Behavioral/Systems/Cognitive

Endogenous κ Opioid Activation Mediates Stress-Induced Deficits in Learning and Memory

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We hypothesized that mice subjected to prolonged stress would demonstrate decreased performance in a learning and memory task attributable to the endogenous activation of the κ opioid receptor (KOR). C57BL/6J mice were tested using the novel object recognition (NOR) assay at various time points after exposure to repeated forced swim stress (FSS). Unstressed mice demonstrated recognition of the novel object at the end of a procedure using three 10-min object interaction phases, with a recognition index (RI) for the novel object of 71.7 ± 3.4%. However, 1 h after exposure to FSS, vehicle-pretreated mice displayed a significant deficit in performance (RI = 58.2 ± 4.1%) compared with unstressed animals. NOR was still significantly reduced 4 but not 24 h after FSS. Treatment with the KOR-selective antagonist norbinaltorphimine (10 mg/kg, i.p.) prevented the decline in learning and memory performance. Moreover, direct activation of the KOR induced performance deficits in NOR, as exogenous administration of the KOR agonist U50,488 [(±)-*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide] (0.3 mg/kg, i.p.) suppressed NOR (RI = 56.0 ± 3.9%). The effect of FSS on NOR performance was further examined in mice lacking the gene for the endogenous KOR agonist dynorphin (Dyn). Dyn gene-disrupted mice exposed to FSS did not show the subsequent learning and memory deficits (RI = 66.8 ± 3.8%) demonstrated by their wild-type littermates (RI = 49.7 ± 2.9%). Overall, these results suggest that stress-induced activation of the KOR may be both necessary and sufficient to produce subsequent deficits in novel object recognition.

Introduction

Brief exposure to stress may induce beneficial adaptative responses to an environmental threat (Cahill and Alkire, 2003). However, chronic exposure to persistent stress may be harmful, producing maladaptive responses resulting in physiological and behavioral disruptions (Carson et al., 1988). These neurobiological and behavioral adaptations have been demonstrated to involve the release of a number of stress-related hormones such as glucocorticoids (Piazza et al., 1990), corticotrophin releasing factor (Koob, 2003), and, more recently, dynorphin (Dyn), the endogenous agonist for the κ opioid receptor (KOR) (Pliakas et al., 2001; Shirayama et al., 2004). For example, C57BL/6J mice exposed to repeated forced swim stress (FSS) or social defeat stress demonstrated stress-induced analgesia, increases in immobility, and a potentiation of cocaine-conditioned place preference (McLaughlin et al., 2003, 2006). These stress-induced behaviors were attenuated by administration of the KOR antagonist norbinaltorphimine (nor-BNI) or disruption of the prodynorphin gene (McLaughlin et al., 2003, 2006), suggesting the involvement of the endogenous KOR system in multiple stress-induced behavioral responses.

Received Dec. 24, 2008; revised Feb. 10, 2009; accepted Feb. 11, 2009.

This work was supported by National Institute on Drug Abuse Grant R03 DA16415 and a Northeastern University Provost Research and Development Fund award (J.P.M.). We thank Dr. Ute Hochgeschwender for generously providing the prodynorphin gene-disrupted mice. We acknowledge the contributions of the students of Psychology U606 (Spring 2006 and 2007) in collecting elements of the NOR data.

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DOI:10.1523/JNEUROSCI.6146-08.2009

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The effect of stress on learning and memory is highly variable, most likely because deficits vary as a function of the severity, type, and timing of the stressful experience (Shors, 2006). However, evidence suggests that stress-induced or exogenous activation of the KOR may mediate changes in learning and memory performance. Shirayama et al. (2004) demonstrated that rats exposed to stress exhibited elevated levels of Dyn in the hippocampus, a brain region associated with learning and memory. Furthermore, chicks administered Dyn or the selective KOR agonist U50,488 [(±)-*trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide] displayed dose-dependent memory impairment when tested 24 h after training in a one-trial peck avoidance paradigm (Colombo et al., 1992).

Given the evidence, we hypothesized that mice exposed repeatedly to stress would show deficits in learning and memory performance mediated by the activity of the endogenous κ opioid system. To test this, C57BL/6J mice were exposed repeatedly to FSS. Learning and memory performance was assessed at different time points after stress using the novel object recognition (NOR) assay, an established animal model for assessing change in learning and memory performance (Save et al., 1992; Genoux et al., 2002). To determine the involvement of the endogenous κ opioid system, prodynorphin gene-disrupted mice or wild-type mice were treated with vehicle or nor-BNI (10 mg/kg, i.p.) before or immediately after forced swimming and subsequent testing in the NOR assay. The effect of exogenous activation of the KOR on learning and memory performance was also examined by instead administering mice U50,488 (0.3 mg/kg, i.p.) before NOR testing. Together, the results suggest that activation of the KOR may

be both necessary and sufficient to impair learning and memory performance, as demonstrated by subsequent deficits in novel object recognition.

Materials and Methods

Animals and housing. Adult male C57BL/6J mice (The Jackson Laboratory), prodynorphin gene-disrupted ($Dyn^{-/-}$) mice, and their wild-type littermates ($Dyn^{+/+}$) weighing 19–27 g were used in all experiments. All mice were group housed (four mice per cage), in self-standing plastic cages (16 × 24 × 12 cm) within the Animal Care Facility at Northeastern University. Housing rooms were illuminated on a 12 h light/dark cycle with lights on at 7:00 A.M. Food pellets and water were available *ad libitum*. Transgenic gene-disrupted mice lacking the functional gene for prodynorphin were provided by Dr. Ute Hochgeschwender (Oklahoma Medical Research Foundation, Oklahoma City, OK). Transgenic mice were backcrossed three generations to C57BL/6J mice, resulting in a mixed strain background of 87% C57BL/6J and 13% 129sv. As such, responses of the prodynorphin gene-disrupted mice were compared with wild-type littermates of the same generation. The presence or absence of the prodynorphin gene was confirmed in genomic DNA isolated from tail tissue samples taken from each mouse using PCR analysis as described previously (Sharifi et al., 2001; McLaughlin et al., 2003), with heterologous prodynorphin mice arising from backcrosses excluded from this study. All procedures using mice were approved by the Northeastern University Institutional Animal Care and Use Committee in accordance with the 1996 National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

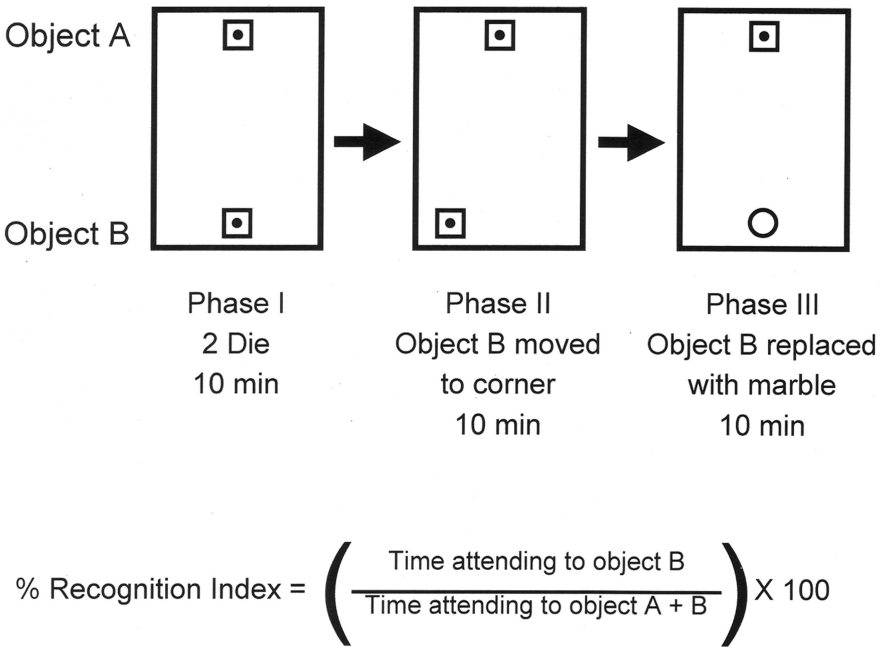

Figure 1. Schematic of NOR assay and calculation of RI. Images represent testing cages as they were arranged in each phase of the assay. Objects A and B were identical in phases I and II, although object B was moved to a different position during phase II testing. In phase III, object B was replaced with a novel object. In all phases, the percentage of time spent attending to object B was calculated as a percentage recognition index by the formula shown. Unless otherwise noted, initial objects were dice and the novel object was a marble, and each phase lasted 10 min, followed by a 10 min ITI.

Chemicals. nor-BNI and U50,488 were provided by the National Institute of Drug Abuse Intramural Drug Program and dissolved in vehicle (saline, 0.9%) for use. The dose of nor-BNI, 10 mg/kg i.p., 1 h before testing, was chosen for use in this study because previous work has demonstrated the selectivity of this antagonist for the KOR in C57BL/6 mice at this dose and pretreatment duration even after repeated daily administration (McLaughlin et al., 2003, their Fig. 2).

Locomotor activity. Locomotor activity of mice was monitored for 60 min immediately after administration of vehicle or U50,488 (0.3 or 1 mg/kg, i.p.). Locomotor activity was captured and digitalized, and the distance traveled was calculated by a Noldus Ethovision Pro locomotor tracking system. Before testing, mice were initially administered vehicle and confined to the locomotor chambers for 60 min to habituate the animals to the apparatus.

Forced-swim stress. To induce stress, C57BL/6J mice were exposed to 2 d of forced swimming. Testing was performed on the basis of methods described previously by Porsolt et al. (1977), as modified by McLaughlin et al. (2003). In this paradigm, mice swam in opaque 5 L beakers filled with 3.5 L of 30°C water. Multiple trials were used to determine the effects of extended exposure to the inescapable stressor. On day 1, the mice received one 15-min trial, whereas on day 2, animals were exposed to four trials, each lasting 6 min, consistent with previous methodology (McLaughlin et al., 2003). The trials were separated by no more than 7 min. On both days, the immobility responses of the mice were recorded during the final 4 min of each trial using the Ethovision Pro locomotor tracking apparatus. After each trial, the mice were dried with towels and returned to their home cages before additional testing. As a result of repeated exposure to inescapable stress, mice typically assumed a posture of immobility in which the forelimbs were motionless in front of the body, the hindlegs displayed limited motion, and the tail was directed outward, consistent with previous observations (McLaughlin et al., 2003). Animals were required to sustain this posture for at least 2 s to qualify as immobile. The criteria for exclusion included difficulty swimming or staying afloat; however, no animals were excluded on this basis.

Antinociceptive testing using the 55°C warm-water tail-withdrawal assay. The 55°C warm-water tail-withdrawal assay was used as described previously (McLaughlin et al., 2003), with the latency of the mouse to withdraw its tail taken as an endpoint. Tail-withdrawal latencies were collected before FSS exposure and again immediately after the conclusion of each day's swimming. A cutoff time of 15 s was used to prevent tissue damage.

Novel object recognition assay. Learning and memory performance was examined using the NOR assay (Save et al., 1992; Genoux et al., 2002). The NOR task for rats and mice is based on the spontaneous exploration of their environment, in which normal animals will spend more time exploring a novel object than a familiar one (Ennaceur and Delacour, 1988; Frick and Gresack, 2003). Because the assay does not require motivation such as food or water deprivation, this assay also minimizes external, physical stressors (such as swimming) that could have confounded the results of this study (Ennaceur and Delacour, 1988). The paradigm was performed over three phases; the first two were acquisition, or training, phases, and the third phase was the retention, or testing, phase (Fig. 1). As such, the NOR is a test for learning and memory, because the final output of the assay is affected by both. During each trial, mice freely explored the environment before being returned to their home cages. In phase I, objects A and B (a pair of dice) were centered across from each other, 2 cm away from the walls of a rectangular cage (16 × 24 × 12 cm) (Fig. 1). In phase II, object B was moved 1 cm from the edge of the cage, whereas object A remained fixed. In phase III, after completion of the training phases, the die (object B) was replaced with a marble, the novel object. Phase II served as a control to demonstrate that subsequent performance in phase III was based on novel object recognition rather than simple changes in object location. In contrast, phase III testing assesses both learning and memory, because mice will recognize and reject previously encountered objects to spend more time with the novel object. For each phase, the time that the mice spent attending to each object was recorded with stopwatches. Attending to the object was

defined as the duration of time the mouse spent in physical contact with the object using any body part other than the tail, or whenever it was within 0.5 cm of the object, facing it, and engaged in active exploration (e.g., sniffing, manipulating). Data are presented as a percentage recognition index (RI) for object B: RI = [(time attending to object B/time attending to object A + B)] * 100 (Fig. 1).

A control experiment was performed to confirm that the increased time spent attending to object B in phase III resulted from the novelty of, rather than the preference for, a particular object. Mice were tested following the described procedure except that the familiar objects were marbles and the novel object in phase III was a die. No differences in performance with either novel object in phase III was detected ($F_{(1,33)} = 0.316$; $p = 0.578$, one-way ANOVA), and there was no significant interaction of stimuli × phase ($F_{(2,100)} = 0.96$; $p = 0.39$, two-way ANOVA), establishing novel object interchangeability.

Statistical analyses. Time spent immobile (seconds) in FSS was analyzed with repeated-measures ANOVA with trial as the within-subjects repeated variable and drug treatment as the between-subjects factor. Individual trial-by-trial analysis between the two drug pretreatment conditions (vehicle vs nor-BNI) was performed using independent samples *t* tests. Antinociception was analyzed by comparing pre- and post-FSS tail-withdrawal latencies using paired-samples *t* tests to test for significant increases in latency after exposure to stress. Locomotor activity was analyzed using one-way ANOVA, with distance traveled as the dependent variable and drug treatment (vehicle, 0.3 or 1 mg/kg U50,488) as the between subjects factor. Optimization of the NOR assay was analyzed using two-way ANOVA, with phase as a repeated measure, to examine the effects of phase or intertrial interval (ITI) duration on NOR. One-way ANOVA was used to determine differences between groups within the individual phases with drug treatment (vehicle, nor-BNI, U50,488) and time of stress exposure included in the model, when appropriate, as between-subjects factors. Simple main effects were analyzed further with Ryan-Einot-Gabriel-Welsch multiple *F* (REGWF) *post hoc* test. The interaction of stress exposure × Dyn genotype ($Dyn^{-/-}$ vs $Dyn^{+/+}$) was examined using a multivariate ANOVA. Within-groups comparisons were made using paired-samples *t* tests to determine whether there was an increase in recognition index from phase I to phase III demonstrated by the group of interest. Data from all experiments were analyzed using SPSS 14.0 statistical package for the social sciences and are presented as mean ± SEM, with significance set at $p < 0.05$.

Results

Optimization of phase and intertrial interval

To optimize the assessment of novel object recognition, NOR was conducted with 1, 5, 10, or 20 min phases with a fixed 10 min ITI (Fig. 2*a*). Results indicated a significant main effect of phase duration ($F_{(3,216)} = 8.6$; $p < 0.001$, two-way ANOVA). Mice tested with 1 min phases displayed significantly less object recognition over three phases compared with mice tested using 5, 10, and 20 min phases ($p < 0.05$ each, REGWF). Mice tested using 5, 10, and 20 min phases spent significantly more time interacting with the novel object in phase III compared with time attending to object B in phase I ($p < 0.01$, $p < 0.001$, $p < 0.05$, respectively; paired-samples *t* test), whereas mice tested with 1 min phases did not [$p = 0.06$, not significant (NS)]. Because a peak RI for the novel object was demonstrated after testing with 10 min phases (71.7 ± 3.4%), this phase duration was used for the remainder of the study.

To establish the optimal ITI for novel object recognition, the duration of the interval between each 10 min phase was examined using 1, 10, or 20 min delays (Fig. 2*b*). A significant main effect of ITI duration was demonstrated ($F_{(2,144)} = 3.74$; $p < 0.05$, two-way ANOVA), but no significant interaction between phase and ITI was found ($F_{(4,144)} = 0.394$; $p = 0.81$). Significant differences in performance were demonstrated between mice tested with a 1 min and 10 or 20 min ITI ($p < 0.05$, REGWF) but not between the mice tested with a 10 and 20 min ITI ($p = 0.48$). Additional analysis found significant increases in the RI values in phase III over phase I when the ITI lasted 10 min ($p < 0.001$, paired-samples *t* test) or 20 min ($p < 0.05$) but not 1 min ($p = 0.20$, NS). Because the peak response was demonstrated using a 10 min delay between phases, this ITI was used throughout the remainder of the study.

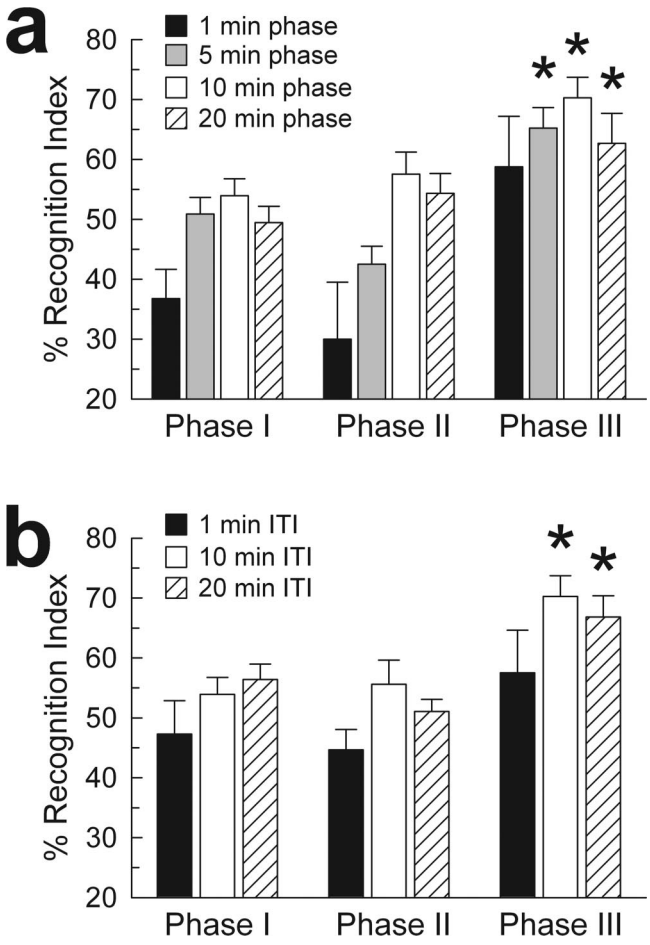

Figure 2. Optimization of conditions for the novel object recognition assay. Data plotted as percentage recognition index ± SEM for each phase. ***a***, Effect of phase duration. Unstressed, vehicle-treated C57BL/6J mice were used in the three phases of the NOR assay. Time in each phase was limited to 1 (black bars), 5 (gray bars), 10 (white bars), or 20 (striped bars) min. Mice demonstrated a significant increase in time spent attending to the novel object in phase III compared with the time spent attending to the equivalent object in phase I, but a peak phase III response was demonstrated with a phase duration of 10 min ($n = 15$–23 mice per bar; $^*p < 0.05$, significantly different from phase I response, paired-samples *t* tests). ***b***, Effect of intertrial interval. The duration of the interval between each 10 min trial was varied from 1 (black bar), 10 (white bar), or 20 (gray bar) min. Mice tested with a 10 or 20 min ITI demonstrated significant increases in recognition index for the novel object in phase III compared with matching phase I responses, with a peak response demonstrated in experiments using a 10 min ITI ($n = 7$–28 mice per bar; $^*p < 0.05$, significantly different from phase I response, paired-samples *t* tests).

Confirmation that FSS-induced immobility and analgesia were mediated by the endogenous κ opioid system

C57BL/6J mice were administered either vehicle or nor-BNI (10 mg/kg, i.p.) 1 h daily before exposure to FSS (Fig. 3) to corroborate the involvement of the KOR system in the response to an inescapable stressor. Analysis indicated a significant main effect of nor-BNI treatment ($F_{(1,47)} = 32.52$; $p < 0.001$), with mice spending less time immobile across the 2 d FSS paradigm compared with vehicle-pretreated mice.

Tail-withdrawal responses were measured before and after

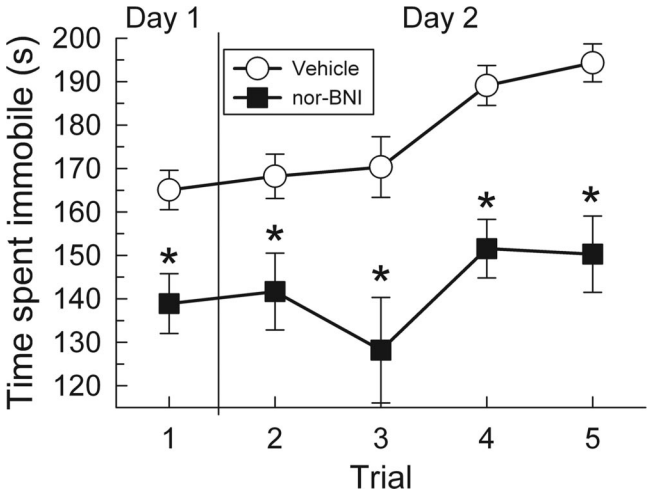

Figure 3. κ opioid antagonist nor-BNI reduced the time spent immobile during forced swimming. C57BL/6J mice were exposed to repeated trials of forced swimming 1 h after daily pretreatment with vehicle (circles) or nor-BNI (10 mg/kg, i.p., squares). Mice pretreated with nor-BNI demonstrated significantly less time spent immobile throughout all trials compared with vehicle-pretreated littermates. (n = 21–28; *$p < 0.05$, significant difference from matching trial, vehicle-treated animals, repeated-measures ANOVA with between-groups comparisons using independent samples t tests).

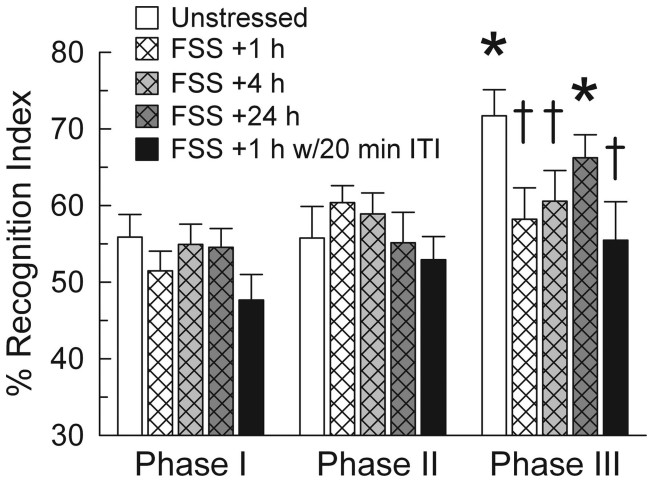

Figure 4. Novel object recognition performance was reduced up to 24 h after exposure to forced swim stress. Vehicle-pretreated, unstressed (white bars), or FSS-exposed (thatched bars) C57BL/6J mice were tested in the NOR assay 1 (white thatched bars), 4 (light gray thatched bars), or 24 (dark gray thatched bars) h after final exposure to forced swimming. Mice tested 1 or 4 h after exposure to stress showed significantly less recognition of the novel object in phase III compared with unstressed mice, whereas mice tested 24 h after the completion of forced swimming demonstrated a statistically similar response to the unstressed mice. Doubling the ITI did not improve the performance of mice tested 1 h after forced swimming (black bars), suggesting that stress-induced deficits were not the result of insufficient training (n = 20–24; *$p < 0.05$, significant difference from phase I response, paired-samples t tests; $^{\dagger}p \leq 0.05$, significant difference from phase III response of unstressed mice, one-way ANOVA with REGWF *post hoc* testing).

exposure to stress to further confirm a stress-induced activation of the KOR. Vehicle-pretreated mice demonstrated a significant increase in tail-withdrawal latency after FSS on day 2 (1.1 ± 0.03 vs 2.4 ± 0.3 s, before and after stress, respectively; $p < 0.001$, paired-samples t test). Conversely, mice pretreated with nor-BNI did not show significant increases in tail-withdrawal latency after forced swimming (1.31 ± 0.09 vs 1.77 ± 0.25 s; $p = 0.06$).

Exposure to stress suppressed novel object recognition in a time-dependent manner

The effects of exposure to FSS on novel object recognition and the duration of stress-induced deficits were evaluated. C57BL/6J mice were pretreated with vehicle 1 h before stress exposure and tested in the NOR assay 1, 4, or 24 h after completion of swim stress (Fig. 4). Comparison of RI between phases I and III revealed a significant increase in time spent exploring the novel object in phase III by unstressed mice ($p < 0.001$, paired-samples t test) and mice tested 24 h after stress ($p < 0.05$). However, mice tested 1 or 4 h after exposure to FSS demonstrated deficits in NOR, with no increases in RI during phase III ($p = 0.21$ and $p = 0.18$, respectively). Increasing the ITI to a 20 min delay between phases did not improve NOR performance in stress-exposed mice, suggesting that stress-induced deficits were not the result of insufficient training conditions ($p = 0.06$). Significant differences between the RI values of individual phases were found between groups in phase III ($F_{(3,76)} = 2.73$; $p = 0.05$, one-way ANOVA) but not phase I ($F_{(3,76)} = 1.40$; $p = 0.24$) or phase II ($F_{(3,76)} = 0.81$; $p = 0.51$). *Post hoc* analyses revealed significant differences in phase III recognition indices in unstressed control mice (71.7 ± 3.4%) compared with mice tested 1 h (58.2 ± 4.1%; $p < 0.05$, REGWF) and 4 h (60.6 ± 4.0%; $p = 0.05$) but not 24 h after stress (66.2 ± 3.0%; $p = 0.29$).

nor-BNI pretreatment prevented stress-induced deficits in NOR

To examine KOR mediation of stress-induced deficits in NOR performance, mice were administered either vehicle or nor-BNI (10 mg/kg, i.p.) 1 h before daily exposure to FSS. Additional mice were likewise treated but then placed in home cages for a matching period of time to provide a comparison of nor-BNI effects on NOR in unstressed control animals. Examination of NOR 1 h after exposure to stress revealed an overall significant difference in phase III RI between the treatment conditions (vehicle or nor-BNI and stress-exposed or unstressed, $F_{(4,84)} = 3.02$; $p < 0.05$, one-way ANOVA) (Fig. 5*a*). Additional analyses identified no significant differences between the phase III RI values of mice pretreated with nor-BNI before FSS and unstressed mice that were pretreated with either vehicle or nor-BNI ($p = 0.98$, REGWF). However, vehicle-pretreated FSS-exposed mice demonstrated significantly less novel object recognition in phase III (RI = 56.4 ± 4.3%) when compared with all other groups ($p \leq 0.05$). Notably, prophylactic administration of nor-BNI 1 h before FSS resulted in a significant increase in RI from phase I to phase III ($p < 0.001$), counteracting the stress-induced deficits in NOR observed in vehicle-pretreated mice.

Administration of nor-BNI immediately after exposure to FSS prevented stress-induced deficits in NOR when examined 1 h later (Fig. 5*b*), because mice did not perform differently from vehicle-treated unstressed mice in phase III ($p = 0.98$) but demonstrated RI values that were significantly greater than vehicle-pretreated, stress-exposed mice ($p < 0.05$).

Exposure to the KOR agonist U50,488 in place of stress produced deficits in NOR performance

Unstressed mice were pretreated with the KOR agonist U50,488 to determine whether KOR activation was sufficient to induce deficits in NOR. A dose of U50,488 that would not significantly impede locomotor activity was first determined (Fig. 6*a*). U50,488 (0.3 or 1 mg/kg, i.p.) dose dependently

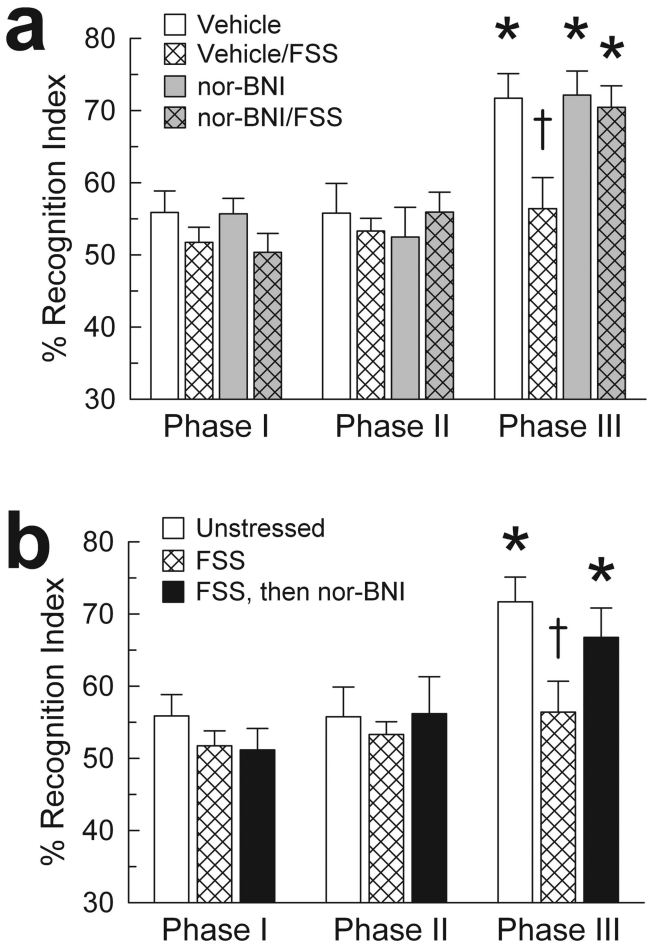

Figure 5. Forced swim stress affected learning and memory through a κ opioid receptor-dependent mechanism. ***a***, Pretreatment with nor-BNI before forced swimming prevented stress-induced NOR deficits. C57BL/6J mice were pretreated (1 h daily, i.p.) with vehicle (white bars) or the KOR antagonist nor-BNI (10 mg/kg; gray bars) and then returned to home cages (open bars) or exposed to FSS (thatched bars). NOR performance was evaluated 1 h after completion of home cage rest or stress exposure. Unstressed, nor-BNI-pretreated mice (gray open bars) showed no difference in NOR performance from unstressed, vehicle-pretreated mice (open white bars). Both groups demonstrated significant increases in percentage recognition index in phase III compared with the time spent attending to the equivalent object in phase I, which were not produced by vehicle-pretreated, FSS-exposed animals (white thatched bars). However, pretreatment with nor-BNI before FSS prevented stress-induced deficits in phase III novel object recognition (gray thatched bars). ***b***, Antagonism of KOR after exposure to forced swimming also prevented stress-induced deficits in NOR. Vehicle-pretreated mice exposed to FSS and treated immediately thereafter with nor-BNI (10 mg/kg, i.p.) demonstrated significant novel object recognition measured 1 h later (black bars) ($n = 17–20$; $^*p < 0.05$, significant difference from phase I response, paired-samples *t* tests; $^\dagger p = 0.05$, significant difference from phase III response of unstressed mice, one-way ANOVA with REGWF *post hoc* testing).

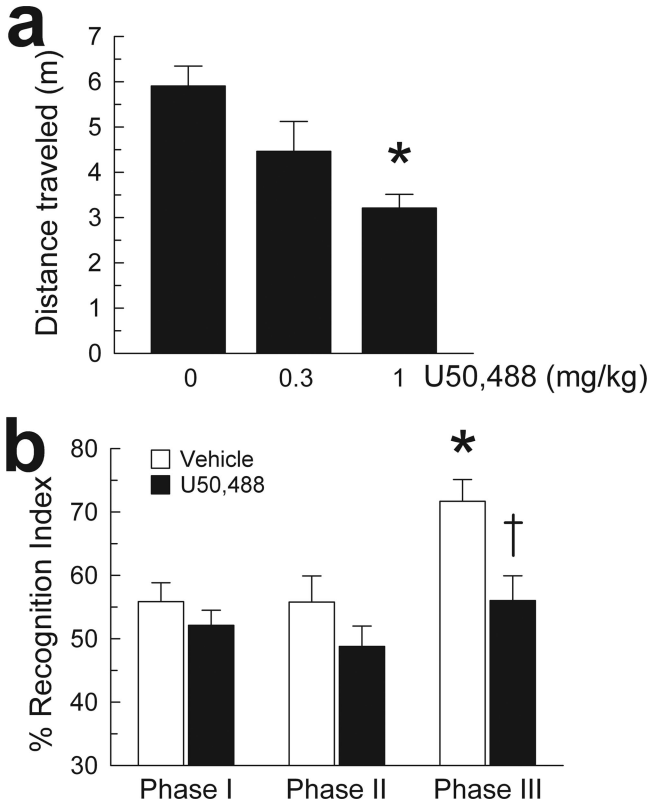

Figure 6. Direct agonist stimulation of the κ opioid receptor impaired novel object recognition. ***a***, Characterization of dose-dependent deficits of locomotion produced by the κ agonist U50,488. C57BL/6J mice, habituated to the locomotor chambers, were administered vehicle or a single graded dose of U50,488, and the total distance traveled in 60 min was determined ($n = 8–16$ mice; $^*p < 0.05$, significant difference from distance traveled after saline administration, one-way ANOVA with REGWF *post hoc* testing). ***b***, U50,488 pretreatment suppressed novel object recognition. U50,488 (0.3 mg/kg; black bars), administered 15 min before object recognition testing, prevented the significant increases in percentage recognition index demonstrated by vehicle-pretreated mice (white bars) in phase III of testing ($n = 17–20$ mice; $^*p < 0.05$, significant difference from phase I response, repeated-measures ANOVA with paired-samples *t* tests; $^\dagger p < 0.05$, significant difference from phase III response of unstressed mice, one-way ANOVA with REGWF *post hoc* testing).

suppressed locomotor activity ($F_{(2,36)} = 6.56$; $p < 0.001$, one-way ANOVA) (Fig. 6*a*), but a dose of 0.3 mg/kg did not significantly decrease locomotion. Mice administered U50,488 (0.3 mg/kg, i.p.) and tested 15 min later in the NOR assay demonstrated no significant increase in RI from phase I to phase III ($p = 0.30$, paired-samples *t* test). The phase III response of U50,488-pretreated mice was also significantly impaired compared with the response of vehicle-pretreated mice ($F_{(2,53)} = 4.55$; $p < 0.05$, one-way ANOVA followed by REGWF) (Fig. 6*b*) and demonstrated no difference from the response of vehicle-treated FSS-exposed mice ($p = 0.99$).

Dynorphin-deficient mice did not demonstrate stress-induced deficits in novel object recognition

Prodynorphin gene-disrupted mice ($Dyn^{-/-}$) and their wild-type littermates ($Dyn^{+/+}$) were injected with vehicle 1 h daily before exposure to FSS and then tested in the NOR assay 1 h after completion of swimming. Additional vehicle-pretreated mice were placed in home cages for a matching period of time to provide a comparison of the effects of prodynorphin gene disruption on NOR in unstressed control animals. There was a significant interaction of stress exposure (FSS-exposed vs unstressed) and genotype ($Dyn^{+/+}$ vs $Dyn^{-/-}$) in NOR performance during phase III ($F_{(1,85)} = 13.75$; $p < 0.001$, multivariate ANOVA) (Fig. 7). Additional analysis ($F_{(3,101)} = 6.40$; $p < 0.01$, one-way ANOVA) demonstrated that $Dyn^{+/+}$ mice exposed to stress spent less time exploring the novel object in Phase III compared with their unstressed littermates ($p < 0.05$, REGWF), consistent with the results from C57BL/6J mice. Notably, whereas stress-exposed $Dyn^{+/+}$ mice demonstrated no significant difference in RI values between phases I and III ($p = 0.09$, paired-samples *t* test), stress-exposed $Dyn^{-/-}$ mice spent significantly more time interacting with the novel object (phase I vs phase III, $p < 0.01$). Importantly, the phase III RI value demonstrated by stress-

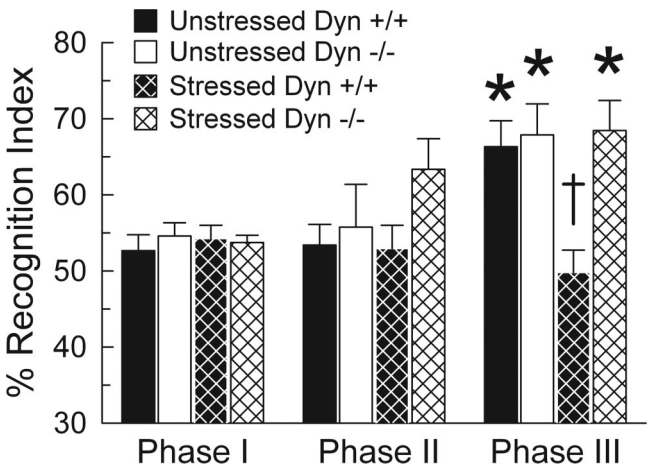

Figure 7. Forced swim stress-induced suppression of novel object recognition is prevented. Prodynorphin gene-disrupted animals ($Dyn^{-/-}$) and their wild-type littermates ($Dyn^{+/+}$) were injected with vehicle 1 h before FSS. One hour after stress, mice were tested in the NOR assay. Unstressed $Dyn^{-/-}$ mice (white bars) and their wild-type littermates (black bars) demonstrated a similar recognition of the novel object in all phases and a significant increase in time spent attending to the novel object in phase III compared with the time spent attending to the equivalent object in phase I. However, whereas wild-type mice showed a significant decrease in NOR in phase III after exposure to stress (black thatched bars), stress-exposed mice lacking the prodynorphin gene (white thatched bars) did not show deficits in learning and memory performance ($n = 19–38$ mice; $^*p < 0.05$, significant difference from phase I response, paired-samples *t* tests; $^\dagger p < 0.05$, significant difference from phase III response of unstressed mice, REGWF *post hoc* testing).

exposed $Dyn^{+/+}$ mice was significantly less than that of unstressed and stress-exposed $Dyn^{-/-}$ mice ($p < 0.05$, REGWF). Moreover, prodynorphin gene disruption in and of itself did not significantly alter novel object recognition, because the RI in phase III was consistent with response of the $Dyn^{+/+}$ mice ($p = 0.91$, NS).

Discussion

In the present study, C57BL/6J mice subjected to forced swim stress demonstrated decreased performance in novel object recognition compared with their unstressed littermates. Novel object recognition was impaired at least 4 but not 24 h after exposure to forced swimming in vehicle-pretreated mice, suggesting that exposure to stress affected learning and memory in a time-dependent manner. The stress-induced decrease in NOR was prevented by nor-BNI pretreatment or prodynorphin gene disruption, suggesting a mediation by the endogenous κ opioid system. Supporting this finding, exogenous administration of U50,488 suppressed NOR when given at a dose that did not have significant effects on locomotor activity.

Reports have shown an inhibition of learning and memory when training sessions were not separated by a sufficient delay for memory consolidation (Genoux et al., 2002). The present results were not an artifact of insufficient training or memory consolidation, because testing conditions were validated against previous reports (Ennaceur and Delacour, 1988; Genoux et al., 2002). C57BL/6J mice showed consistent significant novel object recognition in phase III when tested using 5, 10, and 20 min phases, and an ITI was characterized to allow for sufficient encoding of the experience with the familiar objects during the earlier phases.

Treatment with the KOR antagonist nor-BNI either before or immediately after FSS prevented stress-induced deficits in novel object recognition but had no effect on the task performance of unstressed animals. The nor-BNI sensitivity of the deficit suggested an endogenous activation of the KOR mediated the performance deficit in novel object recognition. Moreover, prodynorphin gene-disrupted mice lacking dynorphin peptides, and therefore the means of endogenously activating the KOR, did not demonstrate stress-induced suppression of NOR. Given that prodynorphin has been shown to also serve as a precursor for Leu^{5}enkephalin (Zamir and Quirion, 1985), these results suggest the alternative possibility of the activation of δ or μ opioid receptors to mediate the stress-induced deficits in novel object recognition. However, the dose of nor-BNI used has been demonstrated previously to be selective for the KOR (McLaughlin et al., 2003), discounting the possible involvement of the other opioid receptors. Overall, these data add to an increasing body of evidence that activity of the endogenous κ opioid system may mediate neurological alterations and behavioral responses to stress (Pliakas et al., 2001; McLaughlin et al., 2003, 2006; Shirayama et al., 2004).

An alternative explanation for the results of this study is that another hormone released in response to stress was responsible for the changes in learning and memory behavior. Notably, glucocorticoids released in response to stress have been reported to both enhance (Der-Avakian et al., 2005) and impair learning and memory performance (de Quervain et al., 1998), although this is further complicated by findings demonstrating that glucocorticoids are not always necessary (Wood et al., 2001) or sufficient (Beylin and Shors, 2003) to produce learning and memory deficits. Nonetheless, the involvement of glucocorticoids or other stress-related hormones cannot be discounted entirely with the present results. The mechanisms underlying learning and memory are complex and likely mediated by a number of factors (Shors, 2006). Importantly, this alternative does not preclude the involvement of the endogenous κ opioid system in learning and memory behavior. Supporting this, our data demonstrated that exogenous activation of the KOR alone was sufficient to induce deficits in novel object recognition, and both prodynorphin gene disruption and nor-BNI pretreatment prevented the stress-induced deficits in NOR. Moreover, previous studies report that exposure to forced swim stress increased the concentration of Dyn A (1–17) in the mouse brain hypothalamus (Nabeshima et al., 1992) and Dyn A and B in the rat brain hippocampus (Shirayama et al., 2004), offering biological evidence supporting the present behavioral findings.

Previous research into the contribution of the κ opioid system to learning and memory performance has produced contradictory results. A number of studies reported improved performance in learning and memory tasks after administration of KOR agonists such as U50,488 (Hiramatsu et al., 1996; Hiramatsu and Kameyama, 1998; Hiramatsu and Hoshino, 2004). Likewise, intracerebroventricular administration of Dyn A (1–17) or Dyn B produced dose-dependent increases in step-through latency in a passive avoidance test (Kuzmin et al., 2006). However, administration of the prodynorphin-derived peptide BigDynorphin produced increases in step-through latency that were blocked by MK-801 [(+)-5-methyl-10,11-dihydro-5*H*-dibenzo [a,d] cyclohepten-5,10-imine maleate] instead of nor-BNI, suggesting that this peptide influenced memory through the NMDA receptor (Kuzmin et al., 2006). In a more direct test of learning and memory, hippocampal infusion of Dyn A (1–13) was reported to ameliorate mecamylamine-induced learning impairments (Hiramatsu and Watanabe, 2006). However, nor-BNI did not fully reverse the preventative effect of Dyn A (1–13), suggesting that it might also have acted through a non-opioid site (Hiramatsu and Watanabe, 2006). This was consistent with other

findings using enantiomers of pentazocine (Hiramatsu and Hoshino, 2004, 2005) or other synthetic KOR agonists (KT-95) (Hiramatsu et al., 2006) to demonstrate that the reversal of memory impairment was not mediated by KOR activity but instead the activation of σ receptors. Notably, the dose of U50,488 administered before NOR testing here was similar to doses of U50,488 reported previously to prevent a drug-induced impairment in Y-maze performance (Hiramatsu et al., 1996; Hiramatsu and Hoshino, 2004). However, there are significant procedural differences between the present and previous studies, including the utilization of exogenous scopolamine to induce memory deficits in a spatial learning and memory task, which may possibly account for the discrepancy in results. Overall, these results suggest that reported KOR agonist-mediated improvements in learning and memory performance may in fact be attributable to nonspecific activity at non-opioid receptor sites.

Conversely, other reports suggest that KOR agonists suppress performance in learning and memory tasks through a direct activation of the KOR, consistent with the present results. Chicks administered Dyn A or U50,488 after training in a one-trial peck avoidance paradigm displayed a subsequent dose-dependent memory impairment, and administration of nor-BNI facilitated performance in a dose-dependent manner (Colombo et al., 1992). Similarly, microinjection of U50,488 into the CA3 region of the rat hippocampus suppressed learning and memory performance in the Morris water maze in a nor-BNI-sensitive manner (Daumas et al., 2007). These results are consistent with the present observation that U50,488 administration induced deficits in novel object recognition.

Evidence of a stress-induced release of dynorphin in the hippocampus (Shirayama et al., 2004) suggests a means by which κ opioid activity may impair learning and memory performance (Daumas et al., 2007). Notably, reports suggest that different types of learning and memory are subserved by distinct brain regions (Bussey et al., 2001; Broadbent et al., 2004). The ability of animals to recognize a novel object from objects previously encountered has been attributed to the activity of the perirhinal cortex in the medial temporal lobe (Murray and Richmond, 2001), a region known to receive input from and provide output to the hippocampus. Although the precise density of KOR within the perirhinal cortex remains uncharacterized in C57BL/6J mice, anatomical studies of rodent brain have demonstrated KOR labeling present in the deep layers of the temporal lobe (Mansour et al., 1996) as well as the hippocampus (Tempel and Zukin, 1987), suggesting that neural correlates exist through which the endogenous κ opioid system may modulate novel object recognition. Overall, the pairing of the established anatomical work with the present behavioral data provides a basis by which the activity of the endogenous κ opioid system may influence different types of learning and memory behavior, although this requires additional investigation.

Notably, assays of learning and memory depend on the integrity of systems mediating attention. It is conceivable that the deficits in novel object recognition may be attributable to endogenous κ opioid-mediated deficits in attention. Reports have shown that administration of exogenous KOR agonists suppress attention in a model of fear conditioning (Iordanova et al., 2006) and increase omissions and correct response latencies in a five-choice serial reaction time task (Paine et al., 2007), although KOR antagonist (nor-BNI) administration was also shown to disrupt aspects of attention related to working memory in mice (Wall and Messier, 2002). The novel object recognition test may be configured differently to examine attention (Silvers et al., 2007), but the present study did not examine this effect. Additional study is required to determine the impact of attention on stress-induced deficits in learning and memory.

In summary, the results of this study demonstrated that prolonged exposure to stress impaired learning and memory performance in a time-dependent manner mediated by the endogenous activation of the KOR. Impairments of novel object recognition were deterred by antagonism of the KOR or prodynorphin gene disruption. Moreover, these data add to the growing body of evidence suggesting that the activity of endogenous κ opioid system may contribute to maladaptive behaviors observed after exposure to a stressful event. As such, the data may offer a new connection between stress-related disorders of mood and learning and memory and suggest new therapeutic approaches in their treatment.

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