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The α_{2A}-Adrenergic Receptor Plays a Protective Role in Mouse Behavioral Models of Depression and Anxiety

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The noradrenergic system is involved in the regulation of many physiological and psychological processes, including the modulation of mood. The α_2-adrenergic receptors (α_2-ARs) modulate norepinephrine release, as well as the release of serotonin and other neurotransmitters, and are therefore potential targets for antidepressant and anxiolytic drug development. The current studies were undertaken to examine the role of the α_{2A} subtype of α_2-AR in mouse behavioral models of depression and anxiety. We have observed that the genetic knock-out of the α_{2A}-AR makes mice less active in a modified version of Porsolt's forced swim test and insensitive to the antidepressant effects of the tricyclic drug imipramine in this paradigm. Furthermore, α_{2A}-AR knock-out mice appear more anxious than wild-type C57 Bl/6 mice in the rearing and light–dark models of anxiety after injection stress. These findings suggest that the α_{2A}-AR may play a protective role in some forms of depression and anxiety and that the antidepressant effects of imipramine may be mediated by the α_{2A}-AR.

Key words: antidepressant; adrenergic receptor; anxiety; forced swim; imipramine; light–dark test

α_2-Adrenergic receptors (α_2-ARs) bind norepinephrine (NE) and epinephrine, effecting a number of CNS-mediated responses. These include lowering of blood pressure, attenuation of pain perception and opiate withdrawal, anesthetic sparing, sedation, and modulation of mood and cognitive processes (Ruffolo and Hieble, 1994; Lakhlani et al., 1997; Arnsten, 1998).

The role of the α_2-ARs in the modulation of mood has been modeled in rodents, using several variations of the Porsolt forced swim test (Porsolt et al., 1978a,b), a widely accepted predictive model of the efficacy of antidepressant drugs. Infusion of α_2-AR agonists and antagonists into the locus ceruleus of rats, for example, can increase or decrease, respectively, activity in this test (Simson et al., 1986; Weiss et al., 1986).

More recently, investigators have focused on the complementary roles of the serotonergic and noradrenergic signaling systems in modulating depression in humans and responses to antidepressant agents in rodent models. In the rodent forced swim test, noradrenergic agents typically increase climbing behaviors, whereas serotonergic agents typically increase swimming behaviors (Kirby and Lucki, 1997; Reneric and Lucki, 1998; Page et al., 1999). A similar functional duality exists in the modulation of depression in human patients: noradrenergic agents (i.e., reboxetine) tend to improve drive or motivation, whereas serotonergic agents (i.e., fluoxetine) tend to improve mood (Dubini et al., 1997a,b; Charney, 1998).

Interestingly, α_2-ARs are expressed in both serotonergic and noradrenergic brain regions and can regulate the release of both neurotransmitters (Baraban and Aghajanian, 1980, 1981). Thus, α_2-ARs potentially are valuable therapeutic targets for antidepressant drug development, because they could affect both the mood and motivational characteristics of the disease.

When we initiated these studies, the role of particular α_2-AR subtypes in depression-related behaviors was unclear. Fortunately, mouse strains lacking the α_{2A}-AR, α_{2B}-AR, or α_{2C}-AR subtypes (Link et al., 1995, 1996; Hein et al., 1999) and a mouse line containing a point mutation (D79N) in the α_{2A}-AR (MacMillan et al., 1996) now permit the delineation of many α_2-AR subtype-specific functions (Rohrer and Kobilka, 1998). Sallinen et al. (1999) have used the forced swim test (Porsolt et al., 1977, 1978a) to evaluate the role of the α_{2C}-AR subtype in stress-related responses. Sallinen and his colleagues (1999) observed that the absence of the α_{2C}-AR in knock-out mice appeared stress-protective, leading to an increase in activity, whereas overexpression of α_{2C}-AR in transgenic mice reduced activity in the forced swim test, indicative of increased stress susceptibility.

The present studies were undertaken to assess the role of the α_{2A}-AR subtype in depression-related settings, particularly because of the known role of catecholamines and the α_2-ARs in the stress response (Bliss et al., 1968; Abercrombie and Jacobs, 1987a,b; Nukina et al., 1987; McEwen and Sapolsky, 1995; Quirarte et al., 1997) and potentially in stress-related depression (Tejani-Butt et al., 1994). When compared with Sallinen's results, the present findings reveal the importance of subtype-selective α_2-AR agents as potential antidepressant agents.

We compared the behavior of wild-type (WT) and α_{2A}-AR knock-out mice in the forced swim test. We selected the tricyclic antidepressant imipramine, a compound that blocks neurotransmitter reuptake primarily at norepinephrine transporters (Ordway et al., 1997) as the test agent to validate our forced swim test. We observed that the effects of imipramine in the forced swim test were dependent on the presence of the α_{2A}-AR. Comparing these results with Sallinen's results using α_{2C}-AR altered mice, it seems that the α_{2A}-AR appears to be stress-protective, whereas

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the α_{2C}-AR seems to mediate stress susceptibility. Thus, subtype-selective α_2-AR agents might prove to be valuable targets for antidepressant therapy.

MATERIALS AND METHODS

Animals. Wild-type male and female C57 Bl/6 mice were purchased from Charles River Laboratories (Wilmington, MA), and a breeding colony was maintained in the Vanderbilt University Animal Care Facility. Male and female α_{2A}-AR-knock-out (α_{2A}-AR-KO) mice in the C57 Bl/6 genetic background were provided by Brian Kobilka (Stanford University), and a breeding colony was maintained in the Vanderbilt University Animal Care Facility. All procedures were approved by the Vanderbilt University Animal Care and Use Committee in accordance with the Animal Welfare Act and the 1989 amendments to this act. The generation of the α_{2A}-AR-KO line has been reported previously (Altman et al., 1999). It is important to note that the α_{2A}-AR-KO mice did not demonstrate any detectable changes in circadian rhythms or nocturnal activity (L. MacMillan and B. K. Kobilka, unpublished observations). Animals in groups of two to five were housed in $11 \times 7 \times 9$ inch cages with standard rodent chow and water available *ad libitum*. The animals were maintained on a 12 hr light/dark cycle, with lights on from 6:00 A.M. to 6:00 P.M.

A group of 56 wild-type C57 Bl/6 mice and 57 α_{2A}-AR-KO mice in the C57 Bl/6 genetic background, aged 7–10 weeks, were used for the forced swim test, open field mobility test, and the light–dark test. The mice were first tested in either the open field mobility paradigm or the light–dark test. Then, after 1 week of rest in their home cages, the mice were re-randomized and examined in the Porsolt forced swim test. No mice were evaluated in the forced swim test without first being evaluated in either the open field mobility or the light–dark test, to control for any potential testing order effects. Mice were moved from the animal care facility to the testing room and allowed to rest for at least 1 hr before the tests were performed.

Porsolt forced swim test. Mice aged 7–10 weeks were placed in a Plexiglas cylinder (diameter, 15 cm) containing water at a temperature of 22–24.5°C and a depth of 14–16 cm so that they could not escape and could not touch the bottom. The animals were placed in the cylinders for observation and videotaping in a 5 min test swim. Two to four mice at a time were videotaped from the side. A cardboard divider separated the cylinders so that the mice could not see each other during the trials. After the trials, the mice were placed in a rewarming cage surrounded by a heating pad on medium setting for 15–20 min. To score the activity, behaviors recorded on the videotapes were categorized into one of six classes: 0, Floating, the mouse is completely still in the water, except for isolated movements to right itself; 1, Twitching, rhythmic, seemingly involuntary movement of one hind leg; 2, Kicking, movement of both hind legs; 3, Swimming, movement of all four legs with body aligned horizontally in the water; 4, Climbing, movement of all four legs with body aligned vertically in the water; 5, Thrashing, rapid alternation between climbing, swimming, and efforts to right itself.

The experimenter was blind to the genotype, drug, and preswim conditions of the mice when watching the videotapes. The order and duration of episodes of each behavior were recorded in an Excel spreadsheet that was used then to calculate the total duration of each individual behavior and combinations of the behaviors (see Table 1). The data were imported into StatView statistical software (SAS Institute, Cary, NC) for more complex statistical analysis. The sum of the durations of floating, twitching, and kicking behaviors during the 5 min test swim was used as the index of immobility. Results in Figure 1 are presented as the mean of this sum plus the SEM for each group. The durations of the individual behaviors are listed in Table 1.

For each experiment, the mice were divided into two groups on the morning of day 1. Animals in one group were placed in the cylinders for a 2.5 min preswim, after which they were toweled off gently and allowed to dry in the rewarming cage. Animals in the other group remained in their home cages. Then they were administered an intraperitoneal injection of either saline or 10 mg/kg imipramine or given no injection and returned to the animal care facility. That evening, between 7:00 and 8:00 P.M., the saline- or imipramine-treated animals again were administered an injection of either saline or 10 mg/kg imipramine. The next morning, the animals were administered a third injection of either saline or the drug and allowed to rest in their home cages for 1 hr before the 5 min test swim. Thus, the animals treated with imipramine received the drug 24, 12, and 1 hr before behavioral assessment. It is common to observe changes in immobility in the forced swim test (the "antidepressant effect") when drugs are administered relatively acutely (within 24 hr before the swim assessment). In humans, antidepressant administration often requires 2–3 weeks before patients experience an alleviation of their depressive symptoms. It is for this reason that the forced swim test is used as a predictive measure of antidepressant efficacy in humans and not a phenomenological model of antidepressant action.

Open field mobility. This test is used as a measure of general activity level. Mice are placed in 27×27 cm chambers with clear Plexiglas sides that are 20 cm in height. Infrared beams located 1 cm from the bottom of the chamber on both the *x* and *y* axes of the box are used to monitor the animals' movement in the floor of the chamber. Infrared beams that are located 5.5 cm above the floor on two sides of the chamber record vertical activity. Software and chambers were purchased from MED Associates (Georgia, VT). A box size (the area, in units of infrared beams, that is occupied by the mouse itself) of two units was used, with a resting delay (the amount of time that must elapse without movement for the animal to be considered "resting") of 50 msec. Three dependent variables recorded by the MED Associates software are reported in Results: (1) the total distance traveled, in centimeters; (2) the number of rearings or raising up on the hind legs, scored as breakages of the "vertical" beams; and (3) the relative amount of time spent in the center versus the perimeter of the chamber. Total distance traveled is indicative of general activity level. Rearing and center–perimeter residence time are used as measures of anxiety (Treit and Fundytus, 1988; Carli et al., 1989; Meng and Drugan, 1993; Steiner et al., 1997; Angrini et al., 1998; Heisler et al., 1998; Nasello et al., 1998; Ramboz et al., 1998; Zhuang et al., 1999).

Light–dark paradigm. This test is used as a measure of the animals' anxiety level (Crawley and Goodwin, 1980; Hascoet and Bourin, 1998). Mice are normally nocturnal but are also exploratory. When placed in a novel environment in which they can choose to be in a dark or brightly lit place, they spend most of their time in the dark place. However, habituation to the novel environment or treatment with anxiolytic drugs increases the percentage of time the mice spend in the brightly lit half of the chamber.

MED Associates software and open field chambers were used with an insert (also purchased from MED Associates) that made half of the area of the chamber dark. An arched opening (5.5×7 cm) allowed the animal to pass freely between the two halves of the chamber. Again, the data were collected in 20 min sessions divided into 5 min blocks. Then the data were analyzed for the time spent in each of the two zones and the number of transitions between these zones. In preliminary experiments, the ambient light in the room was adjusted so that wild-type mice spent 75% of their time in the dark half of the chamber. This adjustment allows us to observe either increases or decreases in the time the mice spend in the dark, on the basis of the limits of this test (spending 50% of the time in the dark is indicative of no preference for either side of the chamber, whereas spending 100% of the time in the dark is the maximum amount of "anxiety" measured by this test).

Drug administration. Where indicated, mice were injected intraperitoneally with either saline or 10 mg/kg imipramine (hydrochloride salt) dissolved in sterile saline at a volume of 10 ml/kg at 22–24 hr, 12–14 hr, and 1 hr before the indicated tests. This dose of imipramine was selected on the basis of observations in the literature that the appropriate dose of this drug is in the range of 1–30 mg/kg (Porsolt et al., 1978b; Baamonde et al., 1992; Layer et al., 1995). Preliminary experiments in which 30 mg/kg was injected revealed a striking sedative effect of this high dose of the drug. Some of the mice demonstrated reduced home cage activity within minutes of administration of 30 mg/kg imipramine, which would confound the interpretation of all of the experiments described above. Therefore, the dose of 10 mg/kg was selected for the studies reported here. The three-dose regimen was selected in an effort to minimize variation in the levels of imipramine in the appropriate target tissues in light of the 12 hr half-life of this drug *in vivo*, while avoiding the sedative effects described above. Imipramine was purchased from Research Biochemicals, Natick, MA.

Statistical analysis. Results were calculated using ANOVA or repeated measures ANOVA where appropriate, using StatView Statistical Software (SAS Institute, Cary, NC). Bonferroni–Dunn follow-up tests were performed when necessary.

RESULTS

Porsolt forced swim test

Because we were evaluating the impact of a manipulation of the noradrenergic system, we examined the effect of imipramine on

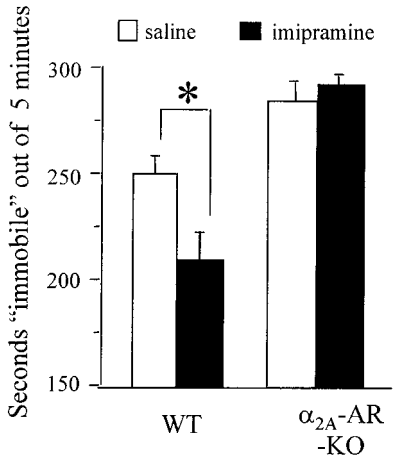

Figure 1. Reduced immobility after a 2.5 min preswim defines the antidepressant effect of imipramine in the forced swim test, and α_{2A}-AR-KO mice are insensitive to this effect. WT and α_{2A}-AR-KO mice in the C57 Bl/6 genetic background were subjected to a 2.5 min preswim as described in Materials and Methods and by Sallinen et al. (1999). After the preswim, the mice were injected intraperitoneally with the indicated agents (saline, *open bars*; imipramine, *filled bars*). Twenty-four hours after the preswim, the mice were evaluated in a 5 min test swim that was videotaped and scored as described in Materials and Methods. The sum of the durations of floating, twitching, and kicking (immobile) behaviors (mean ± SEM) is presented as the dependent variable; $n = 8$–9 mice per group. [ANOVA results: genotype effect, $F_{(1,30)} = 38.9$, $p < 0.0001$; drug effect, $F_{(1,30)} = 3.08$, $p = 0.0896$; drug × genotype interaction, $F_{(1,30)} = 6.57$, $p = 0.016$. WT animals only, ANOVA results: drug effect, $F_{(1,16)} = 6.54$, $p = 0.021$ (indicated by *).] The detailed observations from the videotaped sessions are provided in Table 1.

the forced swim test. (Imipramine is a tricyclic antidepressant that acts primarily on the NE transporter but also has some serotonergic effects.) We first sought to establish the antidepressant effect of imipramine in our system and in C57 Bl/6 WT mice. We observed, as shown in Figure 1 and Table 1, that imipramine caused a significant decrease in immobility, compared with saline, when administered after a 2.5 min preswim in the dosing regimen described in Materials and Methods. After the preswim, imipramine-treated WT mice were immobile for 210 ± 13.4 sec (mean ± SEM), $n = 9$; saline-treated WT mice were immobile for 250 ± 8.5 sec, $n = 9$ (Fig. 1) ($F_{(1,16)} = 6.54$; $p = 0.021$).

When α_{2A}-AR-KO mice were examined after the preswim when treated with either saline or imipramine, we observed that imipramine had no effect on their immobility ($F_{(1,14)} = 0.751$; $p = 0.40$). After the preswim, imipramine-treated α_{2A}-AR-KO mice were immobile for 293.0 ± 3.6 sec, $n = 8$; saline-treated α_{2A}-AR-KO mice were immobile for 285.4 ± 8.0 sec, $n = 8$ (Fig. 1). Thus, the antidepressant effect of imipramine requires the presence of the α_{2A}-AR.

Interestingly, the α_{2A}-AR-KO mice exhibited more immobility than WT mice regardless of preswim or drug treatment ($F_{(1,62)} = 58.4$; $p < 0.0001$). WT mice that were treated with saline or imipramine without a preswim were immobile for 187.3 ± 10.3 and 186.0 ± 10.8 sec, respectively, whereas α_{2A}-AR-KO mice treated with saline or imipramine without a preswim were immobile for 232.0 ± 9.2 and 234.1 ± 9.6 sec, respectively (Fig. 1, Table 1). This observation suggests that the loss of the α_{2A}-AR has a "depressant" effect and that the α_{2A}-AR-KO mice have a greater response to stressful stimuli than WT mice.

WT mice that were not injected demonstrated considerable variability in their duration of immobility (data not shown), so that the uninjected group was not statistically different from either the saline- or imipramine-treated group. This finding suggests that the stress of being injected intraperitoneally has a normalizing effect on the behavior of WT mice, allowing examination of the specific effects of the drugs. Not surprisingly, α_{2A}-AR-KO mice that were not injected behaved similarly to α_{2A}-AR-KO mice injected with either saline or imipramine (data not shown). Furthermore, they were immobile for longer periods than WT mice. This suggests that the loss of α_{2A}-AR expression is correlated with an altered stress response, whether the source of stress is injection or placement in an inescapable tank of water.

The alterations in immobility in both imipramine-treated and α_{2A}-AR-KO mice appear to be mediated by the noradrenergic system. First, the decrease in immobility of imipramine-treated mice is attributable to an increase in climbing and not swimming behaviors (Table 1). Second, preliminary examination of the effects of fluoxetine in this test demonstrated no effect on activity. This result is not completely surprising; some studies involving rats in the forced swim test have also demonstrated no significant effect of fluoxetine (Paul et al., 1990; Millan et al., 1997; Berton et al., 1999), although several studies have demonstrated an anti-immobility effect of fluoxetine in rats (Duncan et al., 1996; Kirby and Lucki, 1997; Reneric and Lucki, 1998; Moser and Sanger, 1999; Page et al., 1999). In mice, fluoxetine seems to be less efficacious than imipramine (Sanchez and Meier, 1997; Khisti and

Table 1. Durations of individual behaviors in 5 min test swim

	Floating	Twitching	Kicking	Swimming	Climbing	Thrashing	Immobility (floating + twitching + kicking)
No preswim							
WT, saline	5.1 ± 2.5	49.3 ± 17.8	132.9 ± 16.3	40.6 ± 8.6	27.7 ± 8.9	44.4 ± 8.0	187.3 ± 10.3
WT, imipramine	1.7 ± 1.1	61.8 ± 18.3	122.6 ± 22.4	62.2 ± 15.1	7.8 ± 5.2#	44.0 ± 11.5	186.0 ± 10.8
KO, saline	55.0 ± 18.6*	112.7 ± 22.6*	64.3 ± 10.7*	33.8 ± 10.5	8.1 ± 5.5*	26.1 ± 7.2	232.0 ± 9.2*
KO, imipramine	189.7 ± 16.1*,#	16.4 ± 8.0#	28.0 ± 9.1*	42.7 ± 9.0	6.8 ± 4.5	16.4 ± 7.4*	234.1 ± 9.6*
2.5 min preswim							
WT, saline	53.9 ± 11.6	119.7 ± 24.1	77.2 ± 24.8	26.6 ± 6.0	19.2 ± 7.1	3.4 ± 1.4	250.8 ± 8.5
WT, imipramine	23.2 ± 8.3	129.0 ± 16.8	57.9 ± 11.7	21.1 ± 5.8	51.9 ± 14.7#	16.9 ± 7.8#	210.1 ± 13.4#
KO, saline	185.9 ± 28.5*	55.6 ± 18.7*	43.9 ± 14.1	13.6 ± 7.3	0.75 ± 0.75	0.25 ± 0.25	285.4 ± 8.0*
KO, imipramine	260.8 ± 24.3*,#	14.3 ± 9.5*	18.0 ± 14.0	14.9 ± 2.6	1.5 ± 1.5*	0.625 ± 0.625*	293.0 ± 3.6*

Behaviors were defined as described in Materials and Methods. Durations (in seconds) were quantified by blinded monitoring of videotaped behaviors and are expressed as mean ± SEM, $n = 8$ or 9 mice per group. *$p < 0.05$, compared with WT, same drug and preswim treatment; #$p < 0.05$ compared with saline, same genotype and preswim treatment (Fisher's PLSD).

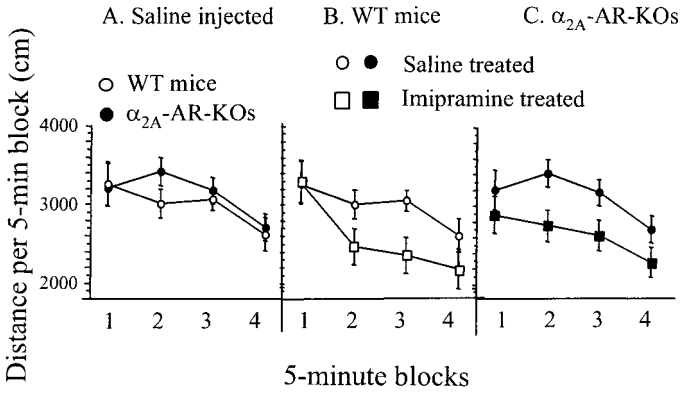

Figure 2. Distance traveled in the open field reveals no hypoactivity or lack of mobility in α_{2A}-AR-KO mice compared with WT mice but a sedative effect of imipramine in both genotypes. WT (*open symbols*) or α_{2A}-AR-KO (*filled symbols*) mice were injected with either saline (*circles*) or imipramine (*squares*) (see Materials and Methods) and placed in the open field mobility chambers for 20 min sessions. Distance traveled in centimeters was recorded in each of four 5 min blocks across the session (mean ± SEM, n = 11–16 mice per group). Genotype effect, $F_{(1,49)} = 0.324$, $p = 0.5715$. Drug effect, $F_{(1,49)} = 5.48$, $p = 0.0233$. Time block effect, $F_{(3,147)} = 16.5$, $p < 0.0001$. The interaction effects were not significant.

Chopde, 2000), which may explain why we failed to see an effect. It is possible that this strain of mice is less responsive to manipulations of the serotonergic system or that our test was not sensitive enough to detect it. Because of this lack of effect in our laboratory and others, we did not evaluate fluoxetine further in these studies.

Open field mobility

Distance traveled

One possible explanation for the reduction in activity of α_{2A}-AR-KO mice compared with WT mice in the forced swim test is a general hypoactivity or lack of mobility of the animals lacking expression of the α_{2A}-AR. Similarly, the apparent antidepressant effect of imipramine in WT mice might, instead, be attributable to a psychostimulant effect of imipramine. To assess these possible explanations for the findings in the forced swim test, the mice were examined in the open field mobility paradigm.

There was no main effect of genotype on open field activity ($F_{(1,49)} = 0.324$; $p = 0.512$). There was, however, a significant drug effect ($F_{(1,49)} = 5.5$; $p = 0.023$). The drug × genotype interaction was not significant ($F_{(1,49)} = 0.003$; $p = 0.96$).

Specifically, after saline injection, the distance traveled by the α_{2A}-AR-KO mice was indistinguishable from that of the WT mice (Fig. 2*A*) ($F_{(1,26)} = 0.202$; $p = 0.657$). Thus, we can be confident that the decrease in active behaviors of α_{2A}-AR-KO mice after saline injection in the Porsolt forced swim test is not attributable to impaired mobility or general hypoactivity in the α_{2A}-AR-KO strain.

Furthermore, for both WT and α_{2A}-AR-KO mice, imipramine caused a decrease in activity in the open field compared with saline ($F_{(1,49)} = 5.48$; $p = 0.0233$), the opposite of its effect in the forced swim test (Fig. 2). Thus, the reduced immobility or antidepressant effect of imipramine in the forced swim test is not attributable to a possible stimulant effect of imipramine. In fact, imipramine appears to have a sedative effect in the open field mobility paradigm. This effect must be mediated by molecules other than the α_{2A}-AR, because it is evident in both WT and α_{2A}-AR-KO mice.

All groups, regardless of drug or genotype, showed habituation to the novel environment, as demonstrated by decreasing activity throughout the duration of the test ($F_{(3,147)} = 16.5$; $p < 0.0001$).

Rearing behavior

An unexpected but interesting difference between WT and α_{2A}-AR-KO mice was noted when rearing behavior in the open field was quantified. The α_{2A}-AR-KO mice exhibited significantly fewer episodes of this behavior than WT mice when injected with either saline or imipramine (Fig. 3*A*,*B*) ($F_{(1,38)} = 12.6$; $p = 0.0011$). However, when uninjected, there was no difference between WT and α_{2A}-AR-KO mice in terms of the number of rearing episodes ($F_{(1,18)} = 0.127$; $p = 0.725$) (Fig. 3*C*). This finding suggests a possible difference in the anxiety levels between the two genotypes as manifest by "injection stress" but not when injection stress is absent. We further addressed this possible difference in anxiety level by examination of the center–perimeter residence time of the mice.

Center–perimeter residence time

The behavior of mice in the open field mobility paradigm can be characterized further by quantifying the amount of time the mice spend in the center versus the perimeter of the open field space. It is assumed that the mice feel safer in the perimeter regions of the open field chambers, close to the walls. More ventures into the center of the chamber are therefore interpreted as a decrease in anxiety. This measure of anxiety has been validated predictively by the demonstration that known anxiolytic agents (specifically diazepam, chlordiazepoxide, and pentobarbital) dose-dependently decrease the amount of time spent in the perimeter

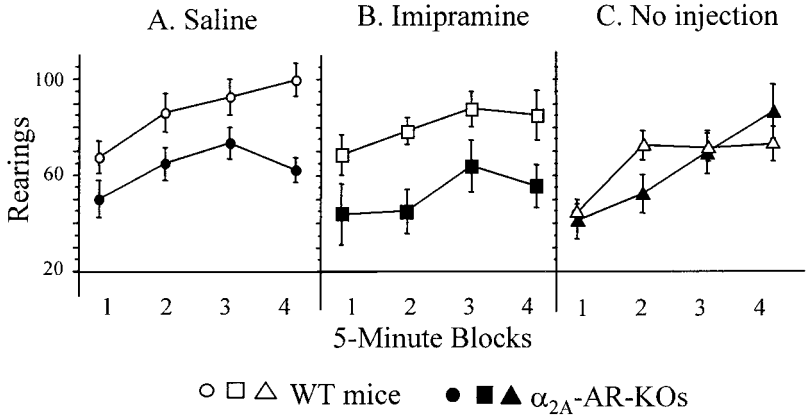

Figure 3. Analysis of rearing reveals an anxiogenic effect of the loss of the α_{2A}-AR after injection stress but no effect of imipramine in WT or α_{2A}-AR-KO mice. WT (*open symbols*) and α_{2A}-AR-KO (*filled symbols*) mice were subjected to injection with saline (*A*, *circles*), imipramine (*B*, *squares*), or no injection (*C*, *triangles*) and placed in the open field chambers for 20 min sessions. The number of episodes of rearing (vertical beam breaks) is recorded for each of four 5 min blocks throughout the 20 min session (mean ± SEM, n = 8–16 mice per group). Repeated measures ANOVA results: genotype effect, $F_{(1,56)} = 9.97$, $p = 0.0026$; drug treatment effect, $F_{(2,56)} = 1.51$, $p = 0.2298$; time block effect, $F_{(3,168)} = 22.9$, $p < 0.0001$.

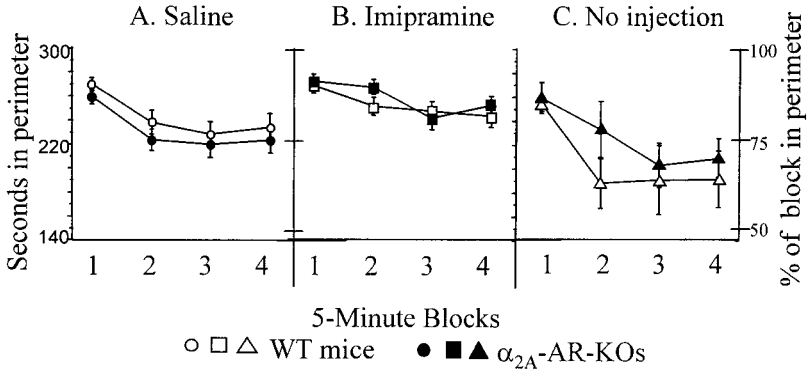

Figure 4. Center–perimeter analysis reveals no difference between WT and α_{2A}-AR-KO mice but an apparent anxiogenic effect of imipramine in both genotypes. WT (*open symbols*) and α_{2A}-AR-KO (*filled symbols*) mice were subjected to injection with saline (*A*, *circles*), imipramine (*B*, *squares*), or no injection (*C*, *triangles*) and placed in the open field chambers for 20 min sessions. The time spent in the perimeter (outer four infrared beam widths, 75% of the floor area of the chamber) is recorded for each of four 5 min blocks throughout the 20 min session (mean ± SEM, $n = 8$–16 mice per group). Genotype effect, $F_{(1,64)} = 0.321$, $p = 0.57$. Drug effect, $F_{(2,64)} = 7.318$, $p = 0.0014$. Time block effect, $F_{(3,192)} = 34.8$, $p < 0.0001$. Bonferroni–Dunn *post hoc* test: imipramine versus no injection, mean difference = 40.23, critical difference = 15.8, $p < 0.0001$; imipramine versus saline, mean difference = 20.8, critical difference = 13.7, $p = 0.0003$.

of the open field (Treit and Fundytus, 1988), and genetically modified mice lacking particular 5-HT receptor subtypes (Heisler et al., 1998; Ramboz et al., 1998; Zhuang et al., 1999) or dopamine receptors of the D3 subtype (Steiner et al., 1997) show the expected change in center–perimeter residence time based on their demonstrated roles in mediating or attenuating anxiety. As shown in Figure 4*A*, *C*, both WT and α_{2A}-AR-KO C57 Bl/6 mice habituate normally in this paradigm when injected with saline or not injected, initially spending most of their time in the perimeter of the chamber, then gradually spending more and more time in the center of the chamber (no main effect of genotype: $F_{(1,64)} = 0.321$; $p = 0.573$). By the end of the 20 min session, the time spent in the perimeter region of the chamber is proportional to the area of the floor covered by that region (75%) (Fig. 4*A*,*C*) ($F_{(3,123)} = 26.7$, $p < 0.0001$). Imipramine injection increases the amount of time in the perimeter over in the 20 min session for both genotypes (Fig. 4*B*) ($F_{(2,64)} = 7.318$; $p = 0.0014$; Bonferroni–Dunn *post hoc* test: imipramine vs no injection, mean difference = 40.23, critical difference, 15.8, $p < 0.0001$; imipramine vs saline, mean difference = 20.8, critical difference = 13.7, $p = 0.0003$).

The α_{2A}-AR-KO mice show behavior similar to WT mice in this paradigm. They habituate normally when treated with saline or no injection and to a lesser extent when treated with imipramine. The effect of imipramine to increase perimeter residence time may represent an "anxiogenic" effect of the drug or be another manifestation of the apparent sedative effect of imipramine in the open field mobility test, as noted above and shown in Figure 2. However, regardless of the interpretation, this effect of imipramine on perimeter residence time is not mediated by the α_{2A}-AR subtype, because it is present in both WT and α_{2A}-AR-KO C57 Bl/6 mice.

Light–dark paradigm

As another measure of anxiety, the mice were tested in the light–dark paradigm. Mice prefer to spend most of their time in the dark. Mice that are more anxious will spend more time in the dark half of the chamber, whereas mice that are less anxious or habituated to the chamber will spend approximately equal time exploring both the light and dark halves of the chamber. The light–dark paradigm in rodents has been validated predictively as a measure of anxiety using agents that are known to have either anxiolytic or anxiogenic effects in humans (Crawley and Goodwin, 1980; Bilkei-Gorzo et al., 1998; Hascoet and Bourin, 1998).

When α_{2A}-AR-KO and WT animals are examined in the light–dark paradigm, the α_{2A}-AR-KO mice appear more anxious than WT mice after injection stress (Fig. 5). In the first 5 min block of the 20 min session, there is no difference between injected WT and α_{2A}-AR-KO mice in terms of the time spent in the dark half of the chamber. However, as the session progresses, the WT mice explore the light half of the chamber to a greater extent than do α_{2A}-AR-KO mice (Fig. 5*A*) ($F_{(3,168)} = 2.91$; $p = 0.0363$). Interestingly, for both WT and α_{2A}-AR-KO mice (Fig. 5*B*), treatment with imipramine causes an apparent anxiogenic effect, such that both genotypes remained in the dark half of the chamber for longer periods of time when treated with imipramine than when treated with saline (Fig. 5) ($F_{(1,35)} = 5.46$; $p = 0.025$). This finding may be attributable to the sedative effect of imipramine seen in the open field distance traveled, because there is a trend toward a decrease but no significant difference in the number of transitions between the light and dark halves of the chamber for mice treated with imipramine versus saline. [Total number of dark→light transitions in 20 min (mean ± SEM): WT, no injec-

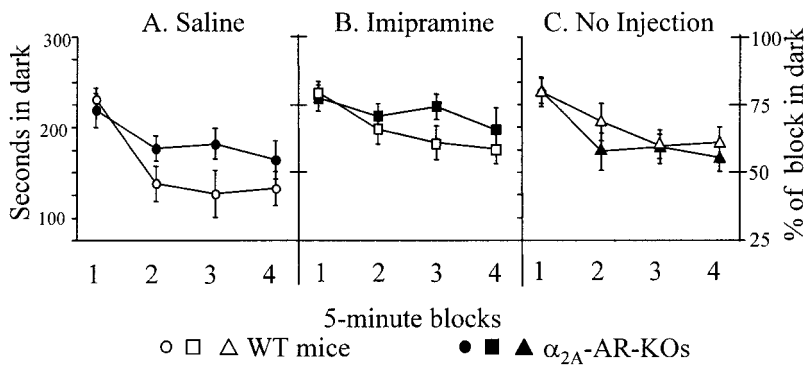

Figure 5. Light–dark analysis reveals an apparent anxiogenic effect of the knock-out of the α_{2A}-AR after injection stress and an anxiogenic effect of imipramine. WT (*open symbols*) and α_{2A}-AR-KO (*filled symbols*) mice were subjected to injection with saline (*A*, *circles*), imipramine (*B*, *squares*), or no injection (*C*, *triangles*) and placed in the open field chambers with the light–dark insert included (see Materials and Methods) for 20 min sessions. The time spent in the dark half of the chamber is recorded for each of four 5 min blocks throughout the 20 min session (mean ± SEM, $n = 8$–14 mice per group). Repeated measures ANOVA results, all groups: genotype effect, $F_{(1,56)} = 0.774$, $p = 0.383$; drug effect, $F_{(2,56)} = 2.77$, $p = 0.072$; time block effect, $F_{(3,168)} = 40.24$, $p < 0.0001$; time block × genotype interaction, $F_{(3,168)} = 2.91$, $p = 0.0363$. Saline plus imipramine groups only, repeated measures ANOVA results: genotype effect, $F_{(1,35)} = 2.15$, $p = 0.152$; drug effect, $F_{(1,35)} = 5.46$, $p = 0.025$; time block effect, $F_{(3,105)} = 26.3$, $p < 0.0001$; time block × genotype interaction, $F_{(3,105)} = 4.66$, $p = 0.0042$.

tion, 159.6 ± 34.8; WT, saline, 152.2 ± 21.6; WT, imipramine, 123.2 ± 18.5; α_{2A}-AR-KO, no injection, 153.9 ± 28.3; α_{2A}-AR-KO, saline, 119.6 ± 18.8; α_{2A}-AR-KO, imipramine, 108.5 ± 24.8; $F_{(2,49)} = 1.33$; $p = 0.274$]. Nevertheless, both WT and α_{2A}-AR-KO animals experience the same effect of imipramine, indicating that the effects of imipramine in this paradigm must not be mediated by the α_{2A}-AR subtype. When WT or α_{2A}-AR-KO mice are not injected before testing in this paradigm, there is no difference in their initial time spent in the dark or in their degree of habituation, similar to the results obtained in the rearing analysis.

DISCUSSION

In the forced swim test, the loss of the α_{2A}-AR caused by deletion of the α_{2A}-AR gene causes an increase in immobility. The increase in immobility in α_{2A}-AR-KO mice cannot be attributed to a general hypoactivity of the mice, as assessed in the open field mobility assay. Because in wild-type animals an increase in immobility can be reversed by an antidepressant agent and because of the ability of the forced swim test to predict antidepressant drug efficacy, we shall henceforth refer to a decrease in immobility as a depressant response. Because the loss of the α_{2A}-AR elicits a depressive response, we suggest that the α_{2A}-AR normally acts as a "suppressor of depression." Alternatively, the increase in immobility could be attributable to increased despair, defeat, fear, or other unmeasurable "mood" modulation. However, the reversal of this behavior by the antidepressant imipramine in WT mice suggests that the increased immobility in α_{2A}-AR-KO mice is indeed a depressant response. Furthermore, the knock-out of the gene encoding the α_{2A}-AR renders C57 Bl/6 mice insensitive to the effects of the NE-directed antidepressant imipramine in the forced swim test. This implies that the α_{2A}-AR is required for the antidepressant effect of imipramine in this test.

The role of the noradrenergic system in anxiety is well documented (Charney and Redmond, 1983; Abercrombie and Jacobs, 1987a,b; Nukina et al., 1987; Tejani-Butt et al., 1994; McEwen and Sapolsky, 1995; Quirarte et al., 1997; Arnsten, 1998), and the role of the α_2-ARs in anxiety has been examined using non-subtype-selective agents (Millan et al., 2000). However, the specific role of the α_{2A}-AR subtype in anxiety has not been clarified. The α_{2A}-AR-KO mice are more anxious than WT mice after injection stress in terms of rearing in the open field and in terms of time spent in the dark in the light–dark paradigm. However, there is no difference in the anxiety level of α_{2A}-AR-KO and WT mice when evaluated on the basis of the relative amount of time spent in the perimeter versus the center of the open field. Thus, the α_{2A}-AR may mediate anxiety-related behaviors in some situations but not others. Alternatively, measures of center–perimeter time in our chambers may not be sensitive to subtle differences in stress.

The fact that the differences in rearing and light–dark residence time are only apparent after injection is particularly interesting. Presumably, injection causes a stress-related response that elicits the release of norepinephrine in the central and peripheral nervous system. The increase in anxiety demonstrated in terms of rearing and dark residence time is most likely a result of the inability to modulate the release of NE caused by injection stress, brought about, perhaps, by the loss of feedback inhibition of either NE or 5-HT release, which would normally be mediated by the α_{2A}-AR subtype (Lakhlani et al., 1997; Hein et al., 1999). The loss of the α_{2A}-AR likely renders mice incapable of modulating the sympathetic response elicited by injection stress, and this loss leads to anxiogenic-like behaviors. Consistent with this interpretation is the finding that in measuring center–perimeter residence time, there is no difference between WT and α_{2A}-AR-KO mice, and there also is no impact of injection. It is also possible that α_2-ARs could perform their antianxiety function postsynaptically, perhaps by a mechanism similar to that by which they reduce the spontaneous firing of neurons in the locus ceruleus (Lakhlani et al., 1997).

Combining the results in rearing and light–dark analysis with the results in the forced swim test provides insight into the stressful nature of the forced swim test itself. In rearing and light–dark tasks, the animal is not excessively stressed, so the difference between WT and α_{2A}-AR-KO mice only becomes apparent after the added stress of injection. However, in the forced swim test, there is a difference between WT and α_{2A}-AR-KO mice in both the presence and absence of injection stress and in the presence and absence of a stress-inducing preswim. This suggests that the test swim itself is sufficiently stressful to elicit a difference between the behavior of WT and α_{2A}-AR-KO mice. Thus, the difference in behaviors between WT and α_{2A}-AR-KO mice is generally the result of an inability to handle stress, either the stress of injection or the stress of being placed in an inescapable tank of water.

It has long been understood that catecholamines, including norepinephrine, are rapidly released in response to stressful stimuli in what is known as the "fight or flight" response (McEwen and Sapolsky, 1995; Arnsten, 1998). Prolonged exposure to stress induces decreased levels of α_{2A}-AR binding sites in the amygdala (Nukina et al., 1987; Tejani-Butt et al., 1994) and hippocampus (Nukina et al., 1987) and increased α_{2A}-AR binding sites in the midbrain (Nukina et al., 1987). In α_{2A}-AR-KO mice, the genetic removal of the α_{2A}-AR causes a lifelong absence of α_{2A}-ARs in all brain regions. This is consistent with our behavioral observations in the forced swim test in which α_{2A}-AR-KO mice behave as though they have been subjected to a stress-inducing preswim even when they have not, suggesting that they are already depressed. Additionally, α_{2A}-AR-KO mice have enhanced responses to stressors, such as injection in the light–dark and rearing tasks and the preswim in the forced swim, suggesting that they also lack the ability to modulate the increased release of NE in response to stressful stimuli.

Interestingly, the ability of imipramine to elicit behaviors in mice that might be interpreted as anxiogenic is independent of the presence of the α_{2A}-AR but varies with the measure of anxiety examined (Figs. 3, 4, 5), suggesting that the anxiogenic effects of imipramine are not mediated by α_{2A}-ARs. The literature confirms our findings that imipramine varies in its anxiety-related responses in various laboratory paradigms. At least one report in the literature describes an anxiolytic effect of imipramine in the light–dark test (de Angelis, 1996), whereas others report neither an anxiolytic nor an anxiogenic effect in the light–dark test (Shimada et al., 1995) and the elevated plus maze (Lister, 1987) models of anxiety.

We undertook our studies of the forced swim test so that we could directly compare them with those recently published by Sallinen et al. (1999) in which the authors demonstrated that mice lacking the α_{2C}-AR perform as though they were medicated with antidepressants even in the absence of drugs. These mice swam for a longer period of time than their WT counterparts on day 2 of the forced swim test, after a 2.5 or 5 min preswim on day 1. Combining our results with Sallinen's (Fig. 6), we can conclude that the α_{2A} and α_{2C}-ARs perform complementary roles in the

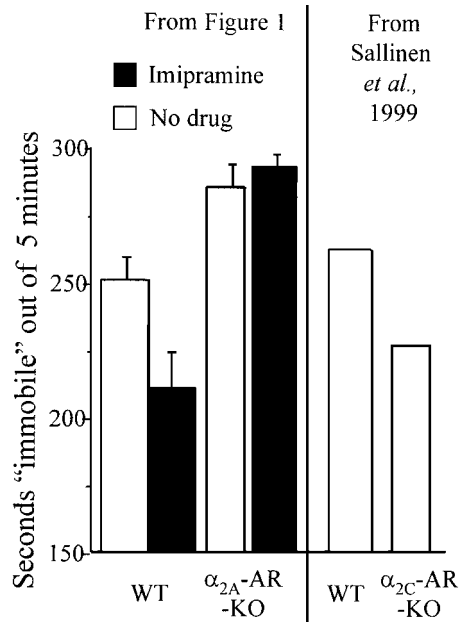

Figure 6. Comparing the behavior of α_{2A}-AR-KO and α_{2C}-AR-KO mice after a 2.5 min preswim reveals complementary roles of these α_2-AR subtypes in the forced swim test. Data from Figure 1 are combined with data from Sallinen et al. (1999). The loss of the α_{2C}-AR has an antiimmobility effect, although the loss of the α_{2A}-AR has the opposite effect, suggesting that the α_{2A}-AR is protective against the depression-related responses measured here, whereas the α_{2C}-AR mediates susceptibility.

signaling pathways that contribute to the behaviors measured in the forced swim test. Our results suggest that a drug that stimulates (or does not inhibit) α_{2A}-ARs while simultaneously inhibiting α_{2C}-ARs might be effective in reducing stress-related depression in humans.

Complementary roles of the α_{2A}-AR and α_{2C}-AR also have been shown to contribute to regulation of NE release in the heart. Hein et al. (1999) have shown that, in isolated atria, the α_{2A}-AR modulates the release of NE at high stimulation frequencies, whereas the α_{2C}-AR modulates release at low stimulation frequencies. Whether similar electrophysiological complementarity exists in the CNS has not been assessed directly, although we do know that the α_{2A}-AR alone cannot fully account for the regulation of NE turnover, at least in the hippocampus (Lakhlani et al., 1997).

The hypothesis that the α_{2A}-ARs endogenously suppress depression suggests that α_{2A}-AR-selective antagonists, or non-subtype-selective α_2-AR antagonists, may not be effective therapeutic tools in human depression. Some, but not all, reports are consistent with this expectation. For instance, Cervo et al. (1990) observed that acute administration of idazoxan, a non-subtype-selective α_2-AR antagonist, had no effect on the immobility of rats on day 2 of forced swim trials. In contrast, idazoxan completely blocked, in a dose-dependent manner, the reduction of immobility induced by chronic administration of desipramine. The authors concluded that the antidepressant effect of desipramine requires the activity of α_2-ARs at the time of the forced swim trial (Cervo et al., 1990). Our results would suggest that the α_{2A}-AR subtype is specifically required for this response, because complete loss of the α_{2A}-AR subtype by genetic knock-out not only reduces activity overall in the forced swim test but also eliminates the antidepressant effect of the tricyclic antidepressant imipramine. The importance of subtype-selective agents has also been demonstrated in reversal of another stress response, anxiety-induced working memory deficits (Birnbaum et al., 2000).

The present findings provide strong evidence that the α_{2A}-AR subtype plays an important role in the modulation of depression and anxiety that may, in some settings, be counteracted by activity at the α_{2C}-AR subtype. Consequently, subtype-selective agonists directed toward the α_{2A}-AR may represent a successful therapeutic intervention for some forms of depression and anxiety.

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