$) mice could be that loss of spinophilin expression was accompanied by a change in receptor density, which can affect responsiveness to α_2-agonists in vivo (Tan et al., 2002). Saturation binding analysis (Fig. 5) revealed that the density of α_{2A}AR in WT and Sp($-/-$) mice was indistinguishable, as was the K_d value for interaction with the radiolabeled α_2AR antagonist, [³H] rauwolscine.

In Sp($-/-$) Mice, α_{2A}AR Coupling to G Proteins Was Enhanced Although the Intrinsic Affinity of the α_{2A}AR for Agonist Was Unaltered. The binding affinity of receptor for an agonist is a critical determinant of the in vivo responsiveness to that agonist. In the framework of the ternary complex model and its extended version (De Lean et al., 1980; Samama et al., 1993; Weiss et al., 1996), receptor affinity for agonist agents is highly regulated by receptor-G protein coupling. Consequently, receptor interactions with agonist in the absence or presence of guanine nucleotides have been widely used as an indirect measure of receptor-G

protein coupling efficiency (e.g., in Hausdorff et al., 1990; Kennedy and Limbird, 1993; Zhao et al., 1998; Piñeyro et al., 2005). As shown in Fig. 6 and summarized in Table 2, the ability of UK14,304 to compete for [³H]rauwolscine binding in the presence of Gpp(NH)p was indistinguishable in WT and Sp($-/-$) mouse brain membrane preparations. Because Gpp(NH)p eliminates G protein regulation of receptor affinity, these data are interpreted to indicate that the intrinsic affinity of the receptor for this agonist ligand is indistinguishable in WT mice and in Sp($-/-$) mice, which was expected, because the α_{2A}AR gene product was not altered in Sp($-/-$) mice. In addition, as expected, competition binding profiles obtained in the presence of Gpp(NH)p fit a one-site model, consistent with the absence of multiple affinity states in the presence of the GTP analog, Gpp(NH)p.

In the absence of exogenously added guanine nucleotide, however, the agonist competition curves for both samples prepared from WT and Sp($-/-$) mouse brains fit a two-site model (Fig. 6). The difference in complexity of these curves in the presence (discussed above) versus absence of guanine nucleotides was consistent with the interpretation that this complexity was due, at least in part, to changes in receptor affinity for agonist as a result of receptor interactions with G proteins. This interpretation was further corroborated by the finding that the K_d value for the "lower affinity state" estimated by computer-assisted analysis of these complex curves obtained in the absence of Gpp(NH)p was comparable with that observed for agonist interactions with the receptor when analyzed in the presence of Gpp(NH)p (Table 2). Computer-assisted analysis of these curves also indicated that there was a greater fraction of receptors interacting with G proteins (i.e., greater fraction manifesting a higher affinity state for agonist) in membrane preparations derived from the brain of Sp($-/-$) versus WT mice (Table 2). The apparent leftward shift of the UK14,304 agonist competition curve in brain particulate preparations from Sp($-/-$) mice was also consistent with the idea that a larger fraction of receptor
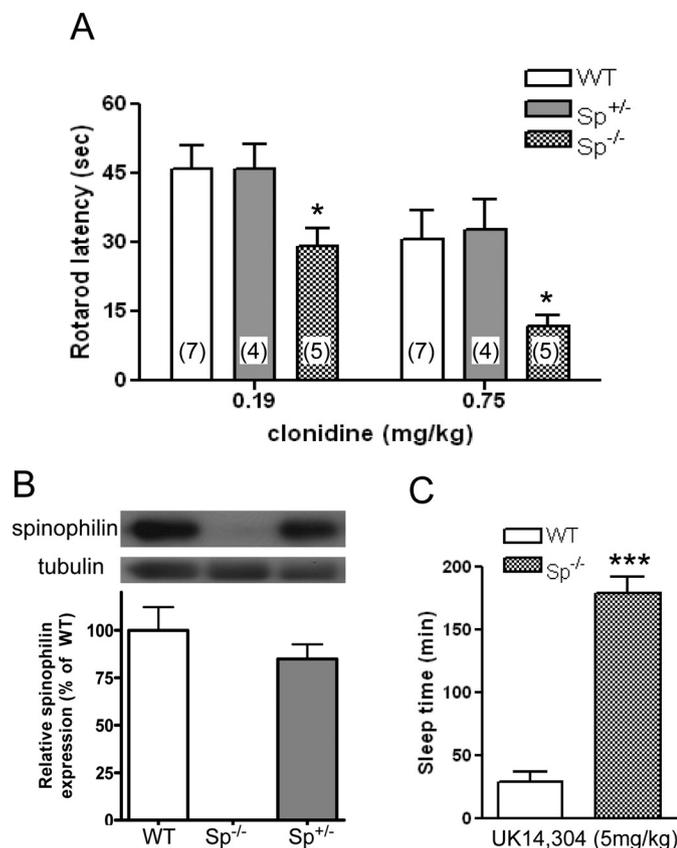

Fig. 4. Enhanced sedative/hypnotic effects in response to α_2AR stimulation in Sp($-/-$) mice. A, hypnotic response was evaluated by Rotarod latency in mice with different genotypes in response to a bolus injection of 0.19 or 0.75 mg/kg clonidine. Values represent mean ± S.E.M. Numbers in parentheses represent the number of mice tested. *, $p < 0.05$. B, relative spinophilin protein content in these mice was detected by Western analysis of brain lysates from WT, Sp($+/-$), and Sp($-/-$) mice. Values represent mean ± S.E.M. $n = 3$ for each genotype. C, prolonged sleep-time in response to UK14,304 observed in mice lacking spinophilin expression. Sleep time, estimated by the duration of LORR, was measured for WT and Sp($-/-$) mice after injection of 5 mg/kg i.p. UK14,304, as described under *Materials and Methods*. Values represent mean ± S.E.M. ***, $p < 0.001$, $n = 5$ for each genotype.
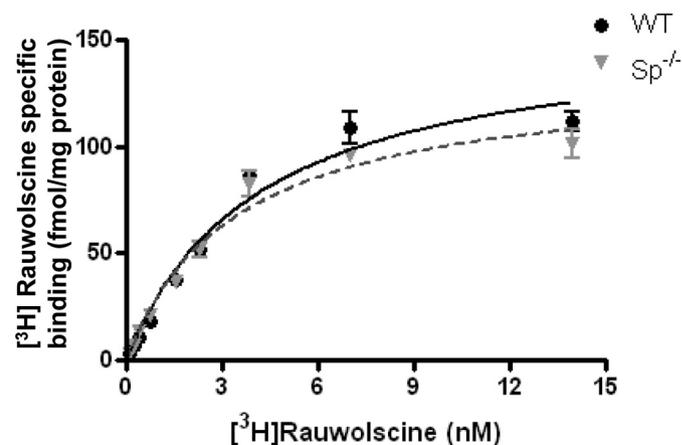

Fig. 5. The density of α_{2A}AR is indistinguishable in particulate brain preparations obtained from WT and Sp($-/-$) mice. Saturation binding of [³H]rauwolscine was performed as described under *Materials and Methods*. Prazosin (1 μM) was added to the incubation to block any binding that would occur to either the α_{2B} or α_{2C}AR subtypes (MacMillan et al., 1996). Values represent mean ± S.E.M.; $n = 3$ for each genotype. The B_{max} values predicted by nonlinear regression fit for α_{2A}AR in WT and Sp($-/-$) brain lysates are 156 ± 9.8 and 134 ± 7.7 fmol/mg protein, respectively.

interacts with G proteins and manifests higher affinity interactions with the agonist ligand than detected in preparations from WT mice (Fig. 6). Taken together, our binding data suggest that endogenous coupling of the α_{2A}AR to G proteins occurred more frequently in mouse brain preparations from Sp(−/−) mice than from WT mice.

Discussion

The present results indicate that spinophilin expression in vivo influences the rate and extent of the α_{2A}AR-mediated decreases in blood pressure, compensatory changes in heart rate, and increased hypnotic effects of two different α_2-adrenergic agonists, UK14,304 and clonidine. These alterations in central nervous system responses to α_2-adrenergic agonists occur in the absence of changes in α_{2A}AR density and intrinsic affinity for agonist. However, our parallel in vitro studies with whole-brain lysates revealed an enhanced efficiency of α_{2A}AR coupling to G proteins in the brain of Sp(−/−) mice compared with WT mice. The enhanced efficiency of α_{2A}AR-G protein coupling in Sp(−/−) mice could
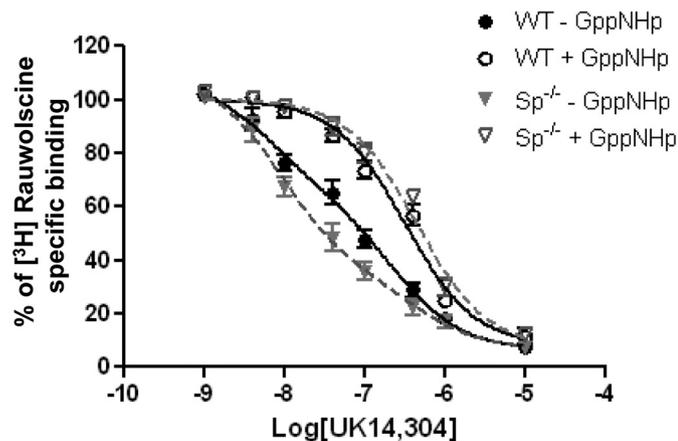

Fig. 6. UK14,304 competition binding curves suggest enhanced coupling of α_{2A}AR to G protein in brain preparations derived from Sp(−/−) mice. Competition binding assays were performed with brain particulate preparations derived from WT or Sp(−/−) mice in the presence or absence of Gpp(NH)p, as indicated. Binding of the [³H]rauwolscine radioligand is given as a percentage of binding without competitors. Curves without GppNHp were best fit via nonlinear regression analysis to two binding sites, whereas curves obtained in incubations performed in the presence of GppNHp were best fit by nonlinear regression analysis to one binding site (see Table 2 for estimates of K_d for each affinity state and percentage of receptors in the higher affinity state for these curves). Values represent mean ± S.E.M. $n = 4$ for each genotype.

TABLE 2
Computer-assisted estimates of K_d values of α_{2A}AR affinity states and percentage of α_{2A}AR in the guanine-nucleotide sensitive higher affinity state

UK14,304 competition for [³H]rauwolscine binding was determined in well-washed particulate fractions, to eliminate endogenous guanine nucleotides, in the absence or presence of Gpp(NH)p, as outlined under *Materials and Methods*. Binding profiles in the absence of Gpp(NH)p were best fit by a two-site model, whereas those obtained in the presence of Gpp(NH)p fit a one-site model. The interaction of the two-state analysis, in terms of receptor interactions with G proteins, is provided in the text.

Genotype	−Gpp(NH)p			+Gpp(NH)p ($-\log K_d$)
	$-\log K_H$	$-\log K_L$	$R_H\%$	
WT	8.2 ± 0.3	6.6 ± 0.2	42 ± 8	6.5 ± 0.1
Sp(−/−)	8.1 ± 0.2	6.4 ± 0.3	72 ± 7*	6.4 ± 0.1

$R_H\%$ indicates the percentage of receptors at a higher affinity state of agonist.
* $P < 0.05$.

thus explain the enhanced in vivo responses in these mice. Given the discrete distribution of spinophilin in particular brain regions, such as hippocampus and thalamus (Allen et al., 1997), the presence of spinophilin would induce an even more profound uncoupling between α_{2A}AR and G proteins in these regions. Thus the effects on receptor-G protein coupling seen in our studies of whole-brain particulate preparations are probably an underestimate of the extent of endogenous modulation of this coupling in spinophilin-rich brain loci.

The impact of eliminating spinophilin in vivo to *enhance* receptor-G protein coupling and to sensitize in vivo signaling pathways, such as those that lead to reduced blood pressure, increased bradycardia, and hypnotic-sedative effects, may seem paradoxical, because arrestin interaction with the GRK-phosphorylated receptor leads to desensitization. Thus, one might have anticipated the opposite outcome, for example, in spinophilin-null mice [in which arrestin interaction with the α_{2A}AR would go unopposed (Wang et al., 2004)] enhanced arrestin-mediated desensitization would occur and manifest as diminished sensitivity for agonist and a briefer duration of agonist-mediated effect, exactly the opposite of what was observed. However, it must be remembered that arrestin has multiple roles in the life cycle and signaling of GPCR. For example, arrestin serves as an adapter of GPCR to clathrin-coated pits thus fostering receptor-mediated internalization. In our previous studies, we also showed that α_{2A}AR internalization is significantly accelerated in cells lacking spinophilin expression (Brady et al., 2003; Wang et al., 2004). Because arrestin-clathrin mediated endocytosis serves as a prelude for receptor recycling and replenishment of the surface receptor pool with "re-sensitized" receptors (Wang et al., 2004; Sorkin and vonZastrow, 2009), this role of arrestin could predominate in the systems that we examined in vivo and thus contribute to the enhanced sensitivity and longer duration of α_{2A}AR-activated processes such as lowered blood pressure and bradycardia. Such a role for receptor endocytosis/recycling for signaling has been observed in other systems, as well. For example, clathrin-mediated internalization and receptor recycling has been shown to be essential for sustained EGFR signaling (Sigismund et al., 2008), and recycling of βAR is required for sustained cardiac responsiveness to catecholamine in vivo (Odley et al., 2004). Furthermore, the endosome can serve as a intracellular platform for assembly of signaling complex, either through receptor-associated scaffolding proteins such as arrestin (Violin and Lefkowitz, 2007) or by recruiting signaling molecules to endosomal resident proteins such as Rab5 (Zoncu et al., 2009). Thus, it is possible that yet-to-be confirmed intracellular arrestin-scaffolded α_{2A}AR–provoked signaling pathways also contribute to the enhanced agonist-elicited responses that we evaluated in vivo. Finally, our finding that elimination of spinophilin also leads to enhanced coupled of α_{2A}AR to G proteins may also contribute in a substantive way to the enhanced hypotensive, bradycardic, and sedative/hypnotic responses that we observed in spinophilin-null mice.

In addition to the sedative/hypnotic and hypotensive effects described here, the antinociceptive response evoked by the α_{2A}AR is also enhanced in spinophilin-null mice (Charlton et al., 2008; Nag et al., 2009). Taken together, these studies suggest that functions of the α_{2A}AR in the central and peripheral nervous system are modulated by

spinophilin in native settings and are thus perturbed by changes in spinophilin expression in vivo. It is of interest to speculate about the impact of α_{2A}AR-spinophilin interactions on G protein coupling and α_{2A}AR signaling in neurons, because spinophilin is enriched in dendritic spines [the basis for its naming by the Greengard laboratory (Allen et al., 1997)]. This would mean that α_{2A}AR interactions with G proteins (and also with arrestin, for which spinophilin is an endogenous antagonist) would vary considerably in the somatodendritic membrane compared with neuronal terminals.

In conclusion, this study demonstrates that elimination of spinophilin in transgenic mice leads to enhanced G protein coupling to the α_{2A}AR, suggesting that peptidomimetic agents that selectively inhibit α_{2A}AR-spinophilin interactions could enhance α_{2A}AR sensitivity to agonists, allowing treatment of hypertension or modulation of pain perception with reduced doses of α_2AR agonists that therefore do not evoke simultaneous sedative or hypnotic side effects.

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