Regulation of anxiety and initiation of sexual behavior by CREB in the nucleus accumbens


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Sexual deficits and other behavioral disturbances such as anxiety-like behaviors can be observed in animals that have undergone social isolation, especially in species having important social interactions. Using a model of protracted social isolation in adult rats, we observed increased anxiety-like behavior and deficits in both the latency to initiate sexual behavior and the latency to ejaculate. We show, using transgenic cAMP response element (CRE)-LaCZ reporter mice, that protracted social isolation also reduces CRE-dependent transcription within the nucleus accumbens. This decrease in CRE-dependent transcription can be mimicked in nonisolated animals by local viral gene transfer of a dominant negative mutant of CRE-binding protein (CREB). We previously showed that this manipulation increases anxiety-like behavior. We show here that it also impairs initiation of sexual behavior in nonisolated animals, a deficit that can be corrected by anxiolytic drug treatment. This local reduction in CREB activity, however, has no influence on ejaculation parameters. Reciprocally, we used the viral transgenic approach to overexpress CREB in the nucleus accumbens of isolated animals. We showed that this local increase in CREB activity completely rescued the anxiety phenotype of the isolated animals, as well as their deficit in initiating sexual behavior, but failed to rescue the deficit in ejaculation. Our data suggest a role for the nucleus accumbens in anxiety responses and in specific aspects of sexual behavior. The results also provide insight into the molecular mechanisms by which social interactions affect brain plasticity and behavior.

Within the CNS, changes in the activity of the transcription factor cAMP response element (CRE)-binding protein, CREB (1), have been related to many adaptive processes, such as learning and memory (2–7), antidepressants effects (8–11), and drug addiction (12–23). Such changes have been identified in several discrete brain areas, among them the nucleus accumbens, a forebrain structure critical for reward and motivation (24–29). Exposure to several forms of stress (18–20) or to drugs of abuse, either psychostimulants (21) or opiates (19, 20), increases CRE-dependent transcription in the nucleus accumbens. The use of virus-mediated gene transfer to manipulate CREB activity specifically within this brain region demonstrated that local CREB overexpression reduces the rewarding effects of psychostimulants and opiates (14, 18, 19), which indicates that activation of this transcription factor may counteract adaptations that intensify drug reward. Our findings with stress responses suggested that, more generally, increased CREB activity in the nucleus accumbens reduces an animal’s behavioral responsiveness to a wide range of emotional stimuli, whether rewarding, anxiogenic, or nociceptive (19).

Although a causal link has not yet been established with certainty, experimental data strongly suggest that not only the activation of CREB but also its inhibition could be instrumental in some adaptive mechanisms. For example, chronic alcohol or nicotine treatment can decrease levels of phosphorylated CREB, the active form of the protein, in the nucleus accumbens (17, 22, 30). Moreover, virus-mediated expression of a dominant negative mutant of CREB revealed that local inhibition of CREB activity in the nucleus accumbens potentiates behavioral responses to emotional stimuli, such as the rewarding effects of drugs of abuse (14, 19), but also increases anxiety-like behavior (19). Transgenic mice deficient in CREB expression in the CNS also exhibit an increased-anxiety phenotype (31).

The nucleus accumbens has been proposed to be a key area not only for responses to drugs of abuse but also for the behavioral response to natural rewards (24–27), such as food and water intake or sexual behavior. In the present study, we determined how local changes of CREB activity in the nucleus accumbens might affect sexual behavior in both naive and sexually experienced males. Microinjections of a herpes simplex virus (HSV) vector allowed us to locally overexpress either CREB itself or the dominant negative mutant mCREB, in which mutation of Ser-133 to Ala prevents its own activation and renders it an inhibitor of endogenous CREB and CRE-dependent transcription (14, 18, 19, 32). We studied the sexual behavior of male rats after the local manipulation of CREB activity in nonisolated animals. We then linked the functional changes we observed to the consequences of an identified physiologic situation, protracted social isolation. Chronic single housing in adulthood, which is associated with deprivation of environmental stimulation, decreases CRE-dependent transcription in the nucleus accumbens, increases anxiety-like behavior, and induces sexual behavior deficits. Last, we show that these anxiety-like behavioral deficits associated with social isolation can be rescued by restoring CREB activity in the nucleus accumbens of isolated animals.

Materials and Methods

Procedures were approved by the Institutional Animal Care and Use Committee of the University of Texas Southwestern Medical Center at Dallas.

Viruses-Mediated Gene Transfer. Surgery was performed on male Sprague–Dawley rats (Charles River Breeding Laboratories) (14, 18, 19). HSV vectors were injected bilaterally (1.5 μl per side) over 7.5 min, into the nucleus accumbens shell (relative to bregma: rat, anterior–posterior = +1.9, lateral = +2.4, dorsal–ventral = −6.7 mm below dura, with a 10° lateral angle). At the

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2Abbreviations: CRE, cAMP response element; CREB, CRE-binding protein; HSV, herpes simplex virus; mCREB, mutant CREB.

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of the two closed arms were 40 cm tall. Testing was carried out open and closed arms of an elevated plus-maze (55 cm from regards to housing conditions or viral injection conditions. We tested the rats for the time spent in the open arms of an elevated plus-maze (120 ± 19 sec vs. 67 ± 14 sec in controls, P < 0.05).

**CRE-LacZ Activity in Reporter Mice.** We used a line of CRE-LacZ mice, wherein β-gal is expressed under the control of CREs (19, 35). The construct, which contains seven CRE consensus sequences in tandem upstream of a minimal somatostatin promoter and the lacZ gene, is flanked by 5′ insulator sequences (35). Adult male mice were either group housed or single housed for 10–12 wk. Perfusion of the animals, brain sectioning (40-μm sections), and immunostaining were performed by using published procedures (19–21). β-gal immunostaining was carried out by using a goat anti-β-gal Ab (1:5,000, Biogenesis, Brentwood, NH), a biotinylated horse anti-goat secondary Ab, and the biotin-streptavidin technique (ABC kit; Vector Laboratories) with 3,3′-diaminobenzidine as chromogen. Density of positive nuclei was determined bilaterally in the nucleus accumbens shell over three sections separated by 240 μm for each animal. The positive cells were counted blindly in regards to the housing conditions of the animals.

**Plasma Corticosterone.** Tail blood samples were obtained at 7:30 a.m. from rats that were single- or double-housed in the room with the shifted light/dark cycle. Each blood sample was collected within 4 min from the time the cage was taken from the animal room. Blood was collected in ice-cold heparin-coated tubes and centrifuged (1,000 × g for 15 min at 4°C). Aliquots of plasma were stored at −20°C until assayed. Plasma corticosterone levels were determined by competitive enzyme immunoassay according to manufacturer’s specifications (ALPCO Diagnostics, Windham, NH) (33).

**Statistics.** Two group comparisons were performed by t test. Multiple group comparisons on experiments using HSV vectors were accomplished by ANOVA followed by a Duncan posthoc analysis.

**Results**

**Manipulation of CREB and Sexual Behavior.** The inhibition of CREB activity by local expression of mCREB profoundly disrupted the initiation of sexual behavior in naïve males, measured either as latency to the first mount (Fig. 1C) or as latency to the first intromission (Fig. 1D). As a direct consequence of this delayed initiation of the behavior, only 18% of the animals with reduced CREB activity in the nucleus accumbens completed a copulatory series within 15 min after their first contact with a receptive female, whereas 68% of control males achieved this goal. This deficit, however, specifically concerned the initiation of the first copulatory series. Neither the total number of mounts necessary to reach ejaculation (Fig. 1F), the latency to ejaculation (Fig. 1E), nor the latency to initiate new copulatory series after completion of the first one (Fig. 1C) was affected by inhibition of CREB activity in the nucleus accumbens.

**Effect of Anxiolytics or Sexual Experience.** Injection of 0.75 mg/kg diazepam 20 min before testing reversed the deficit in initiating sexual behavior in naïve animals with decreased CREB activity in the nucleus accumbens (Fig. 2). This same dose of diazepam had no effect in control animals and did not significantly alter the latencies to initiate a second copulatory series in either control or mCREB-treated animals (Fig. 2). In a separate experiment, males were trained three times for sexual behavior in the week before the viral injection (either HSV-mCREB or
shell (microinjection of HSV-mCREB, but not HSV-CREB, into the nucleus accumbens of receptive female, as measured by latency to the first mount, is impaired after left immunohistochemistry in nucleus accumbens shell after HSV-LacZ (bilateral cannula placement for nucleus accumbens shell injections. (Barrot et al.)

Diazepam reversal of HSV-mCREB phenotype. (Fig. 2.)

Fig. 1. CREB and sexual behavior in double-housed males. (A) Schematic of bilateral cannula placement for nucleus accumbens shell injections. (B) CREB immunohistochemistry in nucleus accumbens shell after HSV-LacZ (Left) or HSV-CREB (Right) injections. (C) The initiation of sexual behavior with a receptive female, as measured by latency to the first mount, is impaired after microinjection of HSV-mCREB, but not HSV-CREB, into the nucleus accumbens shell (F3,44 = 2.83, P < 0.05; *, P < 0.04 against other three groups). The initiation of subsequent copulatory series is not affected by CREB manipulation. (D) Similar results are observed when considering the latency to intromission (F3,44 = 3.92, P < 0.015; * P < 0.015 against other three groups). (E) The delay between the first intromission and the first ejaculation is not affected by CREB manipulation. (F) The total number of mounts necessary to reach ejaculation is not affected by CREB manipulation.

HSV-LacZ). This training procedure also prevented the deficit in initiating sexual behavior normally observed after expression of mCREB (data not shown, HSV-mCREB, n = 9, vs. HSV-LacZ, n = 12; mount latency: P > 0.25; intromission latency: P > 0.75).

CREB Overexpression in Isolated Animals. Using injections of HSV-CREB, we increased CRE-dependent transcription in the nucleus accumbens of sexually naive isolated animals and tested whether this would rescue their behavioral phenotype. We previously showed that overexpression of WT CREB within nucleus accumbens shell increases local CRE-dependent transcription (19). HSV-CREB injection in the nucleus accumbens of isolated animals corrected the anxiety phenotype of the animals in the elevated plus-maze (Fig. 4E) as well as their deficit in the latency to initiate

Social Isolation and CREB-Dependent Transcription. We next studied whether protracted social isolation alters CREB activity in the nucleus accumbens. Indeed, the density of β-gal-positive cells in the CRE-LacZ mice was reduced by 34% in the shell part of the nucleus accumbens after 10–12 wk of social isolation (Fig. 3).
In which CREB inhibition induced a deficit in sexual behavior, morphine (19), and even sucrose (19). Thus, the present results, brain region increases the rewarding effects of cocaine (14, 18), behavior. Previous work has shown that CREB inhibition in this first copulatory series, without affecting ejaculation parameters. These deficits were the result of the local decrease in CRE-dependent transcription (see Fig. 3). However, despite the reversal of these two phenotypes, the increased latency to first ejaculation was still present (Fig. 4H), which suggests that this latter deficit is mediated by a different mechanism.

Discussion

We show in the present study that inhibition of the transcription factor CREB in the nucleus accumbens leads to a deficit in initiating sexual behavior that is associated with an anxiety-like phenotype. Our data also show that a decrease in CRE-mediated transcription in the nucleus accumbens can physiologically result from protracted social isolation, a condition that also causes deficits in initiating sexual behavior and increased anxiety-like behavior. Finally, we show that these anxiety phenotypes in isolated animals can be reversed by experimentally restoring CREB activity in the nucleus accumbens.

Using virus-mediated gene transfer (36, 37), we found that CREB overexpression in the nucleus accumbens shell had no significant effect on male sexual behavior, whereas CREB inhibition profoundly disrupted the initiation of sexual behavior in naive animals. This latter effect involved only the initiation of the first copulatory series, without affecting ejaculation parameters or the initiation of subsequent copulatory series. These findings suggest that a local decrease in CREB activity in the nucleus accumbens affects only a specific aspect of sexual behavior. Previous work has shown that CREB inhibition in this brain region increases the rewarding effects of cocaine (14, 18), morphine (19), and even sucrose (19). Thus, the present results, in which CREB inhibition induced a deficit in sexual behavior, are unlikely to be due to reduced rewarding aspects of sex per se.

Because we previously showed that decreased CREB activity in the nucleus accumbens can also increase anxiety-like behavior (19), we treated sexually naive HSV-mCREB animals with the anxiolytic drug diazepam and found that it reversed the deficit in initiating sexual behavior without significantly affecting control animals or other parameters of sexual behavior. This finding suggests that the deficit in initiating sexual behavior is likely to be a consequence of increased anxiety in animals with decreased CREB activity in the nucleus accumbens shell. The lack of effect of CREB overexpression per se, and the absence of effect of diazepam in control animals, reflect the likelihood that our experimental procedure (test within the animal room and under red light conditions) is nonanxiogenic for normal animals.

Control of anxiety is not viewed as a main function of the nucleus accumbens. However, our previous results (19) and the present data show that this brain area can exert significant influence on anxiety-related behaviors. Moreover, a recent study of deep brain stimulation in human patients with severe anxiety disorders and obsessive-compulsive disorders showed significant reduction in severity of symptoms by targeting the shell of the nucleus accumbens (38). These results probably relate to the key position of the nucleus accumbens shell within specific brain circuits. The nucleus accumbens shell receives massive glutamatergic inputs from limbic areas such as prefrontal cortex, ventral subiculum of the hippocampus, and basolateral amygdala (39), all of which have been associated with processing stressful or anxiogenic stimuli (40–42). The nucleus accumbens shell also receives major inputs from several midline thalamic nuclei, including paraventricular inputs rich in neuropeptide Y, α-melanocyte-stimulating hormone, and catecholamines, which could also provide stress-related information (43). In addition, the shell subregion receives inputs from multiple other brain areas (39) that have been shown to influence stress and anxiety-like responses (44–46), such as lateral septum, lateral habenula, extended amygdala (which includes the bed nucleus of the stria terminalis), lateral hypothalamus, and monoaminergic nuclei such as ventral tegmental area, dorsal raphe, and locus coeruleus. The fact that the nucleus accumbens integrates information from all of the above structures and is an interface to action (24) probably explains its potential role in the behavioral expression of stress and anxiety.

A specific feature of the anxiety-like phenotype associated with initiation of sexual behavior must be noted: We observed the phenotype only in animals naive for sexual behavior. Once a first copulatory series has been completed, or if the animal was previously trained for sexual behavior, the deficit in again initiating the behavior disappeared. The possibility that the nucleus accumbens shell might be specifically involved in processing emotional information with a novelty component has been raised by other studies. During novelty exposure, there is

**Fig. 4.** Influence of social isolation on sexual behavior and anxiety. (A) Isolated animals spend less time in the open arms of an elevated plus-maze (*, P < 0.015). DH, double-housed controls; SH, animals isolated for 10–12 wk. (B–D) Both latency to initiate sexual behavior (*, P < 0.035) (B and C) and latency to ejaculate after intromission (*, P < 0.025) (D) are delayed in isolated animals. (E) In isolated animals, the deficit in the time spent in open arms of an elevated plus-maze is corrected by local microinjection of HSV-CREB. (F–H) In isolated animals, the microinjection of HSV-CREB into the nucleus accumbens shell corrects the increased latency to initiate sexual behavior (*, P < 0.04) (F and G), but it does not correct the increased latency to ejaculate (H).
a transient increase in dopaminergic activity in the nucleus accumbens shell (47, 48). We previously showed that the simple fact of manipulating and injecting an animal induces dopamine and Fos responses in the nucleus accumbens shell, whereas the same manipulation repeated 2 hr later has no effect (49). Similarly, an unfamiliar appetitive stimulus such as sweet chocolate taste induces a dopamine response in the nucleus accumbens shell that habituates after a single preexposure (50). Our present results might indicate that CREB inhibition in the nucleus accumbens shell affects reactive anxiety only in response to novelty, rather than inducing a constitutive anxiety state. It is, however, important to raise the alternative possibility that sexual experience in itself could be anxiolytic and thereby modify subsequent behavior. Further experiments are needed to understand the precise role the nucleus accumbens might play in anxiety and novelty responses.

CREB activity in the nucleus accumbens appears to be controlled by environmental information. In the present study, we used transgenic mice with a CRE-LacZ reporter gene (19, 35) to show that protracted social isolation reduces CREB-dependent transcription in the shell of the nucleus accumbens. Many stimuli have been previously shown to increase CREB phosphorylation or activity within this brain area. This is the case with drugs of abuse such as cocaine, amphetamine (12, 21), or opiates (19, 20).

Different physical stressors, such as forced-swim (18), foot-shock, restraint stress, social stress, or repeated unpredictable stress (19) also increase CREB activity in the nucleus accumbens shell. The fact that social isolation decreases CREB activation, the opposite of what is found with several active stressors (e.g., foot-shock and swim stress) is probably due to the nature of the stimulus. Indeed, the nucleus accumbens integrates sensory and limbic inputs, making social isolation likely to be processed differently from active stressors because social isolation involves the removal of environmental sensory stimuli instead of the addition or imposing of external stimuli. The difference between social isolation and other forms of stress is also reinforced by the lack of effect of our isolation procedure on corticosterone levels. This later finding is in marked contrast to the active stressors, all of which increase circulating corticosterone levels. Interestingly, decreased levels of phosphorylated CREB have also been observed in the nucleus accumbens after chronic treatment with alcohol (17) or nicotine (22, 30). Decreased CREB activity in the amygdala has been proposed to contribute to the anxiety resulting from alcohol withdrawal (51).

Our results raise the possibility that CREB activation, as measured with a CRE-LacZ reporter gene, is decreased in the nucleus accumbens following social isolation. These findings are consistent with previous studies showing that anxiety (in particular anxiety to novelty), and related deficits can also follow isolation in adult animals (54, 55). Here, we focused on corticosterone levels. This later finding is in marked contrast to the active stressors that increase corticosterone levels. Interestingly, decreased levels of phosphorylated CREB have also been observed in the nucleus accumbens after chronic treatment with alcohol (17) or nicotine (22, 30). Decreased CREB activity in the amygdala has been proposed to contribute to the anxiety resulting from alcohol withdrawal (51).

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In conclusion, our findings show that decreased CREB-mediated transcription within the nucleus accumbens is one of the possible mechanisms from which key behavioral consequences of protracted social isolation may result. Our data reveal that anxiety (in particular anxiety to novelty), and related deficits in initiating sexual behavior, could be associated with a decrease in CREB activity. These findings are consistent with previous studies showing that anxiety (in particular anxiety to novelty), and related deficits can also follow isolation in adult animals (54, 55).

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