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Antidepressant-Like Effects of κ-Opioid Receptor Antagonists in Wistar Kyoto Rats

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The Wistar Kyoto (WKY) rat strain is a putative genetic model of comorbid depression and anxiety. Previous research showing increased κ-opioid receptor (*KOR*) gene expression in the brains of WKY rats, combined with studies implicating the KOR in animal models of depression and anxiety, suggests that alterations in the KOR system could have a role in the WKY behavioral phenotype. Here, the effects of KOR antagonists in the forced swim test (FST) were compared with the WKY and the Sprague–Dawley (SD) rat strains. As previously reported, WKY rats showed more immobility behavior than SD rats. The KOR antagonists selectively produced antidepressant-like effects in the WKY rats. By contrast, the antidepressant desipramine reduced immobility in both strains. Brain regions potentially underlying the strain-specific effects of KOR antagonists in the FST were identified using c-fos expression as a marker of neuronal activity. The KOR antagonist *nor*-binaltorphimine produced differential effects on the number of c-fos-positive profiles in the piriform cortex and nucleus accumbens shell between SD and WKY rats. The piriform cortex and nucleus accumbens also contained higher levels of KOR protein and dynorphin A peptide, respectively, in the WKY strain. In addition, local administration of *nor*-binaltorphimine directly into the piriform cortex produced antidepressant-like effects in WKY rats further implicating this region in the antidepressant-like response to KOR antagonists. These results support the use of the WKY rat as a model of affective disorders potentially involving KOR overactivity and provide more evidence that KOR antagonists could potentially be used as novel antidepressants.

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INTRODUCTION

Depression is a debilitating illness that exerts a large cost, emotionally and economically, on society (Mauskopf *et al*, 2009). Despite the availability of a number of clinically effective treatments, a large segment of the population diagnosed with depression exhibits a treatment-resistant form of the disorder (Rush *et al*, 2006). Patients with comorbid depression and anxiety suffer from poorer overall outcomes in response to antidepressant treatment (Fava *et al*, 2008). These data suggest that potential antidepressant compounds should be tested in animal models of comorbid depression and anxiety to produce treatments that better address the unique aspects of this subtype of depression.

The Wistar Kyoto (WKY) rat strain was first developed as a normotensive control for the spontaneously hypertensive rat strain (Okamoto and Aoki, 1963). Evidence later emerged that the WKY strain exhibits a unique behavioral phenotype characterized by passive coping behavior and increased sensitivity to stress. For example, WKY rats exhibit increased development of stress-induced ulcers (Pare, 1989b), prolonged elevation of corticosterone after swim stress (Rittenhouse *et al*, 2002), exaggerated depression-like behavior in the forced swim test (FST) and learned helplessness test (Pare, 1994; Lopez-Rubalcava and Lucki, 2000), and increased anxiety-like behavior in the open-field test (Pare, 1989a), elevated plus maze (Pare, 1992), and defensive burying test (Pare, 1992, 1994). These results support the characterization of the WKY strain as a putative genetic model of comorbid depression and anxiety. In addition, this strain shows resistance to the antidepressant efficacy of selective serotonin reuptake inhibitors suggesting that it may provide insight into mechanisms that confer resistance to frontline antidepressant treatment (Lopez-Rubalcava and Lucki, 2000; Tejani-Butt *et al*, 2003; Will *et al*, 2003).

Given the potential utility of the WKY strain as a unique model of psychiatric dysfunction, the neurobiology of its phenotype could provide important understanding of the

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basis for depression or leads to novel targets for treatment. Quantitative trait loci and microarray analyses have been used to identify genes that are differentially expressed in WKY rats that could contribute to their behavioral phenotype (Ahmadiyeh *et al*, 2003, 2005; Solberg *et al*, 2004, 2006; Pearson *et al*, 2006). A key finding was the increased expression of the κ-opioid receptor (KOR) in the locus coeruleus of WKY rats compared to Sprague–Dawley (SD) rats, a result confirmed by real-time PCR (Pearson *et al*, 2006). These data are intriguing in the light of substantial literature linking the dynorphin–KOR system with stress and depression. Specifically, KOR activation mediates some endogenous neurobiological aspects of aversion and also comprises an important part of the response to environmental stressors (Iwamoto, 1985; Bals-Kubik *et al*, 1993; McLaughlin *et al*, 2003). Also, stressors that produce end points of depression in behavioral models (eg, learned helplessness and the FST) increase dynorphin expression in limbic regions (Shirayama *et al*, 2004) and, conversely, KOR agonists reproduce end points of depressive behavior (Newton *et al*, 2002; Mague *et al*, 2003; McLaughlin *et al*, 2006a). Consistent with a role for this system in stress-induced depression, disruption of the prodynorphin gene produces resistance to stress-induced immobility (McLaughlin *et al*, 2003). Finally, KOR antagonists produce antidepressant-like effects in a number of rodent models (Newton *et al*, 2002; Mague *et al*, 2003; McLaughlin *et al*, 2003, 2006b; Shirayama *et al*, 2004).

In this study, we examined the hypothesis that increased KOR function is a contributor to the depression-like features in WKY rats. The potential antidepressant-like effects of KOR antagonists in the FST were compared between WKY and SD rat strains. The SD strain was chosen because of its use as the comparison strain in the previously discussed microarray study (Pearson *et al*, 2006) and in the previous behavioral studies conducted by our laboratory (Lopez-Rubalcava and Lucki, 2000; Rittenhouse *et al*, 2002) and others (Tejani-Butt *et al*, 2003; Ma and Morilak, 2004). Using c-fos expression as a marker of neuronal activity after swim stress and KOR antagonist treatment, we identified the nucleus accumbens and piriform cortex as brain regions in which differences in KOR function may differentiate the strains. Consequently, KOR and dynorphin A protein levels in the nucleus accumbens and piriform cortex were compared in behaviorally naïve rats from both strains. Moreover, the local infusion of *nor*-binaltorphimine dihydrochloride (*nor*-BNI) in the piriform cortex of WKY rats was sufficient to produce antidepressant-like effects in this strain.

MATERIALS AND METHODS

Animals

Male SD (Charles River Laboratories, Wilmington, MA) and WKY (Taconic, Germantown, NY) rats, weighing 225–250 g on arrival, were used in these experiments. The subjects were housed two per cage in a temperature-controlled (22°C) colony room under a 12 : 12 h light–dark schedule (lights on at 0700 hours). Food and water were freely available. All rats were handled daily for a week before behavioral testing. The care and use of animals were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Drug Treatments

nor-BNI (Tocris Bioscience, Ellisville, MO), desipramine hydrochloride (Sigma-Aldrich, St Louis, MO), and sodium pentobarbital (Sigma-Aldrich) were dissolved in distilled water. 2-(3,4-Dichlorophenyl)-*N*-methyl-*N*-[(1S)-1(3-isothiocyanatophenyl)-2-(1-pyrrolidinyl)ethyl]acetamide hydrochloride (DIPPA; Tocris Bioscience, Ellisville, MO, USA) was dissolved in a mixture of 20% DMSO and 80% distilled water. All injection solutions were administered at a volume of 2 ml/kg. All systemic injections were given subcutaneously except for pentobarbital, which was given intraperitoneally. In the piriform cortex microinjection study, *nor*-BNI was dissolved in artificial cerebrospinal fluid (aCSF).

Forced Swim Test Procedures

The modified rat FST used in these experiments was similar to the protocol used previously in this laboratory (Detke *et al*, 1995). Rats were placed into a glass cylinder (21 cm diameter) filled with water (23–25°C; 30 cm depth) for 15 min. After the 15 min swim session, rats were removed, dried with paper towels, and placed into a polycarbonate cage located on a heating pad for 15 min. The rats were then returned to their home cage. A 5 min swim test was conducted 24 h after the 15 min session. This test was videotaped and scored for the frequency of climbing, swimming, and immobility behaviors by an observer masked to treatment using a time-sampling technique previously described (Detke *et al*, 1995). Climbing was defined as upward-directed movement of the forepaws aimed toward the sides of the cylinder. Swimming was defined as horizontal movement throughout the cylinder. Immobility consisted of minimum movement required to keep the rat's head above water in the absence of the other two behaviors.

In the first two experiments, owing to their long duration of action (Jones and Holtzman, 1992; Chang *et al*, 1994), *nor*-BNI (1, 5, and 10 mg/kg) and DIPPA (1, 2.5, 5, and 10 mg/kg) were administered only once, 0.5 h after the first swim session and 23.5 h before the 5 min swim test. The study with desipramine (20 mg/kg) used the traditional antidepressant treatment regimen with injections given at 23.5, 5, and 1 h before the 5 min swim test. Rats in the control groups received 0.9% saline injections.

nor-BNI Microinjection in the Piriform Cortex

Rats used in this study were anesthetized using sodium pentobarbital (60 mg/kg), mounted into a stereotaxic device (Kopf, San Diego, CA), and bilateral stainless steel guide cannulae (22 gauge; Plastics One, Roanoke, VA) were implanted within the piriform cortex (AP + 1.5, ML ± 4.2, DV −7.0 mm, relative to bregma (Paxinos and Watson, 2005). The cannulae were kept in place using cranioplastic cement anchored to the skull by two screws. Stainless steel dummy cannulae were inserted after completion of the surgery to prevent blockage.

Rats were given 7 days to recover from the surgery before being tested in the modified FST. After being exposed to the

first 15 min swim session, *nor*-BNI (2.5 and 10 μg per side) was infused 23.5 h before the 5 min test, the same time point as the systemic *nor*-BNI injections in the previous FST experiment. Rats in the control group received aCSF infusions. *Nor*-BNI or aCSF (1 μl) was infused bilaterally through injection cannulae (26 gauge) extending 1 mm beyond the previously implanted guide cannulae, using polyethylene tubing connected to a 10 μl syringe (Hamilton, Reno, NV). An infusion pump (Instech, Plymouth Meeting, PA) controlled the flow rate (0.1 μl/min) of the solution. The injection cannulae were left in place for an additional 5 min to allow for diffusion and then were replaced by dummy cannulae.

After completion of the FST, rats were anesthetized with an overdose of sodium pentobarbital. Fast Green dye (1 μl) was infused using the same parameters as the aCSF and *nor*-BNI injections given before testing. The rats were then decapitated and their brains were removed and stored at −80°C until processing. Sections through the piriform cortex (40 μm) were obtained using a cryostat and an observer masked to treatment and behavioral performance verified the location of the Fast Green dye.

Locomotor Activity

Locomotor activity was measured in a Plexiglas open-field arena (*L* 46 cm × *W* 38 cm × *H* 38 cm) located in a room lit by two 15 W lightbulbs pointed away from the testing arena (75 lux). Total distance traveled (cm) was measured over a 15 min time period using the SMART video tracking system (San Diego Instruments, San Diego, CA). A single systemic injection of saline, *nor*-BNI (10 mg/kg) or DIPPA (10 mg/kg) was given 23.5 h before testing.

c-Fos Immunohistochemistry

At 2 h after exposure to the 5 min FST session, rats from the saline and *nor*-BNI (10 mg/kg) treatment groups (both strains) were anesthetized with sodium pentobarbital (60 mg/kg) and transcardially perfused with a heparinized saline solution followed by a 4% paraformaldehyde solution. The brains were removed and stored in 4% paraformaldehyde at a temperature of 4°C for 24 h. Brains were then stored in a 20% sucrose solution until sectioning. A second set of subjects used to examine the effects of the FST procedure on c-fos expression did not receive any drug injections (naïve *vs* FST experienced) and were perfused in an identical manner. Both sets of tissues were processed using a protocol previously described (Roche *et al*, 2003).

Coronal sections (30 μm thick) were cut on a cryostat and collected into six–well plates. The sections were rinsed in phosphate buffer (PB; 0.1 M) and stored in cryoprotectant solution at −80°C. For the reaction, sections were rinsed three times in PB and incubated with 0.75% H_2O_2 for 30 min followed by three 10 min rinses in phosphate buffered saline (PBS). Sections were incubated in rabbit anti-c-fos (1 : 1000; Abcam, Cambridge, MA) for 3 days at 4°C. Sections were then rinsed three times and incubated in biotinylated donkey anti-rabbit antiserum (1 : 200; Jackson Labs, West Grove, PA) for 90 min followed by avidin–biotin complex (1 : 600, ABC Elite Kit; Vector Labs, Burlingame, CA) for 90 min at room temperature. Sections were immersed in a solution containing 0.02% 3,3′-diaminobenzidine-4HCl (Sigma-Aldrich), 0.01% H_2O_2, and 0.6% nickel in Tris buffer (0.05 M) for 10–15 min. The reaction was terminated by rinses in Tris buffer.

Sections were mounted and photomicrographs were taken using a Zeiss Axiovert 25 and Leica DFC 480 camera and imaging software by an individual masked to the experimental condition. At least two sections per animal per area were used to count c-fos profiles. For a particular brain region, the number of c-fos profiles per section was averaged for each subject and the group mean determined from these values.

Western Blot Analysis of KOR Levels

Rats were handled for 7 days before study but did not participate in any behavioral experiments. Brains were placed in a block from which 3 mm slices containing the nucleus accumbens and piriform cortex were microdissected using a trephine. The tissue was stored at −80°C until analysis. Samples were homogenized in a RIPA buffer (Sigma, St Louis, MO) with a protease inhibitor cocktail (Halt; Pierce, Rockford, IL) and centrifuged at 15 000 *g* for 15 min (4°C). Protein content was determined using the bicinchoninic acid method. Samples (50 μg per condition) were subjected to SDS–PAGE and proteins were transferred to polyvinylidene fluoride membranes as previously described (Curtis *et al*, 2006). After blocking the membranes with Odyssey buffer (1 h, diluted in PBS 1 : 1), membranes were incubated with rabbit anti-KT2 (KOR tail) antibody (3 mg/ml, provided by Dr Charles Chavkin, University of Washington) for 15 h at 4°C. Membranes were then incubated with the loading control antibody, mouse anti-β-actin (1.5 h, 1 : 5000; Sigma). After rinsing, membranes were incubated with two infrared fluorescent secondary antibodies with different wavelengths for 1 h (1 : 5000, donkey anti-rabbit IRDye 680CW and donkey anti-mouse IRDye 8000CW; LiCor, Lincoln, NE) for simultaneous detection of both primaries.

Membranes were scanned and proteins were detected using the Odyssey Infrared Imaging System (LiCor). Odyssey Infrared Imaging software was used to quantify the integrated intensity of each band and to determine the molecular weights of the proteins (based on Bio-Rad Precision Plus Protein Standards). A ratio of KOR to the loading control was calculated for each sample and the mean ratios were compared between groups.

ELISA Analysis of Dynorphin A Levels

Dynorphin A levels were measured in the same rats that were used for western blot analysis. Brains were placed in a block from which 3 mm slices containing the nucleus accumbens and piriform cortex were microdissected using a trephine. The tissue was stored at −80°C until analysis. Dynorphin A protein levels were quantified using a commercially available ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA). The tissue was homogenized in lysis buffer (100 mM Tris, 1 M NaCl, 4 mM EDTA, 0.1% sodium azide, 2% bovine serum albumin, 2% Triton X-100, 5 μg/ml aprotinin, 0.1 μg/ml pepstatin A, 0.5 μg/ml antipain (pH 7.0)) at a concentration of 20 ml/g of wet tissue weight.

The homogenate was then centrifuged for 30 min (15 000 *g* at 4°C). The supernatant was then processed according to the manufacturer's instructions. Each sample was run in duplicate.

Statistical Analyses

PASW Statistics 17.0 (SPSS, Chicago, IL) software was used for all statistical analysis. The *nor*-BNI and desipramine FST studies were analyzed using two-way ANOVAs (strain × treatment). The DIPPA FST studies were analyzed using one-way ANOVAs investigating the effects of treatment on behavior within each strain. The c-fos immunohistochemistry experiments were analyzed using two-way ANOVAs (strain × treatment). Pair-wise follow-up comparisons were conducted for regions with a significant or trend for a strain × treatment interaction ($p<0.10$) because of predetermined planned comparisons. The KOR western blot and dynorphin A ELISA studies were analyzed using Student's *t*-tests. The piriform cortex microinjection study was analyzed using a one-way ANOVA. Significance was established at $p<0.05$. Fisher's PLSD test was used for *post hoc* analyses.

RESULTS

KOR Antagonists Selectively Decrease Immobility in WKY Rats in the FST

WKY rats exhibited significantly higher counts of immobility ($F(1,65)=26.41$, $p<0.001$) behavior and lower counts of climbing behavior ($F(1,65)=46.68$, $p<0.001$; Figure 1). Saline-treated WKY rats showed about 50% more immobility than saline-treated SD rats ($p<0.001$). *Nor*-BNI treatment significantly reduced immobility ($F(3,65)=4.11$, $p=0.010$) and increased swimming behavior ($F(3,65)=3.80$, $p=0.014$). None of the doses of *nor*-BNI tested (1, 5, and 10 mg/kg) significantly altered the incidence of immobility in SD rats compared to the saline-treated strain control. In contrast, *nor*-BNI (5 and 10 mg/kg) produced significant decreases in immobility counts ($p=0.014$ and 0.013, respectively) and increases in swimming counts ($p=0.023$ and <0.001, respectively) in WKY rats. *Nor*-BNI had no effect on climbing in either strain. *Nor*-BNI (10 mg/kg) also did not produce any significant behavioral effects in SD rats when it was injected more frequently (23.5, 5, and 1 h before testing) according to a standard screening protocol used to measure the effects of tricyclic antidepressants (data not shown).

The second KOR antagonist tested, DIPPA, also produced strain-specific effects in the FST. DIPPA had no significant effect on immobility or swimming behavior in SD rats (Figure 2a). However, DIPPA significantly decreased climbing behavior ($F(2,27)=4.04$, $p=0.029$) in this strain at both the 5 and 10 mg/kg doses ($p=0.028$ and 0.016, respectively). DIPPA decreased immobility behavior in WKY rats ($F(4,64)=3.49$, $p=0.012$; Figure 2b). Treatment groups of 5 and 10 mg/kg showed significantly lower counts of immobility behavior compared to the saline-treated control group ($p=0.021$ and 0.029, respectively). DIPPA treatment also increased swimming ($F(4,64)=3.02$, $p=0.024$). The 10 mg/kg treatment group exhibited significantly more swimming behavior than the control group ($p=0.004$). DIPPA did not significantly alter climbing behavior in WKY rats.

Tricyclic Antidepressant Desipramine is Effective in both WKY and SD Rats

The tricyclic antidepressant desipramine was injected according to a standard screening protocol (23.5, 5, and 1 h before testing) and produced a different profile of effects compared to the KOR antagonists (Figure 3). There were significant effects of both strain ($F(1,32)=16.96$, $p<0.001$) and treatment ($F(1,32)=26.36$, $p<0.001$) on immobility behavior and no significant interaction. *Post hoc* analysis showed that the saline-treated WKY group exhibited significantly higher immobility counts than the saline-treated SD rats ($p<0.001$). Also, desipramine treatment decreased immobility in both SD and WKY rats when compared with their within-strain control groups ($p<0.001$ and $p=0.011$, respectively). There were also significant effects of strain ($F(1,32)=16.88$, $p<0.001$) and treatment ($F(1,32)=23.45$, $p<0.001$) on climbing behavior. As previously shown in the *nor*-BNI FST experiment, saline-treated WKY rats exhibited significantly lower counts of climbing behavior compared to

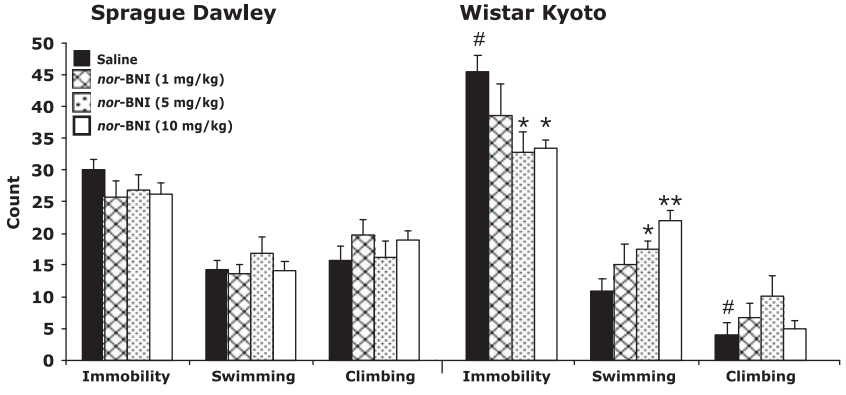

Figure 1 The effects of *nor*-binaltorphimine dihydrochloride (*nor*-BNI) in the forced swim test (FST) in Sprague–Dawley (SD) and Wistar Kyoto (WKY) rats. At baseline, WKY rats exhibit more immobility and less climbing behavior compared to SD rats. Systemic administration of *nor*-BNI (1, 5, or 10 mg/kg, s.c.) had no significant effects on any of the FST behaviors in SD rats. In contrast, *nor*-BNI (5 and 10 mg/kg) reduced immobility and increased swimming behavior in WKY rats. All data are depicted as the mean + SEM. $n=8$–10 per group. The number symbol represents significant differences between the saline-treated groups across strain, $^{\#}p<0.001$. Asterisks represent differences within strain from the saline-treated control group, $^{*}p<0.05$ and $^{**}p<0.01$.

saline-treated SD rats ($p<0.001$). Desipramine treatment significantly increased climbing behavior in SD and WKY rats compared to their within-strain control groups ($p=0.001$ and 0.012, respectively). There were no effects of strain or treatment on swimming behavior. Desipramine did not produce any significant behavioral effects when it was injected only once (23.5 h before testing), the same time that *nor*-BNI was administered (data not shown).

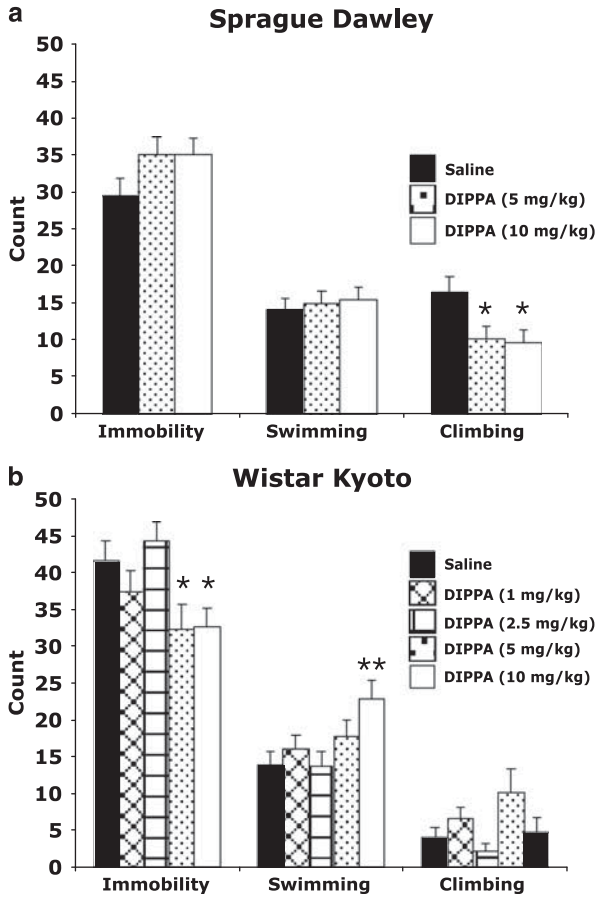

Figure 2 The effects of DIPPA in the forced swim test (FST) in Sprague–Dawley (SD) and Wistar Kyoto (WKY) rats. (a) Systemic administration of DIPPA (5 and 10 mg/kg) did not produce antidepressant-like effects in SD rats. Both doses significantly decreased climbing behavior. $n=9$–11 per group. (b) DIPPA (5 and 10 mg/kg) significantly decreased immobility in WKY rats. The 10 mg/kg dose also significantly increased swimming behavior. $n=12$–16 per group. All data are depicted as the mean + SEM. Asterisks represent significant differences from the saline-treated control group. $^{*}p<0.05$ and $^{**}p<0.01$.

KOR Antagonists do not Produce a Stimulant-Like Increase in Locomotor Activity

Doses of *nor*-BNI and DIPPA (10 mg/kg) that reduced FST immobility in WKY rats were tested for their effects on locomotor activity in both strains. There were significant main effects of strain ($F(1,42)=4.20$, $p=0.047$) and treatment ($F(2,42)=19.92$, $p<0.001$) along with a significant strain × treatment interaction ($F(2,42)=15.11$, $p<0.001$) on locomotor activity (Table 1). Saline-treated WKY rats exhibited a lower level of baseline activity when compared to saline-treated SD rats ($p=0.003$). In SD rats, *nor*-BNI treatment did not alter locomotor activity whereas DIPPA treatment produced a significant decrease ($p<0.001$). Neither KOR antagonist altered locomotor activity in WKY rats compared to the saline-treated control group.

Differential Induction of c-fos by *nor*-BNI in WKY Rats Exposed to Swim Stress

c-Fos expression was quantified in 14 brain regions (Figure 4; descriptive statistics in Table 2) in a subset of the rats (saline and 10 mg/kg groups) from the *nor*-BNI FST

Table 1 The Effects of KOR Antagonists on Locomotor Activity

Treatment	Sprague–Dawley	Wistar Kyoto
Saline	3419 ± 260	2410 ± 113[b]
nor-BNI (10 mg/kg)	3532 ± 387	2484 ± 113
DIPPA (10 mg/kg)	1308 ± 104[a]	2300 ± 130

All data are depicted as the mean distance traveled (cm) ± SEM. $n=8$ per group.
[a] $p<0.001$ compared to the saline-treated SD group.
[b] $p<0.01$ compared to saline-treated SD group.

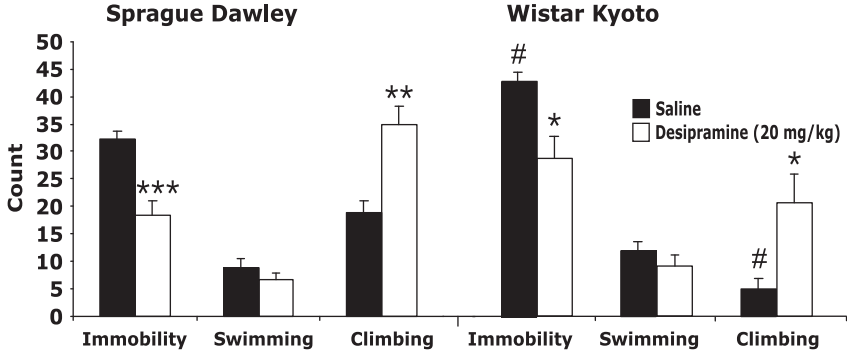

Figure 3 The effects of desipramine in the forced swim test (FST) in Sprague–Dawley (SD) and Wistar Kyoto (WKY) rats. WKY rats exhibited increased immobility and decreased climbing behavior at baseline compared to SD rats. Systemic administration of desipramine (20 mg/kg) significantly decreased immobility and also increased climbing behavior in both strains. All data are depicted as the mean + SEM. $n=8$–10 per group. The number symbol represents significant differences between the saline-treated groups across strain, $^{\#}p<0.001$. Asterisks represent significant differences from the saline-treated control group within strain. $^{*}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

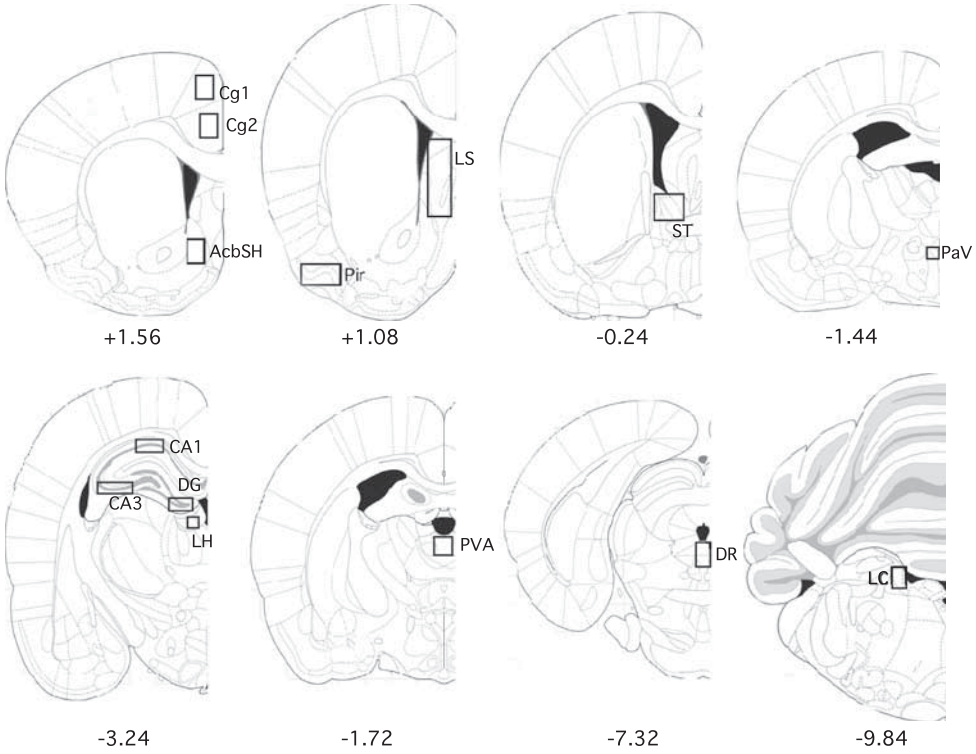

Figure 4 Localization of brain regions analyzed in c-fos studies. These schematics represent the approximate areas used for the quantification of c-fos profiles for each brain region. The images, from the top left image, correspond to Figures 20, 24, 35, 45, 47, 60, 94, and 115, respectively from Paxinos and Watson (2005). The numbers underneath each image represent the anterior/posterior distance from bregma. Abbreviations: AcbSh, nucleus accumbens shell; Cg1, cingulate cortex area 1; Cg2, cingulate cortex area 2; DG, dentate gyrus; DR, dorsal raphe; LC, locus coeruleus; LH, lateral habenula; Pir, piriform cortex; PaV, paraventricular nucleus of the hypothalamus; PVA, paraventricular thalamic nucleus; ST, bed nucleus of the stria terminalis.

Table 2 The Effects of *nor*-BNI on c-fos Activation in the FST

Brain region	Sprague–Dawley		Wistar Kyoto		*p*-Value
	Saline	*nor*-BNI	Saline	*nor*-BNI	
Piriform cortex	**27.53 ± 5.97**	**9.17 ± 5.85**	**21.00 ± 8.65**	**74.22 ± 16.19**	**0.016[a]**
Nucleus accumbens shell	**81.71 ± 5.24**	**92.75 ± 23.22**	**62.12 ± 11.14**	**129.19 ± 16.36**	**0.086[b]**
Cingulate cortex (Cg1)	100.27 ± 13.53	130.78 ± 55.66	152.50 ± 41.93	176.70 ± 37.87	0.940
Cingulate cortex (Cg2)	44.27 ± 7.03	99.50 ± 10.80	110.94 ± 31.56	93.53 ± 25.15	0.179
Lateral septum	251.00 ± 70.38	201.71 ± 66.01	257.19 ± 36.66	263.71 ± 31.11	0.230
Bed nucleus of the stria terminalis	14.43 ± 4.58	20.00 ± 6.08	12.19 ± 1.73	12.02 ± 1.74	0.440
Paraventricular hypothalamic nucleus	175.21 ± 30.80	171.47 ± 23.19	121.74 ± 27.78	161.03 ± 29.07	0.425
Paraventricular thalamic nucleus	230.33 ± 39.39	186.97 ± 46.01	238.03 ± 40.43	232.44 ± 41.69	0.756
Dentate gyrus	21.84 ± 4.05	25.10 ± 5.71	23.20 ± 4.32	31.92 ± 5.90	0.581
CA1	1.85 ± 0.67	1.47 ± 0.74	2.89 ± 1.20	4.48 ± 1.26	0.360
CA3	4.88 ± 3.64	3.17 ± 1.77	5.64 ± 2.32	4.70 ± 0.84	0.824
Lateral habenula	17.48 ± 3.49	25.73 ± 6.82	19.55 ± 4.48	25.19 ± 3.31	0.855
Dorsal raphe	26.00 ± 16.79	31.29 ± 10.00	33.30 ± 23.94	31.15 ± 5.71	0.874
Locus coeruleus	8.57 ± 1.77	12.83 ± 4.25	17.23 ± 3.87	22.88 ± 3.96	0.847[c]

The mean c-fos profiles ± SEM is given for the 14 brain regions analyzed. The regions in which KOR and dynorphin A protein were quantified are shown in bold. The '*p*-value' column shows the *p*-value for the strain × treatment interaction and main effects for each region. $n = 5$–9 per group.
[a]Significant main effect of strain, $p = 0.044$.
[b]Significant main effect of drug, $p = 0.020$.
[c]Significant main effect of strain, $p = 0.014$.

experiment. WKY rats exhibited overall higher numbers of c-fos-positive cells in the locus coeruleus ($F(1,30) = 6.77$, $p = 0.014$) and the piriform cortex ($F(1,22) = 4.58$, $p = 0.044$). *Nor*-BNI treatment did not alter c-fos expression differentially between strains in the locus coeruleus. Only two brain regions, the piriform cortex and nucleus accumbens shell, showed a significant or trend for interaction between strain and drug treatment sufficient to justify follow-up pair-wise comparisons ($p < 0.10$). *Nor*-BNI treatment significantly increased c-fos-positive profiles in the piriform cortex in WKY rats ($p = 0.003$) whereas it produced no significant change in SD rats (representative sections shown in Figure 5a). *Nor*-BNI treatment also increased c-fos-positive profiles in the nucleus accumbens shell in WKY rats ($p = 0.002$) but produced no significant effect in SD rats (representative sections in Figure 5b). None of the other brain regions showed any significant differences in patterns of c-fos expression.

Swim Stress Effects on c-fos Expression

To compare the effects of the FST procedure alone on c-fos expression between strains, we quantified c-fos profiles in a subset of the brain regions from the previous experiment in separate groups of rats that were either FST experienced or FST naïve. These animals did not receive pretreatment injections. There were no significant interaction effects for any of the brain regions between strain and swim stress (Table 3). The FST procedure produced significant increases in c-fos expression in the piriform cortex ($F(1,20) = 14.87$, $p = 0.001$), nucleus accumbens shell ($F(1,20) = 11.34$, $p = 0.003$), dentate gyrus ($F(1,19) = 22.95$, $p < 0.001$), and the CA3 area of the hippocampus ($F(1,20) = 13.54$, $p < 0.001$). There was a significant overall difference in c-fos expression between strains in the piriform cortex ($F(1,20) = 4.54$, $p < 0.046$) and the dentate gyrus ($F(1,19) = 11.54$, $p = 0.003$).

Strain Differences in KOR and Dynorphin A Expression

Baseline levels of the KOR protein in experimentally naïve subjects from both strains were measured in the nucleus accumbens and piriform cortex. KOR protein levels were elevated in the piriform cortex of WKY rats compared to SD rats ($t(28) = 2.87$, $p = 0.008$; Figure 6a and c). There was no difference in the amount of KOR protein in the nucleus accumbens between the two strains ($t(18) = 0.55$, $p = 0.590$; Figure 6b).

Baseline levels of dynorphin A, the main ligand of the KOR, were also measured in the nucleus accumbens and piriform cortex (Figure 7). Dynorphin A levels were greater in the nucleus accumbens of WKY rats compared to SD rats ($t(18) = 4.03$, $p < 0.001$; Figure 7a). There was no strain difference in dynorphin A content in the piriform cortex ($t(18) = 0.14$, $p = 0.89$; Figure 7b).

Infusion of *nor*-BNI Within the Piriform Cortex Produces Antidepressant-Like Effects in WKY Rats

WKY rats were given local infusions of *nor*-BNI within the piriform cortex and subsequently tested in the FST. The infusion of *nor*-BNI into the piriform cortex reduced immobility in the FST ($F(3,19) = 3.76$, $p = 0.028$; Figure 8a). Both doses tested (2.5 and 10 μg per side) produced significant decreases in immobility counts ($p = 0.03$ and 0.01, respectively) compared with the aCSF control group. There were no significant effects on swimming or climbing as the increase in active behavior was split between the two categories. To evaluate the site specificity of the antidepressant-like effect, the data from rats that received 10 μg per side outside the piriform cortex ('misses' group; Figure 8b) were compared with the aCSF controls. There was no difference in immobility between these two groups ($p = 0.586$).

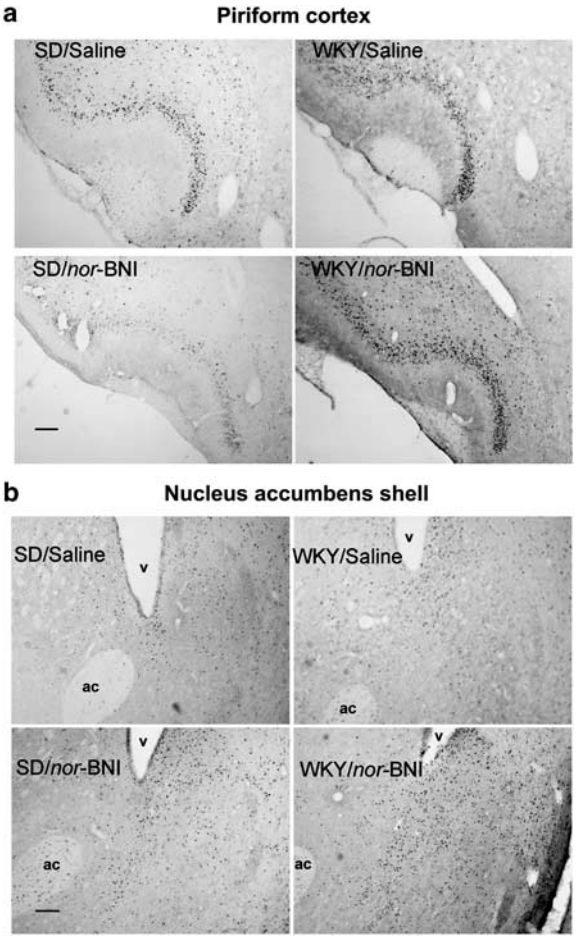

Figure 5 Photomicrographs of the piriform cortex and nucleus accumbens shell. Representative photomicrographs from the four treatment groups in the c-fos activation experiment. (a) Piriform cortex; bar represents a distance of 200 μm. (b) Nucleus accumbens shell; bar represents a distance of 80 μm.

DISCUSSION

This study showed selective antidepressant efficacy of KOR antagonists in the FST in the WKY rat strain compared to the SD strain. Distinct c-fos activation patterns in WKY and SD rats treated with the KOR antagonist *nor*-BNI implicated the nucleus accumbens shell and piriform cortex as sites of action that may be involved in the strain-related pharmacodynamic differences. Consistent with those find-

Table 3 The Effects of the FST Procedure on c-fos Activation

Brain region	Sprague–Dawley		Wistar Kyoto		*p*-Value
	Naïve	Swim stress	Naïve	Swim stress	
Piriform cortex	15.60 ± 4.30	45.60 ± 11.57	10.57 ± 2.56	25.69 ± 3.99	a,b
Nucleus accumbens shell	6.60 ± 2.20	14.60 ± 5.32	8.79 ± 3.21	23.26 ± 2.20	a
Dentate gyrus	2.40 ± 0.60	9.00 ± 2.81	6.50 ± 2.05	17.09 ± 1.13	a,c
CA1	0.75 ± 0.48	2.20 ± 0.97	1.57 ± 0.69	2.84 ± 0.56	
CA3	2.20 ± 0.37	7.60 ± 1.33	3.14 ± 1.03	10.06 ± 2.41	a
Locus coeruleus	1.40 ± 0.68	9.75 ± 6.55	2.80 ± 1.59	4.67 ± 2.40	

The mean c-fos profiles ± SEM is given for the six brain regions analyzed. The '*p*-value' column shows the main effects for either strain or swim stress for each region. There were no significant interactions between strain and swim stress. $n = 5$–7 per group.
[a]Significant main effect of swim stress, $p \leqslant 0.003$.
[b]Significant main effect of strain, $p = 0.046$.
[c]Significant main effect of strain, $p < 0.003$.

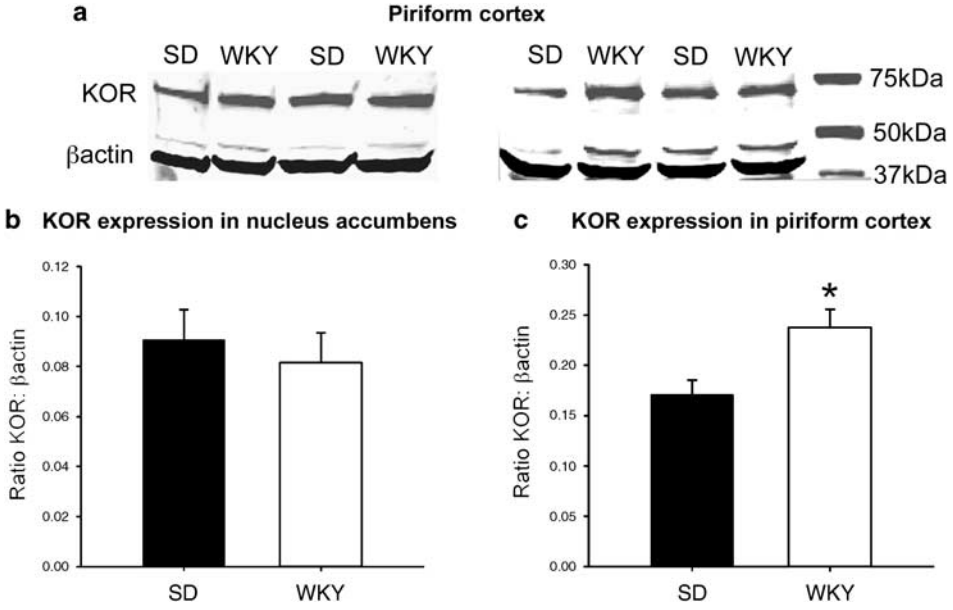

Figure 6 Western blot analysis of κ-opioid receptor (KOR) expression in the nucleus accumbens and piriform cortex. (a) Representative blots show the expression of KOR protein (MW = 72 kDa), and β-actin (MW = 42 kDa) in the piriform cortex. (b and c) Graphs show the mean ratio of the integrated intensity of each band of KOR protein to the corresponding band of β-actin from the same sample. KOR expression was greater in the piriform cortex, but not nucleus accumbens, of Wistar Kyoto (WKY) rats compared to Sprague–Dawley rats. All data are depicted as the mean + SEM. $n = 10$–15 per group. Asterisks represent significant differences between groups, $^*p < 0.05$.

ings, we also measured baseline differences in dynorphin A and KOR protein levels in these regions between SD and WKY rats. In addition, local infusion of *nor*-BNI into the piriform cortex of WKY rats produced antidepressant-like effects in the FST further implicating this region in the behavioral response of the strain to KOR antagonists. Together, these findings suggest that the dynorphin–KOR system contributes to the stress-sensitive phenotype of WKY rats and that KOR antagonists could be a novel therapy for affective disorders.

The KOR system has been previously implicated in preclinical models of depression and KOR antagonists have been investigated as potential antidepressant treatments (Pliakas *et al*, 2001; Mague *et al*, 2003; McLaughlin *et al*, 2003; Shirayama *et al*, 2004). Antidepressant-like effects of KOR antagonists have been detected in SD rats under different conditions. Central administration of *nor*-BNI produced antidepressant-like effects in previous studies (Pliakas *et al*, 2001; Mague *et al*, 2003; Shirayama *et al*, 2004). The results of systemic administration of *nor*-BNI have been mixed. In agreement with our findings, Zhang *et al* (2007) reported that systemic administration of *nor*-BNI did not produce antidepressant-like effects in SD rats in a single trial version of the rat FST. In contrast, Beardsley

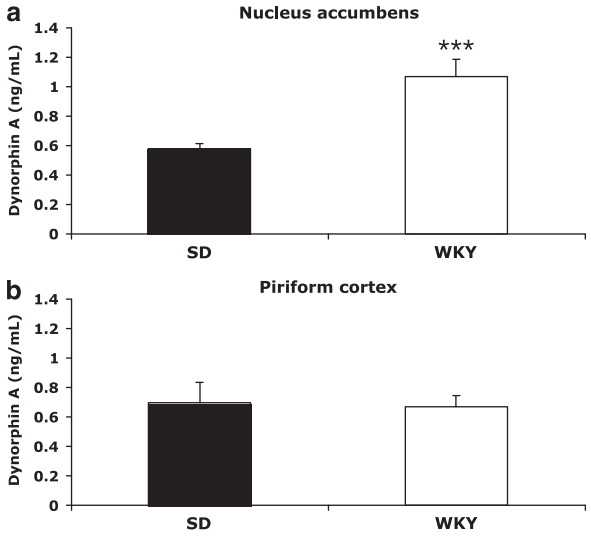

Figure 7 Dynorphin A expression levels in the nucleus accumbens and piriform cortex. (a) Wistar Kyoto (WKY) rats had higher baseline tissue content levels of dynorphin A in the nucleus accumbens. (b) There were no significant differences in dynorphin A content in the piriform cortex. All data are depicted as the mean + SEM. $n = 10$ per group. Asterisks represent significant differences between groups, $^{***}p < 0.001$.

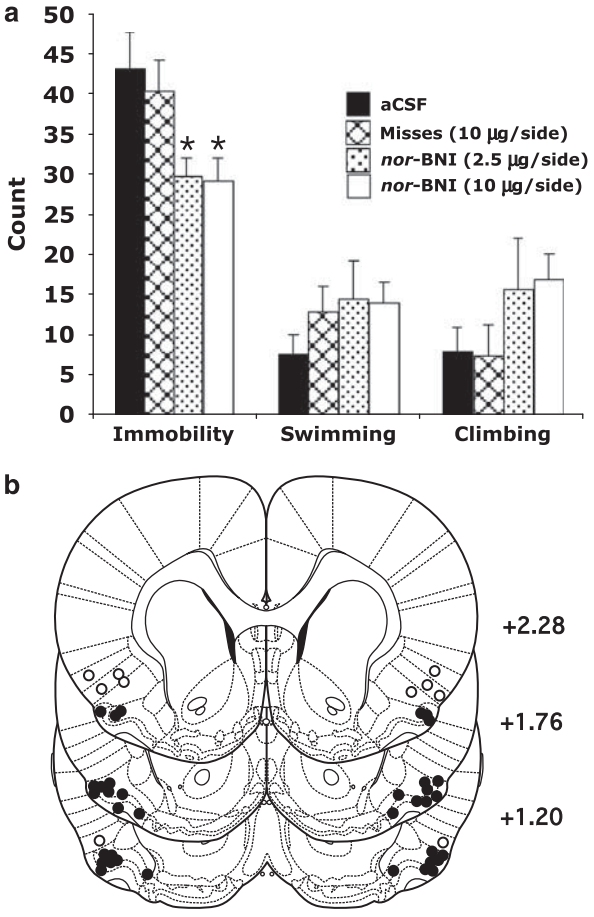

Figure 8 The effects of local infusion of *nor*-binaltorphimine dihydrochloride (*nor*-BNI) into the piriform cortex in the forced swim test (FST) in Wistar Kyoto (WKY) rats. (a) Both doses tested (2.5 µg per side ($n = 4$) and 10 µg per side ($n = 7$)) produced significant decreases in immobility. The 'misses' group ($n = 5$), 10 µg per side dorsal with respect to the piriform cortex, did not exhibit any significant changes in behavior compared to the artificial cerebrospinal fluid (aCSF)-treated controls ($n = 7$). (b) The location of the injections for the rats used in this study. Filled circles represent placements within the piriform cortex and open circles represent placements outside the region. All data depicted as the mean + SEM. Asterisks represent significant differences from the aCSF-treated control group, $^{*}p < 0.05$.

et al (2005) reported that systemic administration of *nor*-BNI produces antidepressant-like effects in the modified rat FST. One possible explanation for the seemingly contradictory findings could be the high level of baseline immobility in their study. The high amount of immobility behavior present in their saline-treated group, compared to the control groups from this study, and the other studies previously cited, could be indicative of increased stress or other environmental conditions that contributed to the antidepressant-like response of the SD rats. These effects could be viewed as additional evidence that KOR antagonists may prove to be effective treatments in other rat models that are characterized by high baseline immobility (Becker *et al*, 2008). In support of this theory, a recent study shows that systemic *nor*-BNI can block the increase in immobility caused by early life methylphenidate exposure (Wiley *et al*, 2009).

Mague *et al* (2003) showed that the KOR antagonist GNTI did not produce antidepressant-like effects when administered systemically, but did produce effects when given centrally. In addition, systemic administration of the KOR antagonist 5′-acetamidinoethylnaltrindole (ANTI), with greater hypothesized central availability, produces antidepressant-like effects in the FST suggesting that insufficient availability in the brain may be a problem for some KOR antagonists. Although a dose of systemic *nor*-BNI higher than 10 mg/kg might still produce antidepressant-like effects in SD rats, administering this dose of *nor*-BNI more frequently to SD rats according to a standard screening protocol (23.5, 5, and 1 h before testing) failed to produce any behavioral effects in the FST. The effects of *nor*-BNI and DIPPA were longer lasting than most antidepressants, producing significant effects in WKY rats when tested 24 h after a single dose, whereas three injections within 24 h using the standard screening protocol are usually required to produce behavioral effects of established antidepressants. The lengthy time course of KOR antagonists in the FST is in agreement with the long-lasting (days) time course for their blockade of KOR agonist-induced analgesia (Jones and Holtzman, 1992; Chang *et al*, 1994).

Our c-fos analysis highlighted the nucleus accumbens shell and piriform cortex as two regions that are activated differentially by *nor*-BNI between the strains and may be involved in the antidepressant-like behavioral response to KOR antagonist treatment. Owing to the limitations associated with the use of c-fos induction as a measure of neuronal activity (Dragunow and Faull, 1989) and the complex nature of FST-associated behaviors, these two regions most likely do not represent the sole areas of functional divergence between the strains. However, previously published research does support a potential role for both regions in the antidepressant-like behavioral response in the FST.

The nucleus accumbens is thought to be involved in the integration of both rewarding and aversive stimuli (Carlezon and Thomas, 2009). This region has previously been implicated in the effects of both KOR agonists and antagonists in the FST (Pliakas *et al*, 2001; Shirayama *et al*, 2004). Therefore, it was not surprising for the area to be highlighted as a region of interest in response to KOR antagonist treatment. Although there was no effect of *nor*-BNI treatment in SD rats, a significant decrease in immobility (33%) in WKY rats coincided with the large increase in c-fos-positive cells in the region. In addition, WKY rats had much higher levels of dynorphin A protein in the region. The behavioral effects of agonists at the KOR are believed to be mediated through presynaptic inhibition of neurotransmitter release (Bals-Kubik *et al*, 1993; Svingos *et al*, 2001; Li and van den Pol, 2006; Kreibich *et al*, 2008). This could lead to a decreased activation of cells in the region that would be counteracted by KOR antagonist treatment. Interestingly, this inhibitory effect of KOR activation in the nucleus accumbens is thought to have a role in the expression of the aversive properties of KOR agonists (Bals-Kubik *et al*, 1993; Hjelmstad and Fields, 2003; Land *et al*, 2008).

The present findings also highlighted the piriform cortex as a potential site of action of KOR antagonists. WKY rats exhibited increased expression of KOR protein in the piriform cortex compared to SD rats and local infusion of *nor*-BNI in the region was sufficient to produce antidepressant-like effects in WKY rats. Although the piriform cortex is primarily considered an olfaction-associated brain region (Haberly, 2001), this is not the first study in which it has been implicated in the behavioral response to antidepressant-like treatments in rodents (Sibille *et al*, 1997; Bechtholt *et al*, 2008; Stone and Lin, 2008). These studies all measured an increase in c-fos activation in the region in response to treatment that produced antidepressant-like effects in their respective behavioral assays. The piriform cortex has also been identified as a region that shows plasticity in response to antidepressant treatment (Sun *et al*, 2005; Zhou *et al*, 2006; Hjaeresen *et al*, 2008). Interestingly, electroconvulsive shock causes the upregulation of brain-derived neurotrophic factor gene expression in the region (Nibuya *et al*, 1995), a growth factor shown to have antidepressant-like properties (Shirayama *et al*, 2002; Hoshaw *et al*, 2005). In addition, degeneration of the piriform is thought to contribute to the behavioral phenotype seen in the olfactory bulbectomy model of depression (Song and Leonard, 2005; Wang *et al*, 2007). The extensive connections of the piriform cortex to the amygdala, nucleus accumbens, thalamus, and prefrontal cortex in both rodents and primates suggest that the region is well placed to influence the behavioral response to stressful situations (Ray and Price, 1992; Carmichael *et al*, 1994; Haberly, 2001).

The locus coeruleus, a region in which WKY rats have been previously reported to show increased *KOR* gene expression in comparison to SD rats (Pearson *et al*, 2006), was also highlighted as a region of interest by the c-fos activation study. Given that the KOR–dynorphin system has been shown to presynaptically inhibit the activity of the locus coeruleus (Kreibich *et al*, 2008), our findings that WKY rats had higher levels of c-fos-positive profiles were initially surprising. However, these results are in agreement with previous research that suggests the regulation of norepinephrine release in WKY rats in response to stress depends on the duration of the stress. After acute stress, WKY rats exhibit a blunted norepinephrine response compared to SD rats (Sands *et al*, 2000; Ma and Morilak, 2004). In contrast, repeated stress leads to an increased norepinephrine response in WKY rats (Pardon *et al*, 2003). The fact that we measured c-fos expression after repeated swim stress may account for the increased number of c-fos-positive profiles in the locus coeruleus. More research into the electrophysiological effects of KOR-specific ligands in WKY rats will need to be conducted.

The WKY rat strain has been proposed as a model of comorbid depression and anxiety. Given the difficulties associated with therapy for comorbid depression and anxiety (Fava *et al*, 2008), it is important to identify novel treatments that may be effective against this subtype of depression. The current studies showed that WKY rats displayed increased sensitivity to the antidepressant-like effects of KOR antagonists. In addition, endogenous alterations in the dynorphin–KOR system in the nucleus accumbens and piriform cortex may have a role in the increased efficacy of KOR antagonists in the strain. Further studies are required to determine if the dynorphin–KOR system is involved in the anxiogenic component of the WKY phenotype. Given the increased difficulty of finding effective treatments for the comorbid depression and anxiety population, genetic animal models that recapitulate this unique behavioral profile can be used to further the development of effective clinical treatments.

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