Anxiolytic-Like Effects of κ-Opioid Receptor Antagonists in Models of Unlearned and Learned Fear in Rats

Allison T. Knoll, Edward G. Meloni, James B. Thomas, F. Ivy Carroll, and William A. Carlezon Jr.

Behavioral Genetics Laboratory, Department of Psychiatry, Harvard Medical School, McLean Hospital, Belmont, Massachusetts (A.T.K., E.G.M., W.A.C.); and Research Triangle Institute, Organic and Medicinal Chemistry, Research Triangle Park, North Carolina (J.B.T., F.I.C.)

ABSTRACT

Endogenous opioid systems regulate neurobiological responses to threatening stimuli. Stimulation of κ-opioid receptors (KORs) produces analgesia but induces prodepressive-like effects in a variety of animal models. In contrast, KOR antagonists have antidepressant-like effects. KORs and their endogenous ligand dynorphin are expressed throughout brain areas involved in fear and anxiety, including the extended amygdala. Here, we examined whether KOR antagonists would affect unlearned fear (anxiety) in the elevated plus maze (EPM) and open field (OF) paradigms and learned fear in the fear-potentiated startle (FPS) paradigm. These studies were designed to accommodate the slow onset (~24 h) and extended time course (>3 weeks) of the prototypical KOR antagonists nor-binaltorphimine hydrochloride (norBNI) and JDTic [(3R)-7-hydroxy-N-[(1S)-1-[[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl]-1,2,3,4-tetrahydro-3-isoquinoline-carboxamide hydrochloride]. Rats received an i.p. injection of norBNI (3.0–30 mg/kg) or JDTic (1.0–10 mg/kg) 48 h before EPM testing. One day later, they were tested in the OF, and 5 and 7 days later, they were trained and tested in the FPS paradigm. Both KOR antagonists dose-dependently increased open arm exploration in the EPM without affecting OF behavior. They also decreased conditioned fear in the FPS paradigm. The anxiolytic-like effects of KOR antagonists were qualitatively similar to those of the benzodiazepine chlordiazepoxide in the EPM. The selective serotonin reuptake inhibitor fluoxetine had no effect in the EPM and anxiogenic-like effects in the OF. Our results indicate that KOR antagonists produce a unique combination of antidepressant- and anxiolytic-like effects and suggest that this class of drugs may be particularly effective for the treatment of comorbid depressive and anxiety disorders.

Although the neurobiology of depression and anxiety disorders is not well understood, evidence suggests that these disorders share common genetic and neuroanatomic components. Depression and anxiety disorders are highly comorbid; more than half of individuals with anxiety disorders also co-present with a lifetime history of depression (Kaufman and Charney, 2000). Furthermore, the development of both

disorders typically coincides with chronic stress or a traumatic experience (Kessler, 1997; Heim and Nemeroff, 1999) and standard antidepressants (e.g., SSRIs) are the most widely used and effective pharmacologic treatments for both disorders. However, antidepressants are associated with delayed therapeutic efficacy, and SSRIs often exacerbate anxiety in humans (Catalano et al., 2000; Grillon et al., 2007) as well as in rodent models of fear and anxiety (Griebel et al., 1994; File et al., 1999; Burghardt et al., 2004; Drapier et al., 2007). An improved understanding of the molecular and neuroanatomic processes that contribute to the manifestation of depressive and anxiety disorders may facilitate the development of more effective treatments.

The endogenous opioid dynorphin acts at κ-opioid receptors (KORs) (Chavkin et al., 1982), and there is increasing evidence that KORs are involved in depressive and anxiety disorders. Brain opioid systems regulate behavioral re-

ABBREVIATIONS: SSRI, selective serotonin reuptake inhibitor; KOR, κ-opioid receptor; NAc, nucleus accumbens; norBNI, nor-binaltorphimine hydrochloride; JDTic, (3R)-7-hydroxy-N-[(1S)-1-[[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethyl-1-piperidinyl]methyl]-2-methylpropyl]-1,2,3,4-tetrahydro-3-isoquinoline-carboxamide hydrochloride; EPM, elevated plus maze; OF, open field; FPS, fear-potentiated startle; ANOVA, analysis of variance; CS, conditioned stimulus; ISI, interstimulus interval; CREB, cAMP response element binding protein; US, unconditioned stimulus; CeA, central nucleus of the amygdala; BLA, basolateral amygdala.

sponses to threatening stimuli (Ribeiro et al., 2005), and KOR activation produces prodepressive and dysphoric effects in humans (Pfeiffer et al., 1986) and rodents (Bals-Kubik et al., 1993; Mague et al., 2003; Todtenkopf et al., 2004; Carlezon et al., 2006). Research in our laboratory and others has demonstrated a role for increased KOR signaling in limbic brain regions such as the nucleus accumbens (NAc) in mediating the depressive-like behavioral effects of numerous stressors (e.g., forced-swim stress, immobilization stress, foot shock) (Carlezon et al., 2005). In contrast, KOR antagonists have antidepressant-like effects in standard behavioral tests (Carlezon et al., 1998; Pliakas et al., 2001; Mague et al., 2003; McLaughlin et al., 2003; Shirayama et al., 2004; Beardsley et al., 2005) and block the prodepressive-like effects of KOR agonists (Todtenkopf et al., 2004). Furthermore, key depressive-like effects are reduced or absent in prodynorphin and KOR knockout mice (Simonin et al., 1998; McLaughlin et al., 2003). The neurobiological mechanisms by which stress stimulates KOR function are not currently known, although there is evidence that stressors can activate the transcription factor cAMP-response element binding protein (CREB) in the NAc, which is sufficient to cause depressive-like behaviors through local increases in CREB-regulated dynorphin function (Carlezon et al., 1998; Pliakas et al., 2001). Regardless, these data suggest that manipulations targeting brain KOR systems might be useful in the study and treatment of depressive disorders.

Considering that KOR antagonists reliably produce antidepressant-like effects in rodents and that depression and anxiety are often comorbid in humans, the present studies were designed to determine whether this class of drugs would also affect anxiety-related behaviors. Dynorphin and KORs are expressed throughout limbic brain areas implicated in fear and anxiety, including the amygdala [basolateral amygdala (BLA) and central nucleus (CeA)] and bed nucleus of the stria terminalis (Fallon and Leslie, 1986; Mansour et al., 1995). Although few studies have assessed the effects of KOR signaling in tests that reflect anxiety and fear-related states, microinjections of KOR agonist into the basolateral amygdala have anxiogenic-like effects in a model of unlearned fear in rats (Narita et al., 2005). We examined whether the prototypical (but structurally unrelated) KOR antagonists nor-binaltorphimine hydrochloride (norBNI) and JDTic would have anxiolytic-like effects on unlearned fear in the elevated plus maze (EPM) and open field (OF) paradigms and learned fear in the fear-potentiated startle (FPS) paradigm. Separate groups of rats were treated with an antidepressant (fluoxetine) or anxiolytic (chlordiazepoxide) before testing in the EPM and OF to compare the acute effects of clinically effective agents to those of the KOR antagonists. These studies were designed to accommodate the slow onset (~24 h) and extended time course (>3 weeks) of KOR blockade by norBNI (Endoh et al., 1992; Jones and Holtzman, 1992) and JDTic (Carroll et al., 2004; Beardsley et al., 2005). We found that the KOR antagonists have anxiolytic-like effects in the EPM and FPS paradigms, suggesting that this class of drugs may be particularly effective for the treatment of comorbid depressive and anxiety disorders.

Materials and Methods

Rats. A total of 170 male Sprague-Dawley rats (Charles River Laboratories, Inc., Raleigh, NC) were used. Rats were group housed

(four rats per cage) and maintained on a 12/12-h light/dark cycle with ad libitum access to food and water except during behavioral tests. Rats were allowed to acclimate to the animal facility for one week before testing and weighed 325 to 375 g at the start of testing. Rats were tested sequentially in each of three behavioral paradigms, with the exception of rats treated with fluoxetine and chlordiazepoxide, which were not tested in the FPS paradigm. Rats received i.p. injections of norBNI or JDTic 48 h before the first behavioral test (EPM test) and did not receive additional treatments. The time point of KOR antagonist administration was selected to ensure maximal and selective KOR blockade at the time of EPM testing; the effects of these drugs peak, approximately 24 h after systemic administration, are maintained at maximal or near-maximal levels for 7 to 10 days and gradually return to control levels over the subsequent 3 to 4 weeks (Endoh et al., 1992; Jones and Holtzman, 1992; Carroll et al., 2004; Beardsley et al., 2005). In vitro binding studies have characterized norBNI and JDTic as highly selective KOR antagonists (Thomas et al., 2004), and in vivo studies have demonstrated that these drugs do not produce μ-opioid receptor antagonism at doses similar to or higher than those used in this study (norBNI, 5–40 mg/kg; JDTic, 1–100 mg/kg) by 24 h after administration (Endoh et al., 1992; Carroll et al., 2004). To assess the acute effects of chlordiazepoxide and fluoxetine, these drugs were administered before both EPM and OF testing. There were 13 to 24 rats per group for the KOR antagonist studies; norBNI and JDTic were tested in parallel and shared a common control (vehicle-treated) group. There were six rats per group for fluoxetine studies and three to nine rats per group for the chlordiazepoxide studies (the inactive low doses had the fewest subjects). The control conditions tended to have more subjects because each group tested always included vehicle-treated rats. Rats were tested in three behavioral paradigms to determine whether KOR antagonism produces acute and long-lasting effects on unlearned and learned fear; these questions can be addressed in the same animals because of the long duration of KOR antagonism produced by norBNI and JDTic. Previous work involving similar paradigms indicates that this type of sequential behavioral testing does not interfere with the detection of drug effects (Weiss et al., 2004). Chlordiazepoxide or fluoxetine were not tested in the FPS paradigm because of limitations in our ability to approximate the type of long-term drug exposure caused by single injections of norBNI or JDTic. For example, daily handling and drug administration can alter baseline anxiety, which would make direct comparisons between standard agents and KOR antagonists impossible. Experimental protocols were approved by McLean Hospital's Institutional Animal Care Committee and were conducted in accordance with the National Institutes of Health (1996).

Drugs. norBNI was generously provided by the National Institute on Drug Abuse Drug Supply Program. JDTic was synthesized at Research Triangle Institute (Research Triangle Park, NC). Chlordiazepoxide hydrochloride (chlordiazepoxide) and fluoxetine hydrochloride (fluoxetine) were obtained from Sigma-Aldrich (St. Louis, MO). Doses of all drugs were based on their salt form. The norBNI, JDTic, and fluoxetine were dissolved in distilled water, and chlordiazepoxide was dissolved in 0.9% saline. All drugs were administered in a volume of 1 ml/kg.

Elevated Plus Maze. Rats received i.p. injections of norBNI or JDTic 48 h before testing. Separate groups of rats received i.p. injections of chlordiazepoxide 30 min, or fluoxetine 60 min, before testing (experimental day 2). Doses and pretreatment times for chlordiazepoxide and fluoxetine were based upon those successfully utilized in previous studies of unlearned fear in rats (File et al., 1999; Prut and Belzung, 2003; Drapier et al., 2007). Rats were tested between 11:00 AM and 5:00 PM on a black plastic maze (Hamilton-Kinder, LLC, Poway, CA) that was 110 × 110 × 85 cm (length × width × height). Each arm of the maze was 10 × 50 cm (width × length), and the intersection of the arms was 10 × 10 cm; the walls of the closed arms were 40 cm high. Rats were transported to a dimly lit holding room at least 1 h before testing. At the time of testing,

each rat was placed in the center of the maze facing the same closed arm and videotaped for 5 min; the testing room was illuminated only by a dim red light (25 W). Videotapes were later scored without knowledge of treatment conditions; time spent in the open and closed arms, open and closed arm entries, and maze crosses were each quantified. Arm entry or exit was recorded when all four of the rat's paws had entered or exited the arm, respectively. Previous work has shown that manipulations that decrease anxiety (e.g., clinically effective anxiolytics) increase the number of open arm entries and the percentage of time rats spend in the open arms, whereas anxiogenic treatments decrease these measures (Pellow and File, 1986).

Data from each drug were analyzed separately using one-way (treatment) analyses of variance (ANOVAs). Significant effects were analyzed using Dunnett's post hoc tests. For studies involving fluoxetine, only one dose (10 mg/kg i.p.) was tested, and differences from vehicle-treated rats were analyzed using Student's t tests.

Locomotor Activity. One day after EPM testing (experimental day 3), locomotor activity was monitored in darkness for 1 h in automated $43 \times 43 \times 31$ cm (length $\times$ width $\times$ height) activity chambers (MED Associates, St. Albans, VT). Rats in the chlordiazepoxide and fluoxetine groups again received i.p. injections before testing exactly as on the previous day; rats that had been treated with norBNI or JDTic (and corresponding vehicle controls) did not receive additional drug treatment due to the long duration of KOR antagonist action (Endoh et al., 1992; Jones and Holtzman, 1992; Carroll et al., 2004; Beardsley et al., 2005). The total number of activity counts (photocell beam breaks) during the test session was quantified. Locomotor activity was assessed based on the mean activity counts over 1 h; the time course of locomotor activity was assessed based on mean activity counts in 5-min increments. Because drugs with anxiolytic or anxiogenic effects can affect the relative amount of time that rats spend in the interior portion of the OF (Prut and Belzung, 2003), the percent distance rats traveled in the interior (25×25 cm) versus exterior portion of the activity chambers was also quantified. These effects were most detectable early in the test session; accordingly, only data from the first 20 min of testing were analyzed for this measure.

Data from each drug were analyzed separately using one-way (treatment) ANOVAs for overall locomotor activity and percentage of distance traveled in the interior of the OF. Two-way (treatment $\times$ time) ANOVAs were used to analyze the time course of locomotor activity in 5-min increments. Significant effects were analyzed using Dunnett's post hoc tests. For studies involving fluoxetine, only one dose (10 mg/kg i.p.) was used, and differences from vehicle-treated rats were analyzed using Student's t tests for locomotor activity and the percentage of distance traveled in the interior of the OF. A two-way ANOVA (treatment $\times$ time) was used to analyze the time course of locomotor activity.

Fear-Potentiated Startle. Beginning 3 days after the OF studies, FPS studies were initiated in the rats treated with KOR antagonists (or vehicle) to determine whether chronic KOR blockade would affect learned fear. As described previously (Meloni et al., 2006), all habituation, training, and testing sessions were conducted in an automated four-chamber fear-potentiated startle apparatus (Med Associates). The rats were held in four identical $19 \times 9 \times 14$-cm Plexiglas cages with steel rod floor bars. Each cage was attached to a load-cell platform and was contained within a $64 \times 40 \times 60$-cm ventilated sound-attenuating chamber. Cage movement resulted in displacement of a transducer in the platform, and the resultant voltage was amplified and digitized by an analog-to-digital converter card interfaced to a computer. Startle amplitude was proportional to the amount of cage movement and was defined as the maximum peak-to-peak voltage that occurred during the first 200 ms after onset of the startle stimulus. Constant wide band background noise (60 dB, 10–20 kHz) and 50-ms startle stimuli (1–32 kHz white noise, 1-ms rise decay) were generated by an audio stimulator and delivered through speakers located 4 cm behind the startle cage. The conditioned stimulus (CS) was light produced by an 8-W (300 lu-

mens) fluorescent bulb located 6 cm behind, and at a 45° angle above, the cage. The unconditioned stimulus (US) was a scrambled foot shock delivered through the floor bars of each cage by an external shock generator. The calibration, presentation, and sequencing of all stimuli were under the control of a computer and customized software. All trials occurred in darkness, except those in which the light was used as a conditioning stimulus.

Three days after the OF test (experimental day 6), rats were placed in startle cages and received a 5-min acclimation period followed by presentation of 27 startle stimuli, nine stimuli at 95, 100, and 105 dB and 30-s interstimulus interval (ISI; defined as the onset interval between successive startle stimuli) in a semirandom order. This session was used to habituate rats to the holding chambers and startle stimuli and to assess baseline startle before training. Two days later (experimental day 8), the rats received a 5-min acclimation period followed by a conditioning session during which they received 10 light-shock pairings (3.7-s light coterminating with a 0.5-s, 0.6-mA foot shock). The mean intertrial interval (defined as the onset interval between successive light-shock pairings) was 3 min (range, 2–4 min). During conditioning, reactivity to the foot shock was quantified to control for possible changes in nociception due to stress or drug treatment.

Two days after training (experimental day 10), rats received a 5-min acclimation period followed by presentation of nine habituating startle stimuli (three stimuli at 95, 100, and 105 dB; 30-s ISI). Rats then received 18 startle stimuli, six at each of three different intensities (95, 100, and 105 dB; 30-s ISI) given in the presence or absence of the light (three startle stimuli at each intensity were preceded by a 3.7-s light stimulus); stimuli were given in a semirandom order with respect to startle intensity and presence of the light stimulus. Animals that have learned to associate the light (CS) with the foot shock (US) typically exhibit a greater startle response in the presence of the light due to increased fear elicited by the CS. Thus, the operational measure of fear was the difference in startle magnitude elicited in the presence and absence of the fear-conditioned cue (percentage of fear-potentiated startle = ([startle in the presence of the light − startle in the dark]/startle in the dark) × 100). Previous work demonstrates that KOR antagonism remains sustained at maximal or near-maximal levels throughout the time points used for FPS training and testing (Endoh et al., 1992; Jones and Holtzman, 1992; Carroll et al., 2004; Beardsley et al., 2005).

Data from each drug were analyzed separately using one-way (treatment) ANOVAs for shock reactivity, baseline startle during habituation trials, startle during the test session, and percentage fear-potentiated startle. Significant effects were analyzed using Dunnett's post hoc tests.

Results

The KOR antagonist norBNI affected behavior in the EPM. The drug dose-dependently altered the percentage of time rats spent in the open arms ($F_{3,55} = 9.97$, $P < 0.01$); rats spent more time in the open arms after treatment with 10 ($P < 0.05$, Dunnett's post hoc tests) or 30 ($P < 0.01$) mg/kg (Fig. 1A). Treatment with norBNI also affected open arm entries ($F_{3,55} = 9.81$, $P < 0.01$); rats made more entries into the open arms of the maze after treatment with 10 ($P < 0.05$) or 30 ($P < 0.01$) mg/kg (Fig. 1B). The drug did not affect closed arm entries or maze crosses (data not shown). Likewise, the structurally dissimilar KOR antagonist JDTic altered the percentage of time rats spent in the open arms ($F_{3,62} = 8.01$, $P < 0.01$); rats spent more time in the open arms after treatment with 10 mg/kg ($P < 0.01$) (Fig. 1C). Treatment with JDTic also affected open arm entries ($F_{3,62} = 6.61$, $P < 0.01$); rats made more entries into the open arms of the maze after treatment with 10 mg/kg ($P < 0.01$) (Fig. 1D).
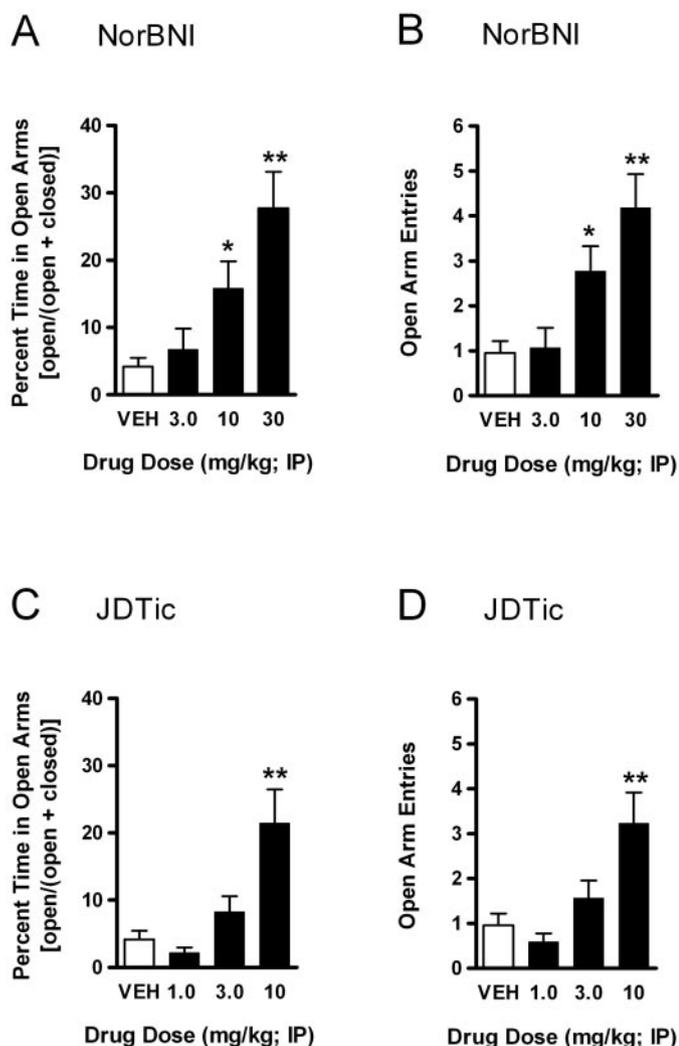
Fig. 1. Effects of KOR antagonists on EPM behavior. Rats received one i.p. injection of KOR antagonists norBNI or JDTic 48 h before testing in the EPM. Treatment with norBNI dose-dependently increased the percentage of time rats spent in the open arms of the EPM (A) and the number of entries rats made into the open arms (mean ± S.E.M.; Dunnett's post hoc tests) (B). Treatment with JDTic dose-dependently increased the percentage of time rats spent in the open arms (C) and the number of entries rats made into the open arms (D). Neither KOR antagonist affected closed arm entries or maze crosses (data not shown). ∗, $P < 0.05$; ∗∗, $P < 0.01$ versus vehicle.

Like norBNI, JDTic did not affect closed arm entries or maze crosses (data not shown). The effects of KOR antagonists on behavior in the EPM were qualitatively similar to those seen with the benzodiazepine chlordiazepoxide, which altered the percentage of time rats spent in the open arms ($F_{5,30} = 5.25$, $P < 0.01$); rats spent more time in the open arms after treatment with 5.0 or 10 mg/kg ($P < 0.01$) (Fig. 2A). Chlordiazepoxide also affected open arm entries ($F_{5,30} = 4.75$, $P < 0.01$); rats made more entries into the open arms of the maze after treatment with 5.0 mg/kg ($P < 0.01$) (Fig. 2B). Although the drug did not affect closed arm entries (data not shown), it affected maze crosses ($F_{5,30} = 4.53$, $P < 0.01$; data not shown); rats crossed the maze more frequently after treatment with 2.5 mg/kg ($P < 0.05$) or 5.0 mg/kg ($P < 0.01$). Unlike these other drugs, the SSRI fluoxetine did not affect open arm exploration; it had no effect on the percentage of time rats spent in the open arms (Fig. 2C) or open arm

entries (Fig. 2D). Fluoxetine decreased closed arm entries ($t_{10} = 2.88$, $P < 0.05$; data not shown) but did not affect maze crosses (data not shown).

In OF studies, norBNI did not affect locomotor activity at any dose, regardless of whether the data were analyzed as 5-min time bins (Fig. 3A) or total activity over the entire 1-h test session (data not shown), nor did it affect the distance rats traveled in the interior of the OF (Fig. 3B). Likewise, JDTic did not affect these measures (Fig. 3, C and D). However, chlordiazepoxide affected the time course of activity (treatment × time interaction, $F_{55,336} = 2.71$; $P < 0.01$); low doses of chlordiazepoxide (0.625, 2.5 mg/kg) increased, whereas higher doses (5.0,10 mg/kg) decreased activity at 5-, 10-, and 15-min time points ($P < 0.05$) (Fig. 4A). Chlordiazepoxide also affected mean activity counts when the data were analyzed as the total activity over the entire 1-h test session ($F_{5,28} = 4.40$, $P < 0.01$; data not shown); rats treated with 10 mg/kg were less active ($P < 0.05$). Chlordiazepoxide affected distance traveled in the interior portion of the OF ($F_{5,28} = 2.68$, $P < 0.05$); rats treated with 2.5 and 5.0 mg/kg traveled farther in the interior portion of the OF (Fig. 4B), consistent with an anxiolytic-like effect ($P < 0.05$). Fluoxetine affected the time course of activity (treatment × time interaction, $F_{11,120} = 2.76$; $P < 0.01$); the drug decreased activity during the first 10 min of the test ($P < 0.01$) (Fig. 4C), although there were no significant effects when the data were analyzed as total activity over the entire 1-h test session (data not shown). However, fluoxetine decreased the percentage of distance rats traveled in the interior OF ($t_{10} = 3.07$, $P < 0.05$), consistent with an anxiogenic effect (Fig. 4D).

Treatment with norBNI before FPS training affected the expression of the conditioned response ($F_{3,55} = 2.93$, $P < 0.05$) (Fig. 5A) without affecting baseline startle or shock reactivity (data not shown); 10 mg/kg reduced FPS ($P < 0.05$). Treatment with JDTic had a similar effect on FPS ($F_{3,64} = 7.88$, $P < 0.01$) (Fig. 5B); 10 mg/kg reduced FPS ($P < 0.01$). JDTic also affected baseline startle ($F_{3,64} = 4.34$, $P < 0.01$; data not shown), although the effect was totally attributable to a small but significant reduction in baseline startle at 1.0 mg/kg, a dose that did not affect FPS.

Discussion

KOR antagonists have anxiolytic-like effects in models of unlearned fear and learned fear. Both norBNI and JDTic increase open arm exploration in the EPM without causing changes in locomotor activity that indicate stimulant or sedative effects. Moreover, both drugs decrease FPS without affecting baseline startle or shock reactivity, which would indicate altered sensory function. The anxiolytic-like effects of KOR antagonists in the EPM are qualitatively similar to those produced by chlordiazepoxide. These results provide evidence that KOR antagonism produces acute and persistent anxiolytic-like effects in addition to antidepressant-like effects (Mague et al., 2003; Beardsley et al., 2005) in rats.

Our primary goal was to examine the effects of a single administration of KOR antagonists on anxiety-related behaviors. These agents have a slow onset and long duration of action, the mechanisms of which are not understood (Endoh et al., 1992; Carroll et al., 2004; Beardsley et al., 2005), making it necessary to design the experiments to accommodate these pharmacologic characteristics. Standard thera-
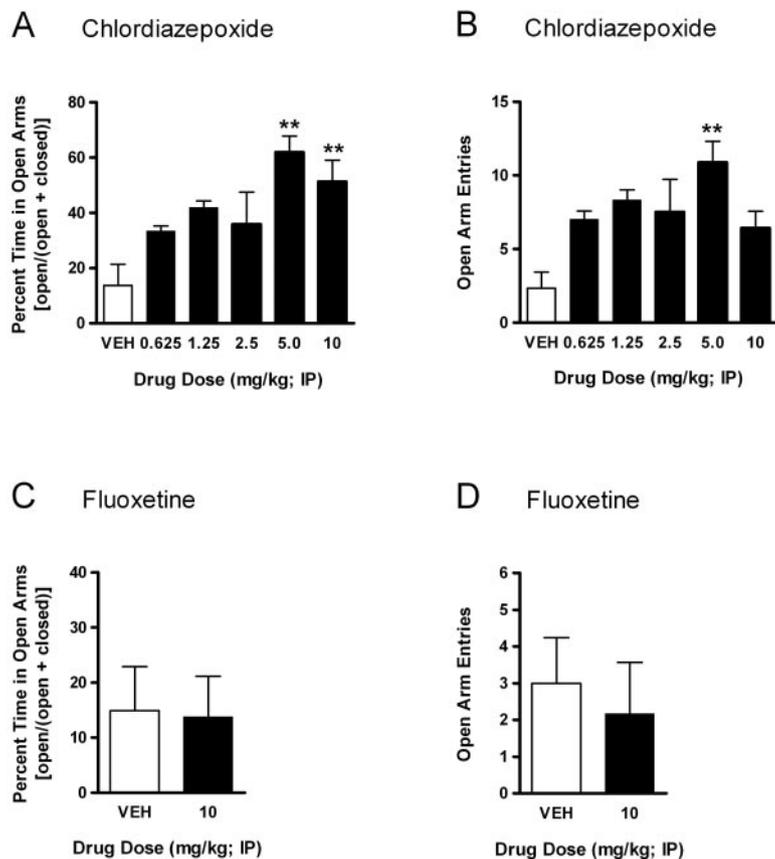
Fig. 2. Effects of chlordiazepoxide and fluoxetine on EPM behavior. Rats received one i.p. injection of chlordiazepoxide 30 min, or fluoxetine 60 min, before testing in the EPM. Treatment with chlordiazepoxide dose-dependently increased the percentage of time rats spent in the open arms of the EPM (A) and the number of entries rats made into the open arms (mean ± S.E.M.; Dunnett's post hoc tests) (B). Treatment with fluoxetine had no effect on the percentage of time rats spent in the open arms (C) or the number of entries rats made into the open arms (Student's t tests) (D). Fluoxetine decreased closed arm entries but did not affect maze crosses (data not shown). ∗∗, $P < 0.01$ versus vehicle.

peutic agents (chlordiazepoxide, fluoxetine) were used to enable qualitative comparisons of effects in the EPM and OF, although their inclusion was not intended to precisely mimic the conditions under which the KOR antagonists were tested; the kinetics of these standards are substantially different from those of norBNI and JDTic, making direct comparisons impossible. Subacute (2-day) treatment with chlordiazepoxide or fluoxetine enables insight on the behavioral effects that these drugs have early in a treatment regimen, although these drugs were not tested in the FPS paradigm because of obvious limitations in our ability to approximate the type of long-term drug exposure caused by single injections of norBNI or JDTic. More direct comparisons of these agents require the development of shorter acting KOR antagonists or longer acting benzodiazepines and SSRIs. Regardless, the longitudinal nature of the KOR antagonist studies demonstrates that the anxiolytic-like effects of these agents persist even after chronic and sustained drug actions.

In the EPM, norBNI and JDTic dose-dependently increase open arm exploration, indicating anxiolytic-like effects. Neither drug affects locomotor activity as assessed by closed arm entries or maze crosses, consistent with previous findings that KOR antagonists lack stimulant effects that can confound data interpretation in tests used to study depression and anxiety (Mague et al., 2003). Although chlordiazepoxide produces greater absolute increases in percent time spent in the open arms, baseline measurements of this parameter were higher in the corresponding control rats. This is probably attributable to differences in the experimental design; rats were administered chlordiazepoxide 30 min before testing, a pretreatment time at which norBNI and JDTic may not produce KOR-specific blockade (Endoh et al., 1992; Carroll et

al., 2004). Accounting for baseline differences, norBNI, JDTic, and chlordiazepoxide produced similar fold increases in open arm exploration (6.6-, 5.1-, and 4.5-fold, respectively), suggesting comparable efficacy. Ideally, identical drug administration regimens would be used to compare these drugs, but such studies require the development of new KOR antagonists.

Acute fluoxetine (10 mg/kg) did not alter open arm exploration, suggesting that this dose does not affect anxiety-like behavior under our testing conditions. Fluoxetine can produce anxiogenic-like effects in various models at comparable doses (5.0–10 mg/kg) (Artaiz et al., 1998; Bagdy et al., 2001; Drapier et al., 2007). It is likely that we did not detect anxiogenic effects because our testing conditions were optimized to detect anxiolytic drug effects (e.g., rats were not handled before testing and were tested during the light phase of their diurnal cycle), thereby preventing further decreases in open arm exploration. However, fluoxetine decreases closed arm entries and decreased activity counts in the OF test, potentially indicating sedation (Mague et al., 2003). Decreases in closed arm entries could also reflect anxiety-induced decreases in exploration (Pellow and File, 1986). Rats treated with 5.0 to 10 mg/kg chlordiazepoxide continue to explore the open arms despite substantial decreases in activity, indicating that dissociations between anxiety-related behavior and locomotor activity are possible in the EPM.

We used the OF as an additional measure of locomotor activity and, indirectly, anxiety-related behavior; drugs with anxiolytic or anxiogenic effects can affect the time rats spend in the interior portion of the OF (Prut and Belzung, 2003). Neither norBNI nor JDTic affects overall activity levels, again confirming that KOR antagonists are devoid of stimu-
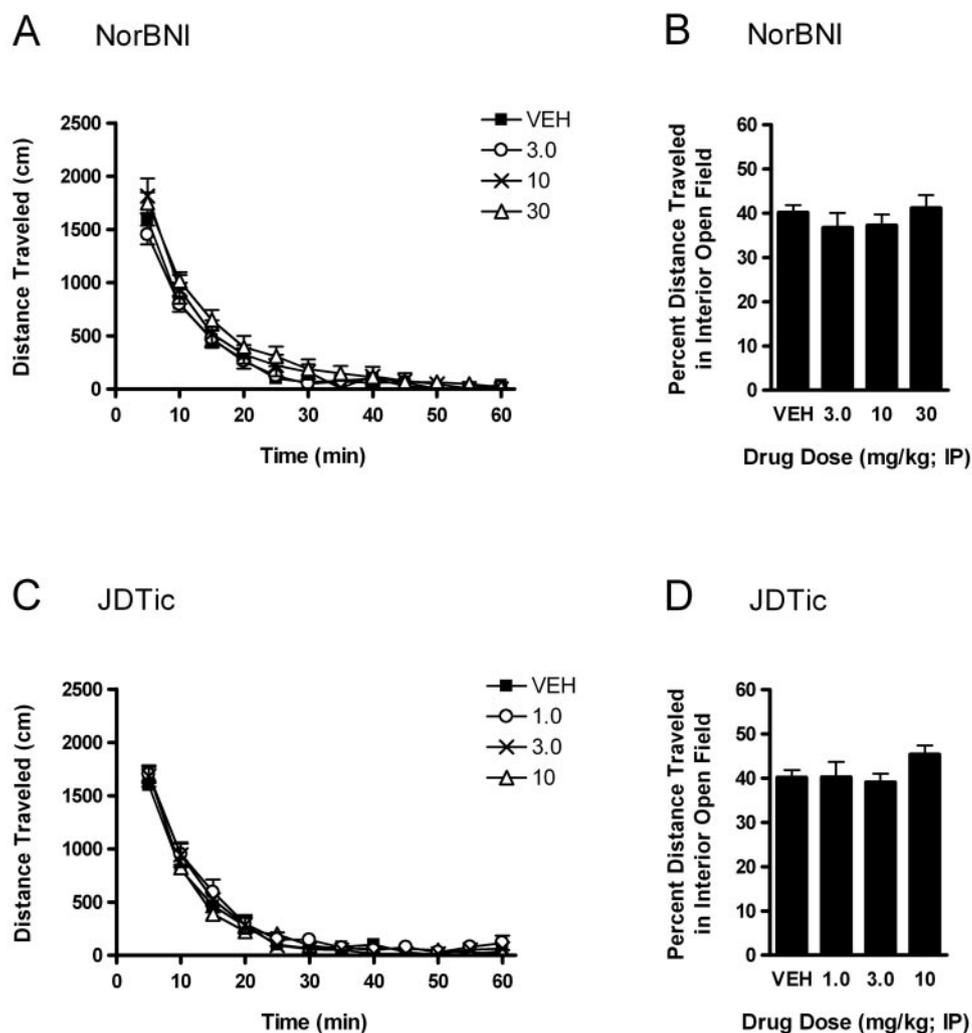
Fig. 3. Effects of KOR antagonists on locomotor activity and interior OF behavior. Treatment with norBNI (A) or JDTic (C) had no effect on the time course of activity analyzed in 5-min time bins over 1 h. Treatment with norBNI (B) or JDTic (D) also had no effect on the percentage of distance rats traveled in the interior of the OF (mean ± S.E.M.; Dunnett's post hoc tests).

lant or sedative effects at active doses (Mague et al., 2003). These drugs also do not affect distance traveled in the interior portion of the OF, which is surprising given their anxiolytic-like effects in the EPM. Chlordiazepoxide increases the percentage of distance rats traveled in the interior OF, consistent with its anxiolytic-like effects in the EPM and other models. One possibility is that the OF paradigm is less sensitive to the anxiolytic effects of nonbenzodiazepine agents; a meta-analysis indicates that only 11% of OF studies detect effects of chronic antidepressant treatment, whereas 67% detect anxiolytic effects of benzodiazepines (Prut and Belzung, 2003). Alternatively, we may not have detected effects of KOR antagonists because our testing conditions were optimized to detect drug effects on locomotor activity (e.g., rats were tested in darkness) and thus were minimally aversive. In contrast, fluoxetine (10 mg/kg) decreases the distance traveled in the interior portion of the OF, suggesting an anxiogenic-like effect. The observation that fluoxetine has anxiogenic-like effects in rats is consistent with reports that SSRIs can initially produce profound anxiogenic symptoms in humans (Grillon et al., 2007), again raising the possibility that our testing conditions in the EPM were not optimal for detecting anxiogenic effects.

Rats treated with KOR antagonists show reduced FPS without alterations in baseline startle or shock reactivity, indicating that these drugs also decrease learned fear. The effects of norBNI were detectable only at the intermediate dose (10 mg/kg), raising the possibility that high doses have nonspecific effects that are incompatible with detecting reduced FPS. Inverted U-shaped functions have been observed in other behavioral tests with KOR antagonists (Todtenkopf et al., 2004; Beardsley et al., 2005) and are often seen in the startle paradigm (e.g., Meloni et al., 2006). Regardless, the fact that JDTic produced reductions in FPS suggests that these fear-reducing actions are a general effect of KOR antagonists. Our experimental design did not enable us to distinguish between the effects of KOR antagonists on the development, consolidation, or expression of FPS. Not all experimental designs are feasible because of the slow onset and long duration of these prototypical KOR antagonists. It is possible to design studies in which expression is examined explicitly by administering the drugs after training so that KORs are blocked only during testing; these experiments are beyond the scope of the present report because it would not be possible to first test the rats in the EPM, the primary goal of these studies. One interpretation of the present data is that KOR antagonists would have protective effects in cases where it is possible to predict when stress will occur. A similar pattern of results was observed in studies involving i.c.v. microinjections of norBNI before exposure to the forced swim test (Pliakas et al., 2001), another potent stressor. Future studies of KOR antagonists in prestressed animals
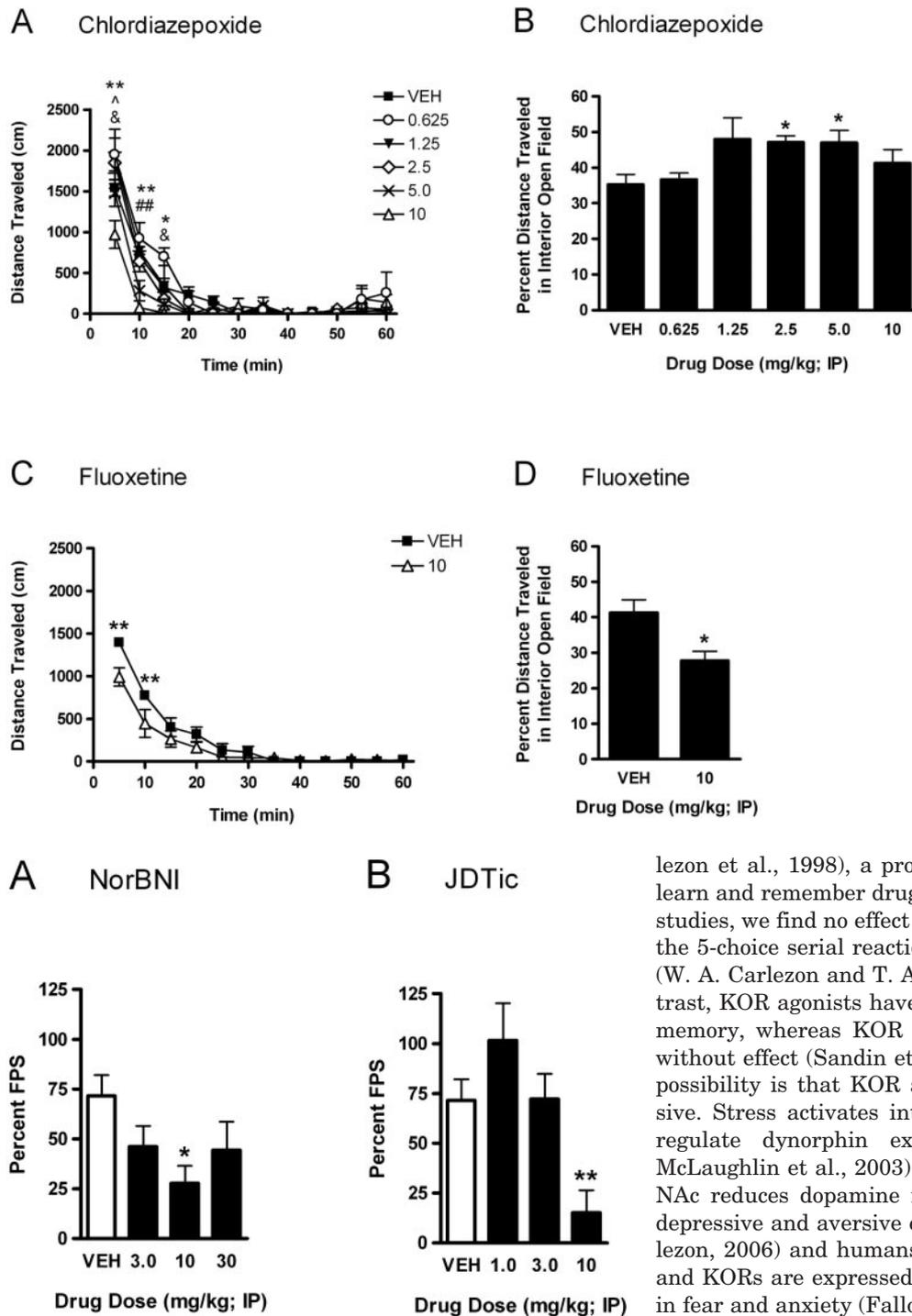
A   Chlordiazepoxide

B   Chlordiazepoxide

C   Fluoxetine

D   Fluoxetine

Fig. 4. Effects of chlordiazepoxide and fluoxetine on locomotor activity and interior OF behavior. Rats received one i.p. injection of chlordiazepoxide 30 min, or fluoxetine 60 min, before testing in the OF. A, treatment with chlordiazepoxide affected the time course of activity analyzed in 5-min time bins over 1 h: low to moderate doses of chlordiazepoxide (0.625–2.5 mg/kg) tended to increase initial locomotor activity, whereas high doses (5.0 and 10 mg/kg) decreased locomotor activity (mean $\pm$ S.E.M.; Dunnett's post hoc tests). &, at 5 and 15 min, 0.625 mg/kg was significantly different from vehicle; ^, at 5 min, 2.5 mg/kg was significantly different from vehicle; #, at 10 min, 5.0 mg/kg was significantly different from vehicle; *, at 5, 10, and 15 min, 10 mg/kg was significantly different from vehicle. One symbol, $P < 0.05$; two symbols, $P < 0.01$ versus vehicle. B, treatment with chlordiazepoxide increased the percentage of distance rats traveled in the interior of the OF. Treatment with fluoxetine decreased locomotor activity in the first 10 min of testing (C) and the percentage of distance rats traveled in the interior of the OF (Student's t tests) (D). *, $P < 0.05$; **, $P < 0.01$ versus vehicle.
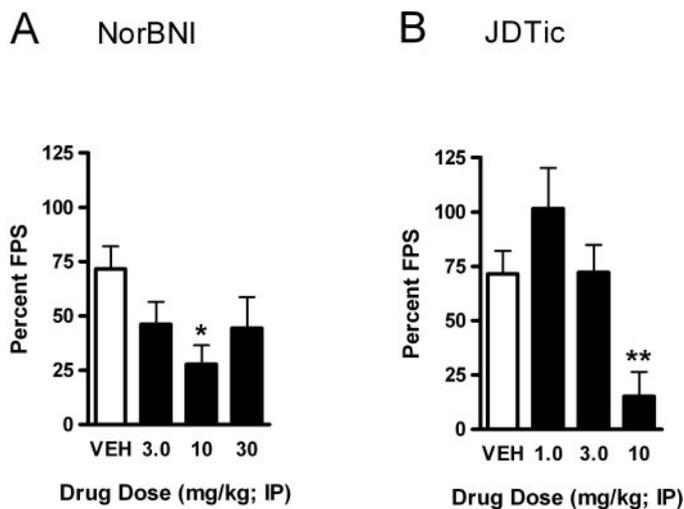
A   NorBNI

B   JDTic

Fig. 5. Percentage of FPS in rats treated with KOR antagonists. Treatment with norBNI (A) or JDTic (B) decreased the percentage of FPS (mean $\pm$ S.E.M.; Dunnett's post hoc tests). *, $P < 0.05$; **, $P < 0.01$ versus vehicle.

(McLaughlin et al., 2003) may help to further characterize how KORs regulate stress-induced anxiety and depressive-like behaviors.

The mechanisms by which KOR antagonists produce anxiolytic-like effects are not understood. There is evidence that this effect is not due to disruption of learning and memory. Treatment with norBNI did not significantly affect the rewarding effects of cocaine in place conditioning studies (Car-

lezon et al., 1998), a procedure that requires the ability to learn and remember drug-environment associations. In pilot studies, we find no effect of KOR antagonists on attention in the 5-choice serial reaction time task in highly trained rats (W. A. Carlezon and T. A. Paine, unpublished data). In contrast, KOR agonists have disruptive effects on learning and memory, whereas KOR antagonists or KOR knockout are without effect (Sandin et al., 1998; Jamot et al., 2003). One possibility is that KOR antagonists make stress less aversive. Stress activates intracellular signaling cascades that regulate dynorphin expression (Pliakas et al., 2001; McLaughlin et al., 2003); increased KOR stimulation in the NAc reduces dopamine function, which is associated with depressive and aversive effects in rodents (Nestler and Carlezon, 2006) and humans (Mizrahi et al., 2007). Dynorphin and KORs are expressed throughout brain regions involved in fear and anxiety (Fallon and Leslie, 1986; Mansour et al., 1995), making drug microinfusion studies a priority for future work.

This is the first report that KOR antagonists have anxiolytic-like effects that accompany their antidepressant-like effects (Mague et al., 2003). Anxiolytic-like effects differentiate this class of agents from standard antidepressants, which can have acute anxiogenic effects in laboratory animals and humans. This work also confirms previous findings that KOR antagonists do not have sedative effects (Mague et al., 2003) that complicate the use of benzodiazepines as anxiolytics in humans. Importantly, KOR antagonists do not possess reward-related effects (Todtenkopf et al., 2004) that contribute to the abuse liability of benzodiazepines. Thus, KOR antag-

onists seem to have a unique behavioral profile that would make them particularly effective for the treatment of comorbid depressive and anxiety disorders.

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Address correspondence to: Dr. William A. Carlezon Jr., Department of Psychiatry, McLean Hospital, MRC 217, 115 Mill Street, Belmont, MA 02478. E-mail: bcarlezon@mclean.harvard.edu