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Cholera toxin B decreases bicuculline seizures in prenatally morphine- and saline-exposed male rats

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Abstract

Prenatal morphine exposure on gestation days 11–18 alters bicuculline-induced seizures in rats during development and in adulthood. Adult, morphine-exposed male progeny exhibit an increased latency to bicuculline seizures, which can be reversed by administration of the opioid receptor antagonist naloxone. In chronically morphine-treated adult mice, cholera toxin B (CTX-B) can reverse the effects of chronic morphine administration. Therefore, the present study investigated whether prenatally morphine-exposed rats show a similar response to CTX-B as chronically morphine-treated adult rodents. Prenatally morphine-, saline- and unexposed male progeny were tested for seizure susceptibility with a 7.5-mg/kg intraperitoneal injection of bicuculline in adulthood. CTX-B or saline was injected subcutaneously at 24, 12, and 0.5 h before bicuculline injection. CTX-B decreased the occurrence of bicuculline-induced seizures in both prenatally saline- and morphine-exposed but not unexposed rats. Furthermore, three, but not one, saline injections administered at 12-h intervals prior to bicuculline administration reversed the increase in seizure latency in prenatally morphine-exposed adult males, suggesting an altered responsiveness of the stress system. The present study demonstrates that CTX-B can decrease the occurrence of bicuculline seizures in prenatally stressed rats and that increased seizure latencies in prenatally morphine-exposed male rats may be related to stress responses.

Keywords: Epilepsy; Convulsions; Prenatal drug exposure; Opiates; GM1 gangliosides; Stressor

1. Introduction

Prenatal opiate exposure has many adverse effects on human offspring (Johnson, 1982; Wilson et al., 1973, 1979), and animal models have been useful in uncovering the alterations in brain neurochemistry that may be involved in these opiate-induced changes (Vathy, 1995). Previous studies in our laboratory demonstrated that exposure of rats to morphine during mid to late gestation (Days 11–18) alters seizure latencies in adult male progeny in a seizure model- and hormone-dependent manner (Schindler et al., 2001; Vathy, 2001). Prenatal morphine exposure decreases the latency to onset of kainic acid (KA) and *N*-methyl-D-aspartic acid (NMDA) seizures (Slamberova et al., 2000), and increases the latency to onset of flurothyl (Vathy et al., 1998) and bicuculline (Schindler et al., 2001) seizures in adult male rats. Opioid receptors are appearing during the prenatal period in which the animals are exposed to morphine in all of our experiments (Kent et al., 1981; Kornblum et al., 1989; Rius et al., 1991). Because prenatal drug exposure is believed to affect the neural systems that are developing at the time of drug exposure (Kellogg, 1992), it is likely that prenatal morphine exposure alters the endogenous opioid system. In fact, we (Rimanoczy et al., 2001; Rimanoczy and Vathy, 1995; Slamberova et al., 2002a,b; Vathy et al., 2000, 2003) and others (Tsang and Ng, 1980; Zadina et al., 1985) demonstrated that prenatal morphine exposure alters μ-, δ-, and κ-opioid receptor characteristics in adult rats in a brain-region-specific manner. Furthermore, we recently showed (Schindler et al., submitted for publication) that the increased latency to bicuculline seizure onset

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is reversed by systemic administration of the opioid receptor antagonist naloxone.

The effect of morphine and naloxone on seizure latency is related to their dose (Calder et al., 1982; Gilbert and Martin, 1975). This is interesting in light of data showing that opioids have a bimodal effect; low doses activate stimulatory guanine nucleotide binding proteins (Gs) (Crain and Shen, 1990), while higher doses activate inhibitory guanine nucleotide binding proteins (Gi and Go) (Childers, 1991). Cholera toxin B (CTX-B) binds selectively to cell surface GM1 gangliosides, which are sialic-acid-containing glycolipids that are abundantly distributed on the surface of all neurons. CTX-B interferes with a putative allosteric GM1 regulatory site on excitatory opioid receptors (Crain and Shen, 1998; Shen and Crain, 1990) and is believed to block the excitatory mode (Gs-coupled) of opioid receptors and thereby prevent tolerance to morphine both in vivo and in vitro (Shen and Crain, 2001). CTX-B can also prevent naloxone-precipitated withdrawal hyperalgesia (Shen and Crain, 2001), which is considered a reliable index of the magnitude of physical dependence after drug withdrawal (Martin et al., 1963; Tilson et al., 1973).

In our model of prenatal morphine exposure, drug administration is discontinued 3 days before birth, thereby exposing the rat pups to withdrawal in utero and perhaps ex utero. Therefore, prenatal morphine exposure might be a model of protracted opiate withdrawal, where opiates act primarily through the Gs instead of the Gi–Go pathway (Crain and Shen, 1992, 1995). Increases in the Gs-coupled action of opioid receptors in brain regions such as the hippocampus, where opioids have disinhibitory actions (Simmons and Chavkin, 1996), would increase opioid inhibition of the inhibitory γ-aminobutyric acid (GABA) interneurons and subsequently lessen susceptibility (decrease incidence or increase time to onset) to seizures in prenatally morphine-exposed animals. Thus, we hypothesized that pretreatment with CTX-B would bring the increased seizure latencies of prenatally morphine-exposed animals down to the level of controls. Therefore, Experiment 1 of the present study investigates changes in prenatally morphine-exposed rats by examining the effects of CTX-B administration on their altered bicuculline seizure latencies.

Seizure onset can also be altered by exposure to stress (Abel and Berman, 1993; Pericic et al., 2001). Even a mild stress associated with handling and/or injection of rats can alter the performance of rats in tasks that are known to be sensitive to stress (Drago et al., 2001). Prenatally morphine-exposed animals have altered behavioral responses to stressors (Castellano and Ammassari-Teule, 1984; Slamberova et al., 2002a,b). To investigate how stress alters the onset of bicuculline seizures in prenatally morphine- and saline-exposed adult male rats, Experiment 2 tested the effect of one or three injections of saline on the latency and incidence of bicuculline seizures.

2. Materials and methods

2.1. Animals

Eight-day pregnant Sprague–Dawley rats from Taconic Farm (Germantown, NY) were maintained in a temperature-controlled colony room with free access to food and water on a reversed 14-h (light):10-h (dark) cycle with lights off at 1100 h. Pregnant rats were randomly assigned to an experimental morphine-treated, control saline-treated, or control untreated group. Both morphine and saline injections were administered subcutaneously twice daily (0800 and 2000 h) on gestational days 11–18 (see (Vathy et al., 1985)). The first three morphine injections were 5 mg/kg each, and the remaining injections were 10 mg/kg each (Vathy et al., 1985).

Rat pups were born on the 22nd day of gestation, and the day of birth was counted as postnatal day (PND) 0. On PND 1, the morphine-exposed pups were tattooed on one footpad with black India ink for identification. Morphine- and saline-exposed pups were sexed, and litters were crossed and reduced to 10 pups/litter (Vathy et al., 1985). Each mother raised 5 of her own and 5 adopted pups who had received the opposite prenatal treatment. Pups were weaned on PND 25, ear-punched for identification, and housed individually. Young adult male rats (PND 68–83) were used in the present study. To avoid litter effects, only one animal was used from each litter. Each experimental group contained 8–24 males. All experimental protocols utilized in this study were reviewed and approved by the Institutional Animal Care and Use Committee and were done in accordance with the National Institutes of Health *Guide for Care and Use of Laboratory Animals*.

2.2. Materials

Morphine sulfate (National Institute on Drug Abuse, Research Technology Branch, Rockville, MD) was dissolved in 0.9% saline. Bicuculline (Sigma, St. Louis, MO) was dissolved in 0.1 N HCl, and the pH was adjusted to 5.5. A stock solution (1 mg/ml deionized, distilled water) of the CTX-B subunit (choleragenoid; Sigma) was heated to 56 °C for 20 min to eliminate possible traces of CTX-A activity without loss of potency of CTX-B (see Shen and Crain, 2001).

2.2.1. Bicuculline-induced seizures

In Experiment 1, rats were injected subcutaneously with 0.9% saline or 1 μg/kg CTX-B at 24, 12, and 0.5 h before bicuculline administration. In Experiment 2, one group of rats was injected with 0.9% saline three times: 24, 12, and 0.5 h prior to bicuculline, another group was injected with 0.9% saline once 30 min prior to bicuculline, and a third group of rats was not injected at all prior to bicuculline injection. The timing of the saline injections corresponds with the timing of the injections that were used in our present and previous

studies. Bicuculline was administered intraperitoneally at a dose of 7.5 mg/kg in all three experiments. The dose of bicuculline was the same as in our previous studies (Schindler et al., 2000, 2001). Each animal was observed and evaluated for seizures for 30 min after bicuculline administration. Bicuculline seizures have two distinct but consecutive phases: clonic and tonic–clonic, each of which originates from a different morphological substrate in the brain (Browning and Nelson, 1986). Clonic seizures usually appear first and are characterized by rhythmic movements of the head and forelimbs. Tonic–clonic seizures often follow clonic seizures and begin with wild running, followed by a short-lasting tensing of all muscles (tonus) and a long-lasting clonus, which usually ends in death. A loss of the righting reflex is observed during tonic–clonic but not during clonic seizures (Veliskova et al., 1990).

2.3. Statistical analysis

The percentage of animals that displayed clonic seizures and the percentage that displayed tonic–clonic seizures in response to the bicuculline injection were analyzed using the chi-square test. The latency to onset of clonic and tonic–clonic seizures was also recorded. An increased latency to the onset of seizure activity indicates a resistance to seizure onset. Only animals that displayed seizures were included in latency measures. Differences in latencies to seizure onset between experimental and control animals were analyzed using a two-way ANOVA (Prenatal Exposure × Adult Treatment) followed by a Fisher's PLSD post hoc test, as necessary. Differences were considered significant if $P < .05$.

3. Results

3.1. Effect of CTX-B injection on bicuculline seizures

There was no effect of CTX-B injections on the latency to onset of clonic (Fig. 1A) or tonic–clonic (Fig. 1B) seizures induced by bicuculline in prenatally morphine-treated, saline-treated or untreated control rats. However, CTX-B significantly decreased the incidence of tonic–clonic ($\chi^2 = 8.869$; $P < .05$) but not clonic bicuculline seizures in both prenatally morphine- and saline-exposed males (but not prenatally untreated controls) relative to control males pretreated with three saline injections (24, 12, and 0.5 h prior to bicuculline administration) (Fig. 1C and D).

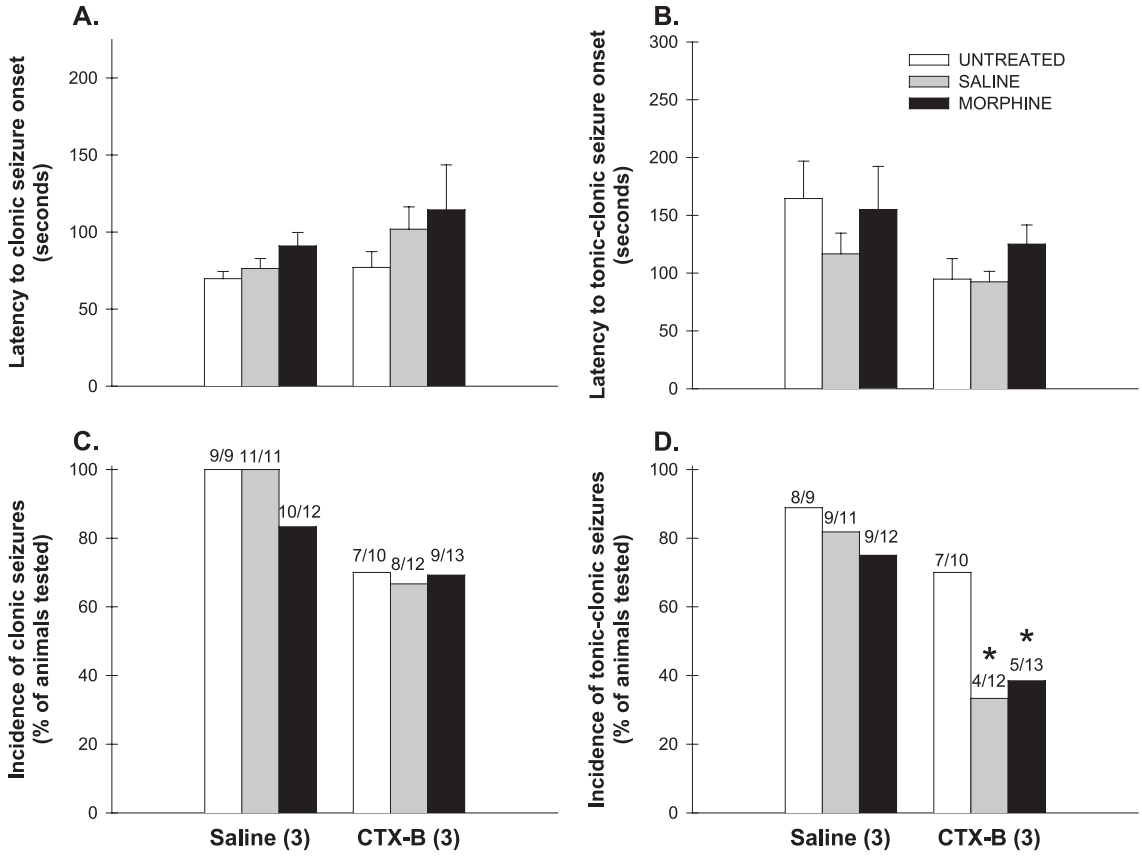

Fig. 1. Cholera toxin B (CTX-B) decreases the incidence of tonic–clonic bicuculline seizures. A = latency to clonic seizure onset, B = latency to tonic–clonic seizure onset, C = percentage of animals tested that had clonic seizures, D = percentage of animals tested that had tonic–clonic seizures. The numbers above the bars indicate the number of animals that had a seizure/total number tested. Values are expressed as means ± S.E.M. * $P < .05$, main effect of CTX-B injection (chi-square test).

3.2. Effect of saline injection on bicuculline seizures

Because control rats that were injected three times with saline prior to bicuculline seizure testing in Experiment 1 did not demonstrate an increase in the latency to onset of tonic–clonic seizures, as was demonstrated in our previous studies (Schindler et al., 2000, 2001, submitted for publication), we hypothesized that three saline injections abrogated the increased latency to tonic–clonic bicuculline seizures in prenatally morphine-exposed males. To test this hypothesis, we injected prenatally morphine- and saline-exposed rats with 0, 1, or 3 saline injections before bicuculline seizure testing. There was a main effect of prenatal morphine exposure on the latency to the onset of clonic [$F(1,66) = 13.613$; $P < .01$] (Fig. 2A) and tonic–clonic [$F(1,51) = 28.579$; $P < .0001$] (Fig. 2B) seizures, and a significant interaction between prenatal morphine exposure and saline injections with respect to tonic–clonic seizures [$F(2,51) = 3.695$; $P < .05$] (Fig. 2B). Post hoc tests revealed that prenatal morphine exposure increased the latency to onset of clonic (Fig. 2A) and tonic–clonic (Fig. 2B) bicuculline seizures with no injection (clonic: $P < .001$; tonic–clonic: $P < .0001$) or a single injection of saline 30 min before bicuculline administration (clonic: $P < .05$; tonic–clonic: $P < .001$). In contrast, in a group of males given three injections of saline (24, 12, and 0.5 h prior to bicuculline administration), the difference between prenatally morphine- and saline-exposed males in the latency to clonic (Fig. 2A) and tonic–clonic (Fig. 2B) seizure onset was no longer apparent. There was no significant effect of prenatal morphine exposure or number of saline injections on the incidence of clonic (Fig. 2C) or tonic–clonic (Fig. 2D) seizures.

4. Discussion

To the best of our knowledge, this is the first report that CTX-B administered systemically has an anticonvulsant effect on seizures in prenatally saline- and morphine-exposed rats. In analgesia studies, treatment of mice with CTX-B unmasks potent inhibitory effects of morphine and other opioid receptor agonists (Shen and Crain, 2001). Similarly, the present study demonstrates that treatment with CTX-B may have enhanced the inhibitory effects of the endogenous opioids on bicuculline seizures.

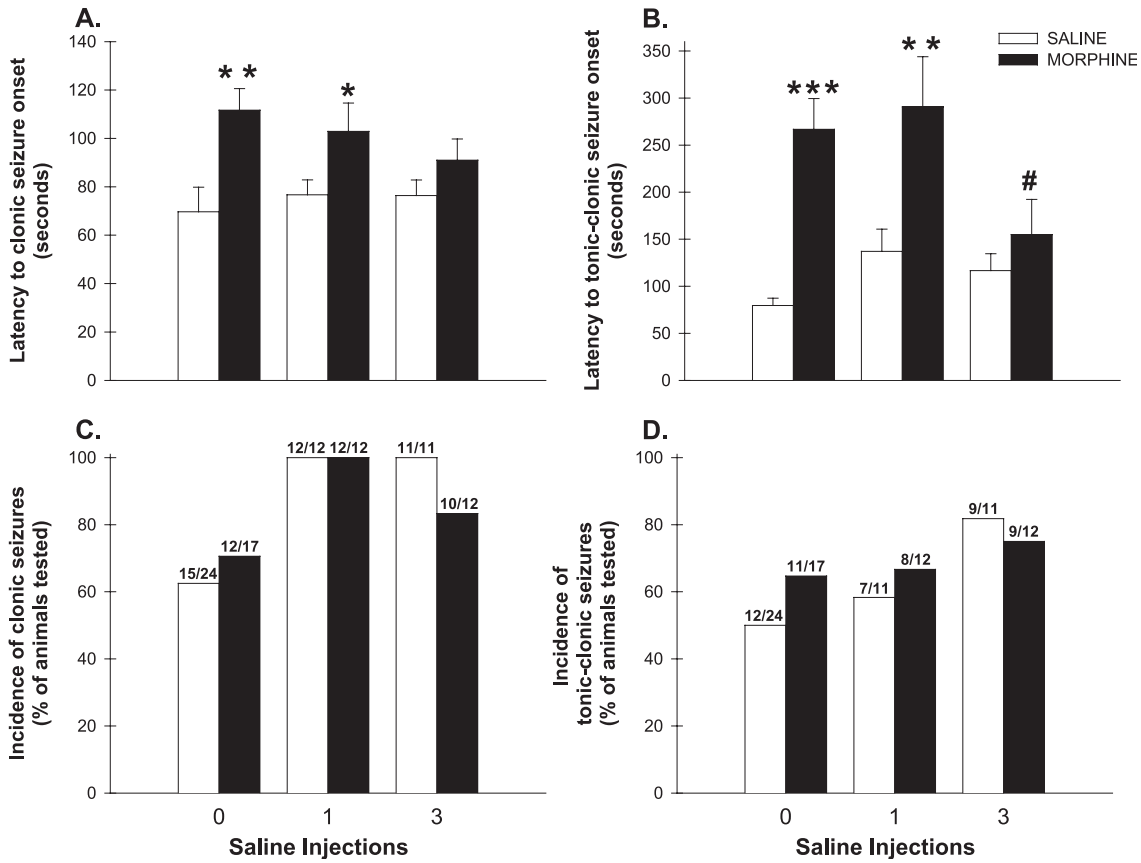

Fig. 2. Prenatal morphine exposure increases the latency to tonic–clonic bicuculline seizures in male rats after zero or one but not three saline injections before seizure testing. A = latency to clonic seizure onset, B = latency to tonic–clonic seizure onset, C = percentage of animals tested that had clonic seizures, D = percentage of animals tested that had tonic–clonic seizures. The numbers above the bars indicate the number of animals that had a seizure/total number tested. Values are expressed as means ± S.E.M. * $P < .05$, * * $P < .001$, and * * * $P < .0001$ vs. prenatally saline-exposed males with the same number of saline injections. # $P < .01$ vs. prenatally morphine-exposed males with zero or one prebicuculline saline injections.

It is noteworthy that CTX-B injections did not alter the incidence of bicuculline seizures in naïve rats—only in prenatally morphine- and saline-exposed rats. This may indicate that CTX-B treatment decreases the occurrence of bicuculline seizures in animals that experience some kind of prenatal stress (maternal saline injection alone inducing stress or maternal injection + drug inducing stress). However, to establish the specificity of CTX-B action on putative Gs-coupled excitatory receptor signaling, comparative studies with ultralow dose of naltrexone or other selective antagonists of Gs-coupled excitatory opioid receptors (Crain and Shen, 2000, #691) may be conducted in order to clarify this ambiguity. This could eliminate the alternative possibilities that the observed CTX-B effect on seizures might be mediated by blocking Gs-coupled nonopioid excitatory receptor signaling.

Other studies show that CTX-B is proconvulsant when administered intracortically (Karpiak et al., 1978) and has no effect when infused directly into the hippocampus or amygdala in vivo or ex vivo in hippocampal slices (Kuriyama and Kakita, 1980; Williams et al., 1993). Systemic injection of CTX-B could potentially affect brain regions involved in seizure initiation and propagation such as the substantia nigra, amygdala, and hippocampus. On the other hand, when CTX-B is injected into a specific brain region, such as the cortex or hippocampus, brain-region-specific effects can be discerned. Therefore, CTX-B that is injected systemically might have proconvulsant effects in the cortex while, at the same time, acting on other brain regions in which it has anticonvulsant effects. Thus, the overall effect of systemic injection of CTX-B is anticonvulsant in prenatally morphine- and saline-exposed rats.

In the present work, control rats that were injected three times with saline prior to bicuculline seizure testing did not demonstrate the increase in the latency to onset of tonic–clonic seizures that had been demonstrated in our previous studies (Schindler et al., 2000, 2001, submitted for publication). Experiment 2 demonstrates a novel and interesting finding that may shed light on the discrepancy between the present and previous results. In prenatally morphine-exposed males, a pretreatment of three injections of saline at 12-h intervals prior to the bicuculline injection reduces their increased seizure latency, while in prenatally saline-exposed males, three saline injections do not affect the latency to seizure onset.

Saline injections are necessary to distinguish the effects of the drug (in this case, CTX-B) and the injection itself. The same vehicle that is used for dissolving the active drug is used as placebo, which is given through the same route of administration as the active drug. On the other hand, many researchers purposely use saline injection of pregnant dams as a form of mild prenatal stressor (Peters, 1982; Ward et al., 2000). Furthermore, animals injected with saline exhibit alterations in stress-sensitive parameters such as corticosteroid levels, core temperature, and swim test (Barrett and Stockham, 1963; Dilsaver and Majchrzak, 1990; Drago et al., 2001). Thus, we conjecture that saline injection (used as placebo) may act as a stressor and induce changes in the hypothalamic–pituitary–adrenal (HPA) axis-regulated stress responses.

Although the present study does not examine changes in the HPA axis in response to saline injections, we found that multiple, but not single, saline injections alter the latency to onset of bicuculline seizures in prenatally morphine-exposed males. It is noteworthy that in prenatally saline-exposed animals, the mild stressor of three saline injections has no effect on bicuculline seizures, while it has proconvulsant effects in prenatally morphine-exposed animals. This may indicate that prenatally morphine-exposed males are more sensitive to stress or respond differently to stressors than controls. This possibility is supported by our previous study, which showed that prenatally morphine-exposed males exhibit increased struggling (a measure of anxiety) in the forced swim test after 2 weeks of daily exposure to a cold water stressor compared to controls (Slamberova et al., 2002a,b).

Several studies (Abel and Berman, 1993; Drugan et al., 1985) demonstrate that stress can alter seizure susceptibility. Some stressful manipulations including inescapable shock (Drugan et al., 1985), handling (Cain and Corcoran, 1985), cold restraint (Rae et al., 1990), and forced swimming (Rae et al., 1990), have proconvulsant effects on seizures, such as those seen in prenatally morphine-exposed males in this study. The present study is, however, the first to demonstrate specifically that a mild stressor of repeated saline injections can decrease the latency to onset of bicuculline seizures, although this stressor is effective only in prenatally morphine- but not saline-exposed rats.

We speculate that the endogenous opioid system, which is involved in altered bicuculline seizure latencies in prenatally morphine-exposed males (Schindler et al., submitted for publication), may also play a role in changes in seizure latencies in prenatally morphine-exposed rats after three saline injections. It has been reported that there are increases in β-endorphin release (Amir et al., 1980) and proenkephalin mRNA (Harbuz et al., 1991) after exposure to a stressor. These changes in the opioid system as a result of stress may modify seizure susceptibility (Cain and Corcoran, 1985; Rae et al., 1990). Further evidence that the opioid system is modulated by stress is that many stress-induced behavioral changes like analgesia (Drugan and Maier, 1983; Grau et al., 1981; Hemingway and Reigle, 1987; Miczek et al., 1982, 1985), immobility in forced swim test (Molina et al., 1994), and learned helplessness (Hemingway and Reigle, 1987) can be blocked by naloxone (Hemingway and Reigle, 1987; Molina et al., 1994).

Stress may also alter seizure latencies in prenatally morphine-exposed animals through nonopioid mechanisms. Stress stimulates the HPA axis and increases the release of corticosterone (CORT). Mineralocorticoids and low concentrations of CORT are excitatory, whereas glucocorticoids and high concentrations of CORT are inhibitory (Roberts et

al., 1995). Therefore, a mild stressor such as saline injections, which does not alter bicuculline seizures in prenatally saline-exposed animals, might elicit a small release of CORT in morphine-exposed males, with its consequent excitatory effect.

Taken together, the present results suggest that prenatal morphine exposure-induced alterations in seizure latencies can be reversed by a mild stressor of three saline injections. In addition, a systemic injection of CTX-B decreases seizure incidence in both prenatally saline- and morphine-exposed rats.

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