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Stress increases dynorphin immunoreactivity in limbic brain regions and dynorphin antagonism produces antidepressant-like effects

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Abstract

Rats exposed to learned helplessness (LH), an animal model of depression, showed a recovery following an intracerebroventricular injection of nor-binaltorphimine dihydrochloride (norBNI; a κ-opioid antagonist). To investigate the potential role of dynorphin A and dynorphin B, we examined the effects of different stress/depression models on dynorphin A and dynorphin B immunoreactivity in hippocampus and nucleus accumbens (NAc). Immobilization stress (3 h) caused an increase in levels of dynorphin A and dynorphin B immunoreactivity in the hippocampus and the NAc. Forced swim stress also temporally increased dynorphin A levels in the hippocampus. Furthermore, exposure to LH produced a similar increase in dynorphin A and dynorphin B in the hippocampus and NAc. Infusions of norBNI into the dentate gyrus or CA3 regions of hippocampus and into the shell or core regions of NAc produced antidepressant-like effects in the LH paradigm. The degrees of norBNI's effects were stronger in the CA3 region and NAc shell and less effective in the dentate gyrus of hippocampus and NAc core. These results indicate that both dynorphin A and dynorphin B contribute to the effects of stress, and suggest that blockade of κ-opioid receptors may have therapeutic potential for the treatment of depression.

Keywords: behavior, depression, hippocampus, κ-opioid receptor, learned helplessness, nucleus accumbens.

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Depression in humans is often precipitated or worsened by physical or psychological stress. This association forms the basis for the use of stress-related models for studies of the neurobiology of depression and antidepressant actions (see Duman *et al*. 1997, 2000; McEwen 1999). There are several limbic brain regions that have been implicated in mood disorders, including the frontal cortex, amygdala, and hippocampus. The hippocampus is particularly sensitive to stress due to the high levels of glucocorticoid receptors expressed in this structure (McEwen 1999). Stress results in neurochemical as well as structural alterations in the hippocampus in animal models, including remodeling of CA3 pyramidal neurons and decreased proliferation of dentate gyrus granule cells (McEwen 1999; Duman *et al*. 2001). Structural alterations similar to the changes that have been observed in animal models could contribute to the atrophy of hippocampus observed in brain imaging studies of depressed patients (Sheline *et al*. 1996; Bremner *et al*. 2000). Recent studies also demonstrate that antidepressant treatment can reduce the atrophy of hippocampus, as well as the

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Abbreviations used: AP, anteroposterior; BDNF, brain-derived neurotropin factor; BSA, bovine serum albumin; CREB, cAMP response element binding protein; DAB, 3,3-diaminobenzidine tetrahydrochloride; DV, dorsoventral; LH, learned helplessness; LTP, long-term potentiation; NAc, nucleus accumbens; norBNI, nor-binaltorphimine dihydrochloride; PBS, phosphate-buffered saline.

cognitive impairments in depressed patients (Riedel *et al*. 2002; Sheline *et al*. 2003; Vermetten *et al*. 2003).

Stress regulates a number of neuropeptides and neurotrophic factors that have been implicated in the etiology and treatment of depression (Duman *et al*. 2000; McEwen 2000; Hokfelt *et al*. 2003). One neuropeptide system that has gained recent attention is dynorphin, which has been primarily studied for its role in the actions of drugs of abuse and is enriched in brain regions involved in drug reward, craving, and withdrawal. However, recent reports demonstrate that infusion of an antagonist of the κ-opioid receptor, the primary receptor for dynorphin, produces an antidepressant effect in two behavioral models of depression, the forced swim test (Pliakas *et al*. 2001; Mague *et al*. 2003) and learned helplessness (LH) paradigm (Newton *et al*. 2002). κ-Opioid receptors are expressed at relatively high levels in the nucleus accumbens (NAc), but are also expressed at relatively high levels in the hippocampus, frontal cortex, hypothalamus, ventral tegmental area and locus coeruleus (Zukin *et al*. 1988; Fan *et al*. 2002). There are higher levels of κ1-opioid receptors in the nucleus accumbens than in the hippocampus and similar levels of κ2-opioid receptors in these two regions.

The brain regions and neurobiological mechanisms underlying the effects of dynorphin in stress and depression models have not been well characterized. Dynorphin is abundantly expressed in the substantia nigra, striatum, nucleus accumbens, hippocampus, cortex and hypothalamus. Previous studies have found that infusion of a dynorphin antagonist, nor-binaltorphimine dihydrochloride (norBNI), into the lateral ventricles results in antidepressant effects in forced swim (Pliakas *et al*. 2001) and LH paradigm (Newton *et al*. 2002). One of these studies also found that microinfusions of norBNI into the NAc produces a similar antidepressant effect implicating this brain region (Newton *et al*. 2002). These results were interpreted in the context of the possible dysphoric actions produced by dynorphin in the mesolimbic dopamine system (Spanagel *et al*. 1992; see Pliakas *et al*. 2001). Increased levels of dynorphin expression are thought to contribute to the negative emotion precipitated by cocaine withdrawal and that can resemble depression.

The focus of the current investigation was to further test the hypothesis that dynorphin could mediate the actions of stress and contribute to the behavioral alterations observed in models of depression. The first goal was to determine if levels of dynorphin immunoreactivity are increased by stress and standard depression paradigms. The second goal was to determine if infusions of the dynorphin antagonist into brain regions where increased levels of dynorphin are found result in an antidepressant response. The results demonstrate that immobilization stress, forced swim, or LH exposure increases dynorphin immunoreactivity in specific subregions of the hippocampus, as well as NAc and that blockade of κ-opioid receptors in these regions produces an antidepressant response in the LH model of depression.

Materials and methods

Animal and treatments

Animals use procedures were in accordance with the Tottori university Guide for the Care and Use of Laboratory Animals and were approved by the Tottori university Animal Care and Use Committee. Male Sprague–Dawley rats (250–300 g) were used. The animals were housed under 12 h light/dark cycle with free access to food and water.

Surgery was performed in a stereotaxic apparatus (Narishige, Tokyo, Japan) under anesthesia with pentobarbital sodium solution [50 mg/kg, intraperitoneal (i.p.) injection, Abbott Laboratories, North Chicago, IL, USA]. We have decided the doses of norBNI due to the previous studies (Spanagel and Shippenberg 1993; Pliakas *et al*. 2001; Wall and Messier 2002; Mague *et al*. 2003). Rats received bilateral microinjection of different amounts of norBNI (2.5 or 0.25 μg/side as indicated) or saline (0.9%) into the dentate gyrus or CA3 region of hippocampus and into the shell or core regions of NAc. A total volume of 1.0 μL was infused into each side over 15 min and the injection syringe was left in place for an additional 5 min to allow for diffusion. The co-ordinates for subregions of the hippocampus and NAc (relative to bregma according to the atlas of Paxinos and Watson 1997) were as follows: − 3.8 anteroposterior (AP), ± 2.0 lateral, − 3.2 dorsoventral (DV) from dura (dentate gyrus hilus); − 3.6AP, ± 3.8 lateral, − 3.0 DV from dura (CA3 stratum radiatum); − 3.6AP, ± 4.0 lateral, − 3.0 DV from dura (CA3 stratum oriens); + 1.7 AP, ± 0.8 lateral, − 7.1 DV from dura (NAc shell); + 2.2 AP, ± 1.6 lateral, + 6.7 DV from dura (NAc core). Brains were sectioned at 17 μm and stained with cresyl violet. Sections were examined with light microscopy for the placement of norBNI infusion.

For antidepressant treatments, imipramine (20 mg/kg, i.p., once per day) or saline (once per day) were administrated for 7 days until one day before the active avoidance behavioral tests were performed.

Stress/depression paradigms

For the immobilization stress, rats were immobilized for 3 h using rodent immobilization cones (Harvard apparatus, Holliston, Massachusetts). Rats were perfused immediately after the last immobilization period.

For the forced swim stress, animals were placed in a container filled with water to a depth of 40 cm (23–25°C) for 15 min. Animals were killed at several different time points, including 0, 2, 5, and 24 h after exposure to swim stress.

For the LH paradigm, animals are initially exposed to an uncontrollable stress. Upon subsequent testing in an escapable conditioned avoidance test, the animals fail to acquire escape responses, and often do not even attempt to escape. This escape deficit is reversed by chronic antidepressant treatment (Chen *et al*. 2001; Shirayama *et al*. 2002). LH behavioral tests were performed with the Gemini Avoidance System (San Diego, CA, USA). This apparatus was divided into two compartments by a retractable door. On day 1 and day 2, rats were subjected to 60 inescapable electric footshocks (0.65 mA, 30 s duration, averaging 20–40 s). On day 3, a two-way conditioned avoidance test was performed as a post-shock test to determine if the rats would show the predicted escape deficits. This screening session consisted of 30 trials in which

electric footshocks [0.65 mA, 6 s duration, at random intervals (mean of 30 s)] was preceded by a 3 s conditioned stimulus tone that remained on until the shock was terminated. Rats with more than 20 escape failures in the 30 trials were regarded as having reached criterion. Approximately 65% of the rats reached this criterion. On day 4, rats were subjected to an additional 30 inescapable electric footshocks (0.65 mA, 30 s duration) to reinforce the LH condition.

On day 5, rats received bilateral microinjections of norBNI or vehicle as described above. On day 8 (3 days after the surgery), a two-way conditioned avoidance test was performed. This test session consisted of 30 trials in which electric footshock (0.65 mA, 30 s duration, at random intervals (mean of 30 s, averaging 20–40 s) was preceded by a 3-s conditioned stimulus tone that remained on until the shock was terminated. The numbers of escape failures and the latency to escape in each of 30 trials was recorded by the Gemini Avoidance System.

Dynorphin immunocytochemistry

All rats were placed under deep pentobarbital anesthesia (80 mg/kg, i.p.) and killed via intracardial perfusion with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Brains were removed, post-fixed overnight in the same fixative at 4°C, and stored at 4°C in 30% sucrose. Serial coronal sections of the brains were cut (35-μm sections) on a freezing Microslicer® (DTK-1000, Dosaka EM, Kyoto, Japan), and sections were stored at 4°C in 0.1 M PBS containing 0.1% sodium azide.

For dynorphin A and dynorphin B immunocytochemistry free-floating sections were washed three times for 5 min in 0.1 M PBS and then incubated for 10 min in 0.1 M PBS containing 0.6% hydrogen peroxide to eliminate endogenous peroxidases. After washing three times for 5 min in 0.1 M PBS, sections were then incubated for 1 h in 0.1 M PBS containing 2% bovine serum albumin (BSA), 5% normal goat serum, and 0.2% Triton X-100 for blocking. Sections were incubated at 4°C for 72 h with primary dynorphin A rabbit polyclonal antibody (1 : 1000; Oncogene, Boston, MA, USA) or primary dynorphin B rabbit polyclonal antibody (1 : 1000; Oncogene). After washing six times for 5 min in 0.1 M PBS, sections were incubated for 2 h with secondary antibody (biotinilated goat anti-rabbit; Vector Laboratories, Burlingame, CA, USA) followed by amplification with an avidin–biotin complex (Vectastain Elite ABC kit; Vector Laboratories) and were visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories). For the pre-absorption test, aliquots of the dynorphin A and dynorphin B antibodies were incubated with dynorphin A (10 μg/mL) and dynorphin B (10 μg/mL), respectively.

For quantification of dynorphin A and dynorphin B expressions, three to four slices at the same anterior–posterior position for hippocampus and NAc were prepared for each rat. The optical density was analysed using a CCD camera and the NIH 1.61 Image program. Average counts were obtained from both sides of each slice for a total of 6–8 counts for each animal. Data are expressed as percentage of control and are the means ± SEM of four to five animals per group.

Statistics

Statistical differences among three groups were estimated by a one-way ANOVA, followed by Scheffe's test. For comparison of the mean values between the two groups, statistical evaluations were done using the two-tailed Student's *t*-test. Differences were set to be significant when *p*-value were less than 0.05.

Results

Stress increases the levels of dynorphin A and dynorphin B immunoreactivity

Dynorphin A and dynorphin B immunoreactivity was examined in the hippocampus and NAc (Fig. 1). In the

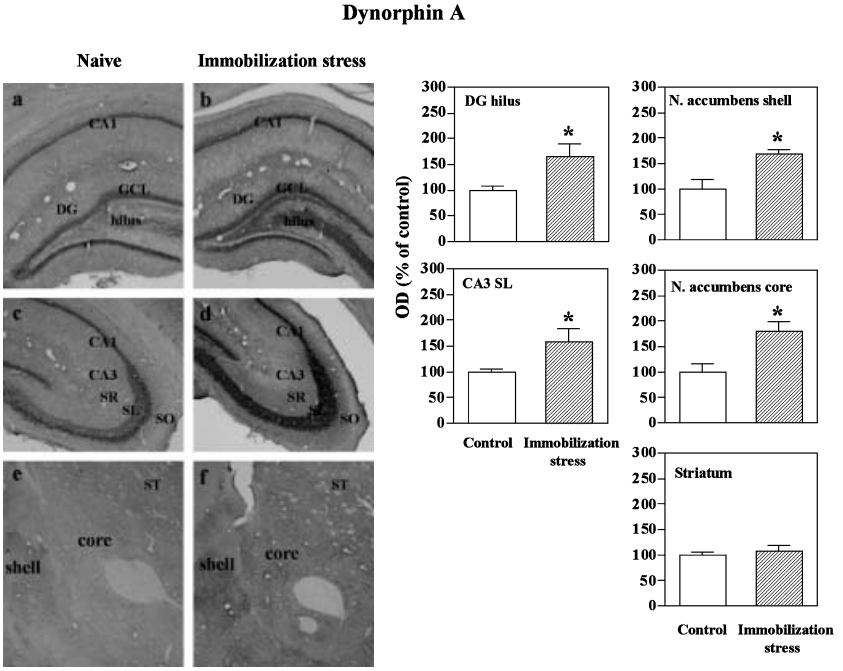

Fig. 1 Acute immobilization stress increases dynorphin A immunoreactivity in subfields of the hippocampus and striatum. Naïve (a, c, e) and stressed (b, d, f) animals. (a and b) The dentate gyrus and CA1 regions of hippocampus; (c and d) the CA3 region of hippocampus; (e and f) the shell and core regions of nucleus accumbens. Shown on the left are representative sections that were subjected to dynorphin A immunohistochemical analysis. On the right are the optical density (OD) readings for the regions indicated. The results are presented as percentage of control and are mean ± SEM ($n = 5$ animals for control and $n = 4$ for stressed). $^*p < 0.05$ compared to control (Student's *t*-test). DG, dentate gyrus; GCL, granule cell layer; CA1, CA1 pyramidal cell layer; SR, stratum radiatum; SL, stratum lucidum; SO, stratum oriens; ST, striatum.

hippocampus, the dentate gyrus and mossy fiber pathway were strongly immunostained. The NAc shell region expressed high levels of dynorphin A and dynorphin B immunostaining, and significant staining was also observed in the core as well as the striatum.

The influence of different stress paradigms, including immobilization, forced swim, and LH, on levels of dynorphin A and dynorphin B immunoreactivity was determined. These three paradigms represent different types of stress with regard to the type of physical stressor, the intensity and duration of stress, and behavioral effects. Examination of these three paradigms will determine if dynorphin immunoreactivity is regulated under different stressful conditions. Acute immobilization stress increased levels of dynorphin A immunoreactivity in the hippocampus and NAc (Fig. 1). Stress-induced increases were observed in the hilus of the dentate gyrus and mossy fiber pathway, including the stratum lucidum of the CA3 region of the hippocampus and the shell and core of the NAc. Similarly, immobilization stress increased dynorphin B in the hippocampus (Fig. 2). Dynorphin B was induced only in the mossy fiber pathway, including the origin of the pathway in the dentate gryus and the stratum lucidum of the CA3 region and the shell and core of the NAc. There was no effect of immobilization stress on levels of either dynorphin A or dynorphin B in the striatum. (Figs 1 and 2).

Exposing animals to forced swim stress, a behavioral model used for testing antidepressant drugs, also increased levels of dynorphin A immunoreactivity in the hippocampus (Fig. 3). This effect was similar to the actions of acute immobilization stress, with increased levels of dynorphin A in the hilus of dentate gyrus and stratum lucidum (Fig. 3). Increased dynorphin A immunolabeling was observed 2 h after swim stress, and was still present at 5 h but not 24 h later.

The influence of another depression paradigm, learned helplessness, on expression of dynorphin immunoreactivity was also determined. Exposure of animals to inescapable footshock results in helpless behavior characterized by failure to acquire active avoidance training (Shirayama *et al.* 2002). Inescapable footshock stress increased levels of dynorphin A immunoreactivity in the hippocampus and NAc (Fig. 4), with a pattern of induction that was similar to that observed after immobilization or forced swim stress. In the hippocampus, induction of dynorphin A was observed in the stratum lucidum of the CA3 region and the dentate gyrus and mossy fiber pathway. In the Nac, the induction of dynorphin A was observed in the shell region, as well as the core. There was no significant effect in the striatum. Exposure to inescapable footshock also increased levels of dynorphin B in the hippocampus, specifically in the mossy fiber pathway (compare Figs 2 and 5). In the Nac, the induction of dynorphin B was observed in the shell as well as the core and there was no significant effect in the striatum (Fig. 5).

The specificity of the dynorphin A and dynorphin B antibodies was determined by pre-absorption with dynorphin A and dynorphin B, respectively. Pre-absorption of the antibodies attenuated dynorphin A and dynorphin B immunostaining in the hippocampus and NAc (Fig. 6). In the hippocampus immunoreactivity in the hilus (subgranular cell layer) and stratum lucidum was blocked by pre-absorption.

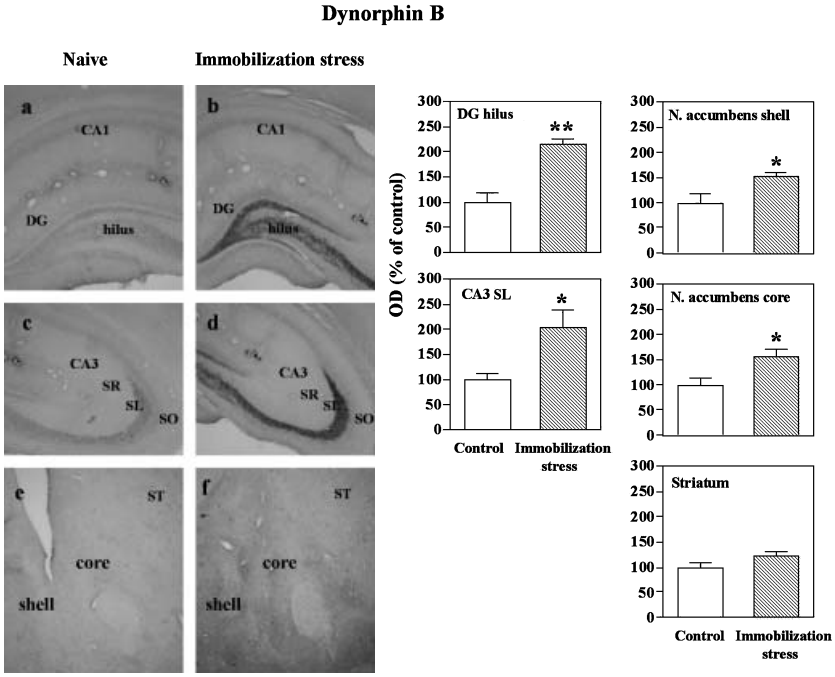

Fig. 2 Acute immobilization stress increases dynorphin B immunoreactivity in subfields of the hippocampus and striatum. Naïve (a, c, e) and stressed (b, d, f) animals. (a and b) The dentate gyrus and CA1 regions of hippocampus; (c and d) the CA3 region of hippocampus; (e and f) the shell and core regions of nucleus accumbens. Shown on the left are representative sections that were subjected to dynorphin B immunohistochemical analysis. On the right are the optical density (OD) readings for the regions indicated. The results are presented as percentage of control and are mean ± SEM ($n = 5$ animals for control and $n = 4$ for stressed). $^{*}p < 0.05$; $^{**}p < 0.01$ compared to control (Student's *t*-test). DG, dentate gyrus; SR, stratum radiatum; SL, stratum lucidum; SO, stratum oriens; CA1, CA1 pyramidal cell layer; ST, striatum.

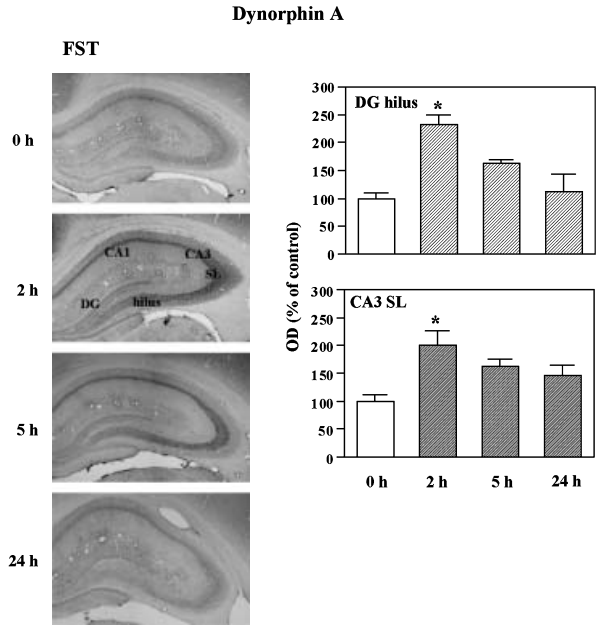

Fig. 3 Forced swim stress increases dynorphin A in hippocampus. Dynorphin A immunolabeling was determined at the time points indicated. An increase in dynorphin A staining was observed in the hilus of dentate gyrus and the stratum lucidum of CA3 region of hippocampus. Sections are shown for each time points, and are representative of separate animals for each time point ($n = 3$ animals per time point). Right top, $F(3, 8) = 9.109$, $p = 0.0059$; right bottom, $F(3, 8) = 4.648$, $p = 0.0366$.*$p < 0.05$ when compared with saline-injected controls (ANOVA followed by Scheffe's test for forced swim stress). DG, dentate gyrus; SL, stratum lucidum; CA1, CA1 pyramidal cell layer.

Blockade of κ-opioid receptors produces an antidepressant effect

Learned helplessness rats exhibit a deficit in escape performance on subsequent conditioned avoidance behavior. Subchronic treatment with imipramine significantly improves the ability of the animals to escape in the avoidance test (Fig. 7). These results demonstrate that the learned helplessness paradigm is responsive to antidepressant treatment as reported previously (Siuciak *et al*. 1997; Chen *et al*. 2001; Shirayama *et al*. 2002).

Previous studies have demonstrated that infusions of a dynorphin/κ-opioid receptor antagonist, norBNI, into the ventricles or NAc produce an antidepressant effect in the learned helplessness paradigm and the forced swim test (Pliakas *et al*. 2001; Newton *et al*. 2002). Because the levels of dynorphin immunoreactivity were robustly induced by stress/depression paradigms in the hippocampus, the influence of direct infusions of norBNI into subregions of hippocampus were tested. The placements of norBNI infusion are shown in Fig. 8. Rats that received bilateral microinjection of norBNI into the dentate gyrus demonstrated a significant improvement in the conditioned avoidance test relative to saline-treated controls (Fig. 9). A dose of 2.5 μg/side was needed to produce a significant antidepressant effect, and the magnitude of the effect was less than observed with imipramine treatment. A dose of 0.25 μg/side did not result in a significant response.

Infusions of norBNI into the CA3 region produced a robust antidepressant effect. Infusion of either dose (0.25 or 2.5 μg/side) produced an effect that was similar in magnitude to the actions of imipramine (Fig. 9). Moreover, microinfusions of norBNI into the CA3 were region-specific. Infusions of norBNI into the stratum radiatum were effective at doses of either 0.25 or 2.5 μg/side. However, infusions of norBNI at the higher dose (2.5 μg/side) into the stratum oriens were not effective. The placements of norBNI infusion are shown in Fig. 8. This is consistent with the selective induction of dynorphin A and dynorphin B in the stratum lucidum but not the stratum oriens by exposure to stress or the depression paradigms.

Microinfusion of norBNI into the NAc, which was previously reported to produce an antidepressant response, was also examined (Newton *et al*. 2002). In addition, the influence of infusions into either the shell or core of the NAc was determined in the present study. The placements of norBNI infusion are shown in Fig. 8. Infusions of norBNI at both the 0.25 and 2.5 μg dose/side into the shell were effective (Fig. 10). Infusions of either the low or high dose of norBNI into the NAc core were also effective although the magnitude of the antidepressant response appeared to be slightly less than observed with infusions into the shell (Fig. 10).

Discussion

In the current study we found that exposure to physical stress (immobilization) or behavioral models of depression increases levels of dynorphin A and dynorphin B in the hippocampus and the NAc. The induction of dynorphin A and dynorphin B immunoreactivity could represent a compensatory adaptation to the stress/depression paradigms, or could contribute to the altered behavioral states produced by uncontrollable stress. To address this question we examined the influence of norBNI, a dynorphin/κ-opioid receptor antagonist, on conditioned responding in the LH model of depression. The results of these behavioral studies demonstrate that the dynorphin antagonist produces an antidepressant-like effect in the LH model that was equivalent to subchronic administration of a chemical antidepressant.

The results of this study demonstrate that all three stress models tested increase the levels of dynorphin immunoreactivity in the hippocampus and NAc. These models differ in the type of stress, as well as the intensity and duration of the stress. Immobilization stress is reported to influence levels of neurotrophic factor expression (Nibuya *et al*. 1995; Smith *et al*. 1995). The forced swim stress is a standard model for screening drugs with potential antidepressant efficacy

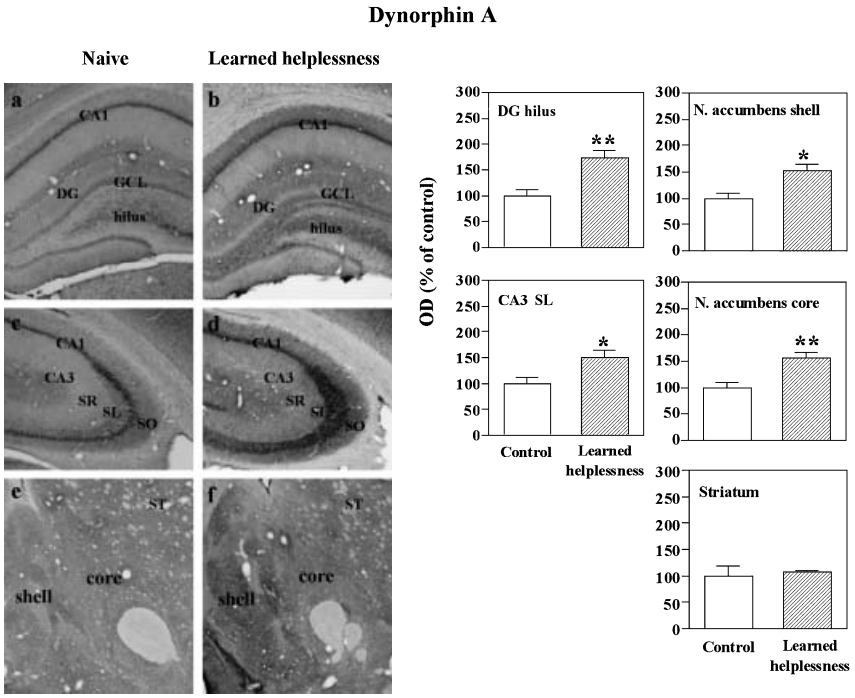

Fig. 4 Learned helplessness increases dynorphin A immunoreactivity in subfields of the hippocampus and striatum. Naïve (a, c, e) and LH (b, d, f) animals. (a and b) The dentate gyrus and CA1 regions of hippocampus; (c and d) the CA3 region of hippocampus; (e and f) the shell and core regions of nucleus accumbens. Shown on the left are representative sections that were subjected to dynorphin A immunohistochemical analysis. On the right are the optical density (OD) readings for the regions indicated. The results are presented as percentage of control and are mean ± SEM ($n = 4$ animals per group). $^*p < 0.05$; $^{**}p < 0.01$ compared to control (Student's *t*-test). DG, dentate gyrus; GCL, granule cell layer; CA1, CA1 pyramidal cell layer; SR, stratum radiatum; SL, stratum lucidum SO, stratum oriens; ST, striatum.

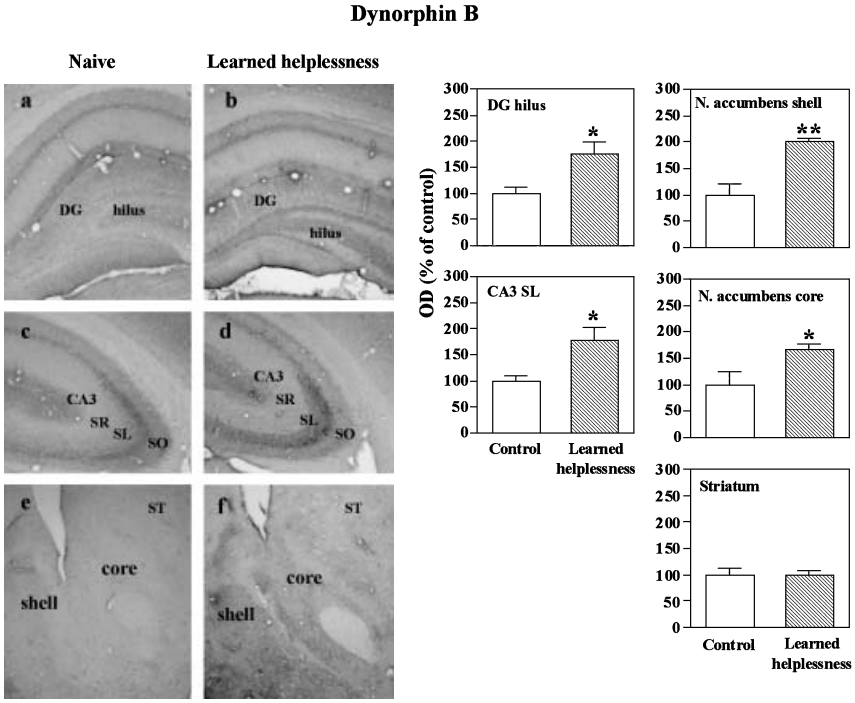

Fig. 5 Learned helplessness increases dynorphin B immunoreactivity in subfields of the hippocampus and striatum. Naïve (a, c, e) and LH (b, d, f) animals. (a and b) The dentate gyrus and CA1 regions of hippocampus; (c and d) the CA3 region of hippocampus; (e and f) the shell and core regions of nucleus accumbens. Shown on the left are representative sections that were subjected to dynorphin B immunohistochemical analysis. On the right are the optical density (OD) readings for the regions indicated. The results are presented as percentage of control and are mean ± SEM ($n = 4$ animals per group). $^*p < 0.05$; $^{**}p < 0.01$ compared to control (Student's *t*-test). DG, dentate gyrus; SR, stratum radiatum; SL, stratum lucidum SO, stratum oriens; ST, striatum.

(Willner 1984; Thiebot *et al*. 1992). LH has also been used for screening antidepressant compounds, and produces a behavioral phenotype that has some validity as a model of depression (Willner 1984; Thiebot *et al*. 1992). All three models resulted in a robust induction of dynorphin A in the stratum lucidum of the CA3 region, the hilus of the dentate gyrus, and immobilizaton and LH stress increased dynorphin A in the core and shell of the NAc. Levels of dynorphin B immunoreactivity were also increased in these same subregions of hippocampus and NAc by immobilization and LH stress. The effects of forced swim stress on dynorphin B were not determined. Neither dynorphin A nor dynorphin B were regulated in the striatum by immobilizaton or LH stress. Taken together, the results demonstrate that induction of

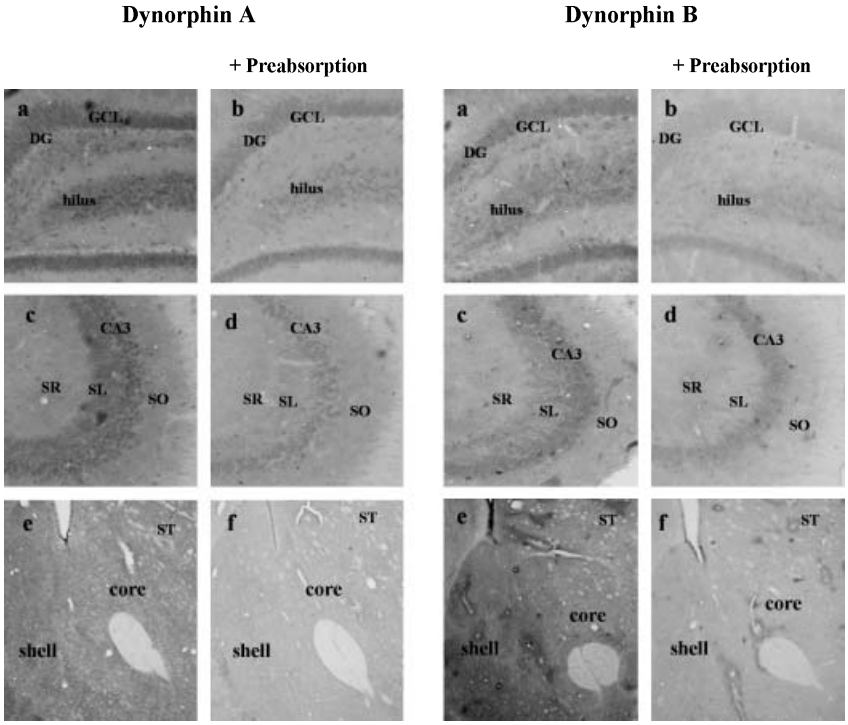

Fig. 6 Pre-absorption blocks dynorphin A and dynorphin B immunoreactivity in subfields of the hippocampus and striatum. LH animals were used. (a and b) The dentate gyrus of hippocampus; (c and d) the CA3 region of hippocampus; (e and f) the shell and core regions of nucleus accumbens. Note that preabsorption blocks dynorphin A and dynorphin B immunoreactivity in the subgranular cell layer of hilus and stratum lucidum. DG, dentate gyrus; GCL, granule cell layer; CA3, CA3 pyramidal cell layer; SR, stratum radiatum; SL, stratum lucidum SO, stratum oriens; ST, striatum.

dynorphin immunoreactivity is an effect that generalizes to different stress stimuli and suggest that this neuropeptide may play a fundamental role in the stress response.

Administration of the κ-opioid antagonist, norBNI, into the dentate gyrus or CA3 region produced an antidepressant response in the LH model. The greater potency and efficacy of the CA3 infusions could be due to the overall higher levels of both dynorphin A and dynorphin B in this subfield of hippocampus. Levels of κ-opioid receptor binding are similar in both regions and do not appear to explain the differential responsiveness to norBNI (Zukin *et al*. 1988; Fan *et al*. 2002). There was also a difference in the actions of norBNI infusions into subfields of the CA3 region, with antidepressant effects observed upon infusions into the stratum radiatum, but not stratum oriens outside the pyramidal cell layer. This is consistent with the subregion induction of dynorphin in the stratum lucidum, but not stratum oriens. In addition, levels of κ-opioid receptors, both κ1 and κ2, are higher in the stratum radiatum than in the stratum oriens (Zukin *et al*. 1988; Fan *et al*. 2002). This subfield specificity could also be explained by the differential inputs from the mossy fiber pathway. The primary target of mature mossy fiber projections is the stratum lucidum that is located near the stratum radiatum, while mossy fiber synaptogenesis is induced by spatial memory or maternal separation in the stratum oriens (Ramirez-Amaya *et al*. 2001; Huot *et al*. 2002). This suggests the involvement of the more mature mossy fiber projections in the induction of dynorphin by stress and in behavioral responses in the LH paradigm. Although the doses of norBNI used in the current study are lower than previous intracerebral (Wall and Messier 2002) or intracerebralventricular (Spanagel and Shippenberg 1993; Pliakas *et al*. 2001; Mague *et al*. 2003) studies, the pharmacological specificity of norBNI has not been confirmed. Future studies with more selective compounds will be required to address this issue.

The mechanisms underlying the actions of dynorphin in responses to stress and models of depression are not known. One possibility is that stress and LH activate hippocampal signaling and increased dynorphin could alter the synaptic responsiveness. Dynorphin is co-localized with glutamate the primary neurotransmitter in granule cells, and synaptic release of dynorphin is reported to cause pre-synaptic inhibition of glutamate release from the mossy fiber and perforant pathway terminals (see refs in Terman *et al*. 2000). These studies demonstrate that dynorphin exerts a potent inhibitory control over glutamate release in the hippocampus, which is demonstrated at a functional level by blockade of mossy fiber or perforant path long-term potentiation (LTP; Weisskopf *et al*. 1993; Terman *et al*. 2000).

Increased levels of dynorphin resulting from stress could block glutamate-mediated functional plasticity of the hippocampal formation, and dynorphin antagonism with norBNI could restore this response. Spatial memory tasks activate hippocampal subfields and plasticity of the mossy fiber projections is involved in and required for the storage of spatial representations (McClelland and Goddard 1996; Vann *et al*. 2000; Ramirez-Amaya *et al*. 2001). Taken together, the

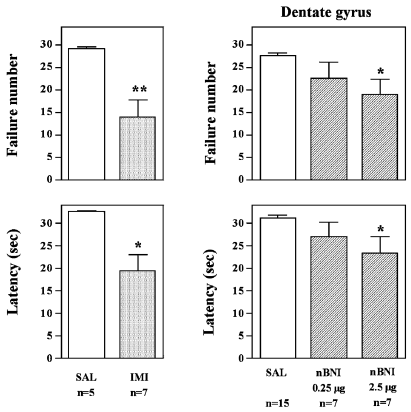

Fig. 7 Infusion of norBNI into the dentate gyrus of hippocampus decreases escape failure in the LH paradigm. NorBNI or saline (SAL) were administered via bilateral infusion into the dentate gyrus, and a conditioned avoidance test was performed 3 days later. Escape failure number and latency to escape were determined. Shown on the left are the results of imipramine administration (7 days) for comparision. The results are expressed as mean ± SEM. Left top, $F(1, 12) = 3.299$, $p = 0.0080$; left bottom, $F(1,12) = 3.018$, $p = 0.0129$; right top, $F(2, 26) = 4.220$, $p = 0.0259$; right bottom, $F(2, 26) = 3.952$, $p = 0.0317$. $^{*}p < 0.05$; $^{**}p < 0.01$ when compared with saline-injected controls (Student's *t*-test for impramine study or ANOVA followed by Scheffe's test for norBNI study).

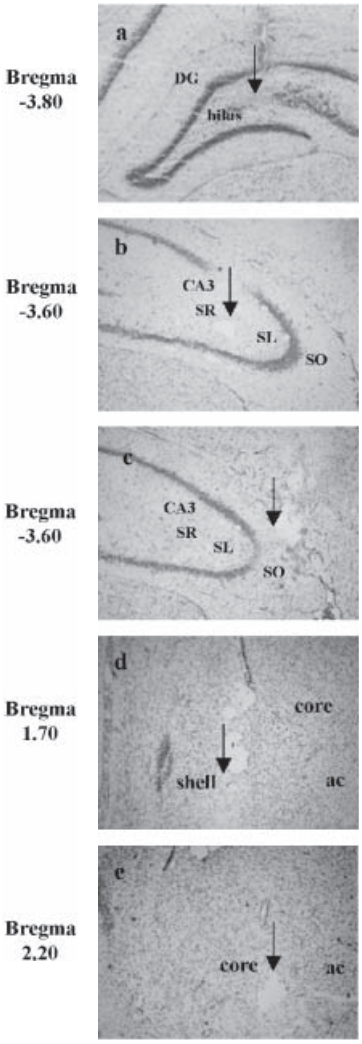

Fig. 8 Location of microinjection sites. (a) is the dentate gyrus of hippocampus; (b and c) the CA3 region of hippocampus; (d and e) the shell and core regions of nucleus accumbens. DG, dentate gyrus; SR, stratum radiatum; SL, stratum lucidum SO, stratum oriens; ac, anterior commissure.

results provide a model whereby the induction of dynorphin by stress could block the plasticity of hippocampal pathways that is required for spatial learning and that could underlie the behavioral deficit observed in the LH model. Evidence for stress-induced dysfunction of hippocampal plasticity is provided by both pre-clinical and clinical studies. Inescapable shock or chronic unpredictable stress has been found to impair LTP in the CA1 pyramidal cell layer and the dentate gyrus granule cell layer (Shors *et al*. 1989; Alfarez *et al*. 2003). Depressed patients were found to perform significantly worse than controls in learning and memory tasks (Riedel *et al*. 2002).

We have previously found that micoinfusions of brain-derived neurotrophin factor (BDNF) into the hippocampus produce a similar effect to blockade of κ-opioid receptors (Shirayama *et al*. 2002). Previous studies have provided evidence of a link between BDNF and dynorphin. Infusions of BDNF are reported to decrease levels of dynorphin (Croll *et al*. 1994), raising the possibility that the actions of BDNF could be accounted for by downregulation of dynorphin. This is also consistent with the reports that stress decreases BDNF, which could result in increased dynorphin, and that antidepressant treatment upregulates the expression of this neurotrophic factor in hippocampus (Nibuya *et al*. 1995; Smith *et al*. 1995; see Duman *et al*. 2000 for review).

GABAergic projection neurons in the NAc receive inputs from ventral tegmental dopamine neurons that express dynorphin. Dynorphin serves a negative feedback mechanism by acting on pre-synaptic κ-opioid receptors to inhibit dopamine neuronal function. There is evidence that the dopamine system is involved in depression and the action of antidepressant treatment. Chronic antidepressant treatment enhances amphetamine-induced increases in dopamine in the NAc (Nomikos *et al*. 1991). In addition, antidepressant administration decreases the number of D-1 dopamine receptors in the limbic system (Klimek and Nielsen 1987; De Montis *et al*. 1990). The NAc dopamine system plays a major role in motivation and reward and could contribute to the anhedonia (decreased pleasure and reward) and decreased motivation that is observed in depressed patients (see Nestler *et al*. 2002). Activation of D-1 dopamine and NMDA receptors within this structure governs the cellular processes that occur with new learning (Smith-Roe and Kelley 2000).

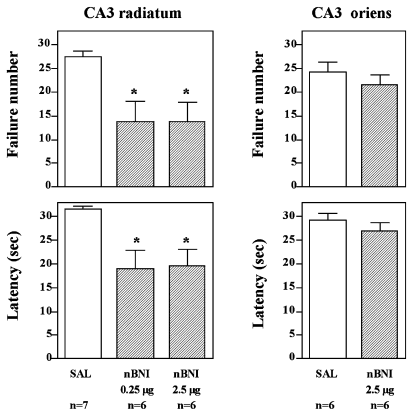

Fig. 9 Infusions of norBNI into the CA3 radiatum decrease escape failures in the LH paradigm. NorBNI or saline (SAL) were administered via bilateral infusion into and CA3 stratum radiatum or stratum oriens, and conditioned avoidance test was performed 3 days later. Escape failure and latency to escape were determined. The results are expressed as mean ± SEM. Left top, F (2, 16) = 6.410, $p = 0.0090$; left bottom, F (2, 16) = 6.463, $p = 0.0088$; right top, F (1, 10) = 0.910, $p = 0.3842$; right bottom, F (1, 10) = 1.102, $p = 0.2964$. $^*p < 0.05$ when compared with saline-injected controls (Student's *t*-test for CA3 oriens or ANOVA followed by Scheffe's test for CA3 radiatum).

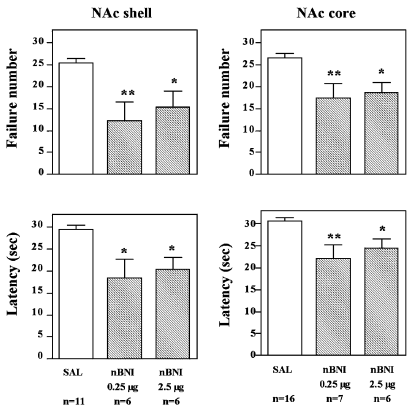

Fig. 10 Infusions of norBNI into the NAc decrease escape failures in the LH paradigm. NorBNI or saline (SAL) were administered via bilateral infusion into the shell or core revigions of the NAc, and a conditioned avoidance test was performed 3 days later. Escape failure and latency to escape were determined. The results are expressed as the mean ± SEM. Left top, F (2, 20) = 7.511, $p = 0.0037$; left bottom, F c(2, 20) = 7.117, $p = 0.0046$; right top, F (2, 26) = 8.186, $p = 0.0017$; right bottom, F (2, 26) = 8.577, $p = 0.0014$. $^*p < 0.05$; $^{**}p < 0.01$ when compared with saline-injected controls (ANOVA followed by Scheffe's test).

The NAc also receives a glutamatergic projection from the hippocampus via the ventral subiculum (Groenewegen *et al.* 1987; Taepavarapruk *et al.* 2000). A recent study reports that glutamate infusions into the NAc shell produce a pro-depressive effect in the forced swim test (i.e. decreased swimming time; Rada *et al.* 2003). However, the relationship between the glutamate and dynorphin systems in the NAc remains unknown.

Rewarding as well as stressful stimuli are reported to increase cAMP response element binding protein (CREB) function in the NAc, and viral expression of CREB can alter responding to anxiogenic, aversive, and nociceptive stimuli (Pliakas *et al.* 2001; Barrot *et al.* 2002). These findings have led to the hypothesis that CREB in the NAc acts as an emotional-gating sensor (Barrot *et al.* 2002). In addition, induction of CREB-mediated transcription in the NAc is reported to produce pro-depressive effects in this brain region (Pliakas *et al.* 2001; Newton *et al.* 2002). One of the gene targets of CREB in the NAc is prodynorphin (Cole *et al.* 1995). The activation of CREB and increased expression of dynorphin seen after stress or drug exposure may contribute to symptoms of emotional numbing or anhedonia (see Nestler *et al.* 2002). κ-Opioid receptor activation has been suggested to play a role in mediating aversion via the mesolimbic dopaminergic pathways (Herz 1997). In addition, it has been suggested that limbic dynorphin systems contribute to cocaine-related dysphoria through activation of the κ-opioid receptor (Spanagel *et al.* 1992; see Carlezon *et al.* 1998). Taken together, these findings suggest that there may be a role for the NAc dynorphin and dopamine systems in certain symptoms of depression.

The NAc receives projections from the basolateral amygdala, ventral pallidum, and the A9, A10 areas. Subfield specific inputs include projections from the subthalamic nucleus to the shell and projections from the bed nucleus of the stria terminals, lateral hypothalamus and medial amygdala to the core (Pulvirenti *et al.* 1994). Dopamine transmission in the NAc shell and core responds differentially to addictive drugs. Cocaine, amphetamine, phencyclidine and morphine preferentially increase extracellular dopamine in the shell (Pontieri *et al.* 1995, 1996; Marcus *et al.* 2001). A recent study reports that the response of NAc shell dopamine undergoes long-lasting habituation whereas no habituation was observed in the NAc core, demonstrating differential expression of motivational stimulus properties in the NAc shell (Bassareo *et al.* 2002). Furthermore, another recent study reports that infusion of a protein synthesis inhibitor into the NAc core, but not the NAc shell, disrupts consolidation of instrumental learning (Hernandez *et al.* 2002). Similarly, the rate of acquisition of instrumental conditioning is decreased in core-lesioned animals (Corbit *et al.* 2001).

These studies suggest that both shell and core could influence responses in the LH model but via different mechanisms. Infusions of the κ-opioid receptor antagonist into the shell could act by enhancing dopamine transmission and reducing the aversive effects of the inescapable stress, resulting in increased response in the active avoidance test. Infusions of the κ-opioid antagonist into the NAc core could

enhance dopamine transmission that is required for instrumental learning and thereby increase responding in the active avoidance test.

Depression is a complex mood disorder that most likely involves several different limbic structures, including the prefrontal cortex and amygdala, as well as the hippocampus and NAc. Some of the primary depressive symptoms, including reduced motivation, inability to experience pleasure and reward, and decreased cognition and learning, could result from alterations of the hippocampus and NAc. The results of the present study demonstrate that antagonism of dynorphin/κ-opioid receptors can produce an antidepressant response, consistent with previous studies (Pliakas *et al*. 2001; Newton *et al*. 2002; Mague *et al*. 2003). In addition, the results raise the possibility that increased dynorphin in subfields of the hippocampus and NAc contribute to the effects of stress that produce a helpless phenotype in the LH model of depression. Additional studies will be required to test this hypothesis further and to determine if the hippocampus or NAc is more critical to the pathophysiology and treatment of depression in animal models, and ultimately to test the therapeutic efficacy of κ-opioid receptor antagonists in clinical trials.

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