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CLOZAPINE PROTECTION AGAINST GESTATIONAL COCAINE-INDUCED NEUROCHEMICAL ABNORMALITIES.

Elena Yablonsky-Alter[1], Eleonora Gashi[1, 2], Theodore I. Lidsky[2, 3], Hoau-Yan Wang[1, 2], and Shailesh. P. Banerjee[1, 2]

[1]Department of Physiology and Pharmacology, The City University of New York Medical School, New York, New York (E.Y.-A., E.G., H.-Y. W., S.P.B).

[2]Depatment of Biology and Neuroscience, Graduate School of the City University of New York, New York, New York (E.G., T.I.L., H.-Y. W., S.P.B.).

[3]Department of Psychobiology, New York State Institute for Basic Research, Staten Island, New York (T.I. L.).

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Corresponding author: Hoau-Yan Wang, Ph.D.

Department of Physiology and Pharmacology

The City University of New York Medical School

Harris Hall, Room 203

138 Street and Convent Avenue

New York, NY 10031

Phone: (212) 650-8813

FAX: (212) 650-7726

E-mail: hywang@sci.ccny.cuny.edu

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ABSTRACT

Clozapine was found to be effective in attenuating cocaine-induced neurochemical effects. We investigate whether clozapine influence *in utero* cocaine exposure induced changes in striatal dopamine levels and cortical NMDA receptor density in mouse and rat brains. Pregnant mice or rats were injected of cocaine (5 or 10mg/kg intraperitoneally) or saline every 24 hour throughout gestation and continued for six weeks following the delivery. Striatal dopamine levels measured by HPLC found to decrease by 24 to 33 % in gestational cocaine exposed between the ages of 3 to 15 days but not in 42 days old pups. The cortical NMDA receptor densities assessed either in the presence of 100 μM glutamate or 30 μM glycine were significantly increased in 15-day-old gestational cocaine-exposed rats. Simultaneous daily administration of 3 mg/kg of clozapine with 5 mg/kg of cocaine to pregnant mice protected against the decrease in striatal dopamine levels or an increase in the concentration of NMDA receptor measured in the presence of 100 μM glutamate in 15- day-old pups. Clozapine did not affect striatal dopamine levels by itself or when co-administered with cocaine in 42 days old pups. The results show gestational cocaine may induce neurochemical abnormalities in brain exhibited as an increased glutamate NMDA receptor density together with a decreased striatal dopamine level. These effects of gestational cocaine exposure may be prevented by simultaneous administration of clozapine. Thus clozapine, which is a partial agonist at the NMDA receptor, may be of value in protecting against gestational cocaine induced adverse effects in brain.

Many studies have documented that cocaine exposure during embryonic development alters subsequent neuro-biological and behavioral functions in the offspring (Dow-Edwards et al., 1990; Henderson and McMillen, 1993). The mechanisms for cocaine-induced neuro-behavioral effects, however, remain to be elucidated. Previous investigators have reported developmental changes in several neurotransmitters including dopamine (Miller et al., 1995; Salvatore et al., 2004), serotonin (Snyder-Keller and Keller, 1993), and acetylcholine (Tyrala et al., 1992) following gestational exposure to cocaine. The mechanism for developmental changes in monoaminergic systems after prenatal cocaine exposure may be attributed to its ability to block neurotransmitter uptake in the fetal brain or the vasoconstrictor activity of the drug. Cocaine is known to inhibit high-affinity neurotransmitter uptake at the presynaptic nerve terminals in both adult animals and fetus brains (Pitts and Marwah, 1987; Meyer et al., 1994). Therefore, cocaine enhances synaptic levels of dopamine, norepinephrine, and serotonin (Ng et al., 1991; Keller et al., 1994) following systemic and gestational administration. Cocaine has also been reported to reduce the fetal blood flow to decrease the supply of oxygen and other nutrients available to the fetus in the pregnant dams (Woods et al., 1987). Hypoxia or diminution of oxygen levels in the fetal brain has been shown to alter development of brain monoaminergic systems (Silverstein and Johnston, 1984).

Numerous studies in fetal and mature animals indicate a role of changes in dopaminergic system in the neurological effects of cocaine. Cocaine-induced increases in synaptic concentrations of dopamine may slow glutamate uptake at its pre-synaptic sites (Kerkerian et al., 1987) resulting in a heightened glutamatergic transmission. Also, it may augment this amino acid transmission at the post-synaptic site by the activation of D_1 receptors (Cepeda and Levine, 1998). In the retinal ganglion and bipolar cells, activation of post-synaptic D_1 receptors enhances glutamate mediated currents by a G protein coupled mechanism involving activation

of adenylyl cyclase (Maguire and Werblin, 1994). Therefore, higher synaptic levels of dopamine may increase glutamatergic transmission by acting both at the pre- and post-synaptic sites of this amino acid. Again, cocaine-induced vasoconstriction due to augmentation sympathetic outflow in mature animals or pregnant dams may cause ischemia with consequent hypoxia, leading to extra release of glutamate (Krajnc et al., 1994). These cocaine-mediated effects may independently or in combination cause cellular damage to the adult and fetal brain.

Thus it is our hypothesis, that cocaine-induced adverse neurobehavioral activity is mediated by the excitotoxicity of glutamate, which may occur by both peripheral and central actions of cocaine. In this investigation, we examine changes in the striatal dopamine levels at different stages of postnatal period following gestational cocaine exposure. In addition, we also measured striatal NMDA receptor concentration. Using changes in dopamine level and NMDA receptor density induced by prenatal cocaine exposure as the guide, we evaluate the ability of clozapine, an atypical antipsychotic agent with partial NMDA agonist activity in blocking cocaine-induced effects.

Methods

Materials

^{3}H-MK801 (24 Ci/mmol) was purchase from Perkin Elmer Life and Analytical Sciences (Boston, MA). Unlabelled MK801, (-) cocaine HCl, Tris base, L-glutamate, L-glycine, monochloroacetic acid were purchase from Sigma (Natick, MA). GF/B glass fiber filters were purchased from Whatman (Kent, England). Micro nylon filters were purchased from Millipore Corporation (Bedford, MA).

Animals

Pathogen-free, male and female CD-1 mice and Sprague-Dawley rats were obtained from Charles River (Wilmington, MA) and were allowed to be acclimated to the colony room with daily handling and weighing for 7 days. Animals were housed individually at 21-23°C with 40 to 60% relative humidity and a 12-hr light/dark photo-period, light on at 0700am. They were maintained on Purina Rodent Laboratory Chow and tap water ad libitum. All animal procedures were in compliance with the Animal Welfare Act, the Public Health Service Policy on Humane Care and Use of Laboratory Animals, the US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training, the NIH guide for the Use and Care of Laboratory Animals and the City University of New York (CUNY) institutional guidelines and were approved by the CUNY Animal Care and Use Committee.

A pair of 10-week-old male and female rats or mice was placed in a cage overnight. The presence of sperm-positive vagina plug is considered E0. The pregnant female animals were housed individually without disturbance other than the daily injection with indicated drugs.

Every experimental series consists of 6 pregnant rats or mice with 2 received one of three treatment paradigms.

In utero cocaine treatment was performed by injecting pregnant mice and rats with 5 mg/kg and 10 mg/kg (-) Cocaine hydrochloride dissolved in sterile physiological saline (2 mg/ml) intraperitoneally (i.p.) at 0900, respectively at an interval of 24 hours throughout 21 days of gestation. Control female mice were injected with equal volume of physiological saline. The dose of cocaine used in this investigation was based on series of preliminary experiments in which this dose of cocaine was found to be effective in a wide variety of behavioral and biochemical parameters comparable to those reported by other investigators without overt growth effects (Dow-Edwards et al., 1990). Extra precaution was made to prevent injecting directly to the womb and injuries to the fetuses. Administration of saline or cocaine to control or drug-treated groups respectively was continued for another 6 weeks following delivery, and the mothers were allowed to feed milk to their newborn pups up to 42 days. The treatment schedule did not alter body weight gains of the dams or pups. To study protective effects of clozapine against cocaine-induced central nervous system (CNS) toxicity, clozapine (3 mg/kg; i.p.) was simultaneously administered with cocaine hydrochloride (5 mg/kg; i.p.) during entire pregnancy (approximately 21 days) and up to 6 weeks after delivery. The control groups received equal volumes of saline. Animals were sacrificed between 3-42 days of age. In general, all animals were decapitated within 12-15 hours of reaching indicated ages.

Receptor binding of ^{3}H-MK-801 to NMDA glutamate receptor.

Pups were decapitated at the ages of 15 days and the brains were removed and placed on a glass plate. Striata and cerebrocortices were dissected out and the tissue was frozen in liquid nitrogen and stored at -100°C until use. We assessed the functional activity of the NMDA

receptor in the frontal cortex of control and experimental groups by measuring the displacement of ^{3}H-MK-801 by non-radioactive MK-801 in the presence of either glycine or glutamate by adopting a procedure previously described (Banerjee et al., 1995). Fifteen-day old rat pups were sacrificed and the brains were removed. The frontal cortices were dissected on ice and immediately frozen at -80°C and thawing at 4°C on the day of the experiment the tissue was washed to reduce residual levels of glutamate and glycine. The tissue was homogenized with a Brinkman Polytron Pt-20 homogenizer in 20 volumes of ice-cold 0.05 M Tris buffer (pH 7.4 at 25 °C) and centrifuged at 49,000 x g for 20 min. The supernatant was discarded and the pellet was re-homogenized in the same buffer and centrifuged as before. This last procedure was repeated twice and the resulting membrane pellet was frozen one more day at -80 °C. For assays, pellet was re-suspended, homogenized in Tris (20 volumes, 5mM) and centrifuged at 49,000 x g for 20 min. and repeated two additional times. Receptor binding assays were carried out by incubating 800µl of crude fractions containing (0.8mg tissue) with radioligand ^{3}H-MK-801 (about 100,000 CPM) with either 100 µM glutamate or 30 µM glycine for 45 min at 25 °C. To determine the non-specific binding, parallel set of samples were incubated with large excess of MK801 ligand (100 µM MK-801). Specific binding is defined as the difference between total binding and non-specific binding. Incubation was terminated with rapid filtration through GF/B glass fiber filters. The filters were rinsed twice with 4ml ice-cold Tris buffer to remove unbound radioactive ligand. They were then be dried and placed in vials containing 3 ml scintillation fluid and counted by scintillation spectroscopy at 60% efficiency. The receptor binding was conducted to develop a displacement curve of ^{3}H-MK-801 binding to the NMDA receptor by conducting the binding assays with eight different concentrations of unlabeled MK-801 in the presence of 100 µM glutamate or 30 µM glycine. The displacement curves were introduced to the Beckman program AccuFit Competitive Two-Site program was used to compute the

Scatchard analysis to estimate the maximal number of binding sites. The program is based on the principle of nonlinear least squares regression analysis to solve the equations describing the binding of labeled ligand to receptor proteins. In a well-washed tissue with no residual endogenous amino acids, ^{3}H-MK-801 binding is minimal to negligible (Reynolds et al., 1987; Banerjee et al., 1995). In accord with a previous report (Reynolds's et al., 1987), addition of either glycine or glutamate increased ionophore opening that cause several fold enhancement of ^{3}H-MK-801 specific binding. This functional assay of NMDA activity was then used to measure specific binding of ^{3}H-MK-801 to cortical tissue obtained from control and experimental pups of 15-day-old in the presence of either 100 μM glutamate or 30 μM glycine.

The protective effect of clozapine on *in utero* cocaine-mediated activities was also assessed by determining changes in striatal NMDA receptor density. Six pregnant rats were divided into three groups: control (saline), cocaine (5mg/kg), and cocaine (5 mg/kg) plus clozapine (3 mg/kg). Pups born to the dams in different groups were sacrificed 15 days after birth. The number of glutamate receptors was estimated by measuring specific binding of ^{3}H-MK-801 to striatal membranes in the presence of 100 μM glutamate.

Determination of Dopamine levels by High Pressure Liquid Chromatography (HPLC)

Pups were decapitated at the ages of 3, 7, 11, 15 or 42 days and the brains were removed and placed on a glass plate. Striatum was dissected out from the sagittal section. The tissue was frozen in 0.07M monochloroacetic acid and stored at -100°C. Each striatum obtained from mouse pup was sonicated in 0.07M monochloroacetic acid using ultrasonic cell disrupter, and centrifuged for 20 min. The supernatant was filtrated through 0.22 μm Micro Nylon filter. Samples were injected in HPLC.

In a separate set of experiments, the effect of clozapine co-treatment on dopamine level was determined. Sixteen pregnant mice were divided into the following four groups: control (saline), cocaine (5 mg/kg, i.p.), clozapine (3 mg/kg i.p.), and cocaine (3mg/kg) plus clozapine (3 mg/kg). Pups born to the dams in each of the treatment groups were sacrificed at either 15 or 42 days after birth and striatal dopamine levels were measured by HPLC.

A Bioanalytical HPLC System (West Lafayette, IN) consisting of a refrigerated CMA/200 autosampler, a LC4B electrochemical detector and a Perkin-Elmer series 410 pump were used for analysis. The column used was a 3 μm reverse phase column (Phase Separation Spherisorb Column S3 ODS2; 10 cmx 4.6 cm). Mobile phase consisted of 0.07M monochloroacetic acid, 0.5M sodium ethylendiaminetetraacetic acid, 1mM sodium dodecylsulfate, and 1.5% acetonitrile adjusted to pH 4.1 and was delivered at a rate of 1.0 ml/min. A 20 μl of each sample was injected onto a column for analysis. A potential on flow cell of electrochemical detector was set at +0.65 V Vs. Ag/AgCl. Chromatograms were integrated, compared to standards and analyzed using Perkin-Elmer Turbochrom Software. The approximate sensitivity limits of the assay with these detector settings and this chromatographic separation were 10 pg for DA and NE, 20 pg for 5-HIAA and DOPAC.

Statistical Analysis

All data are presented as mean ± S.E.M. Two-tailed Student's t test was used to compare particular responses between two groups. In addition, analysis of variance followed by Newman-Keul's test for multiple comparisons was also used to evaluate the differences in each of the age groups. The threshold for significance was $p < 0.05$.

Results

Effect of In Utero Cocaine Exposure on Striatal Dopamine Levels in Mouse Brains.

In order to define a marker for neurochemical effects of cocaine, we measured striatal dopamine levels by HPLC in offspring derived from a group of 20 pregnant mice (10 control and 10 cocaine-exposed). The pups were sacrificed at the ages of 3, 7, 11, 15, and 42 days by decapitation and striatal dopamine levels were measured by HPLC. The average striatal dopamine content of the control pups at the ages of 3, 7, 15 and 42 days were 37.5 ± 4.0 pg/mg, 56.3 ± 7.2 pg/mg, 116.7 ± 7.1 pg/mg, 208.3 ± 5.8 pg/mg, and 185.8 ± 1.9 pg/mg, respectively. Exposure to gestational cocaine decreased striatal dopamine levels by 30%, 28%, 25%, 25% and 15% in the pups at the ages of 3, 7, 11, 15, and 42 days, respectively (Fig. 1). Interestingly, in 42-day-old pups, the levels of striatal dopamine showed no significant difference between the control and the experimental groups. This suggests that the decline of striatal dopamine following gestational cocaine exposure seen at early postnatal period was reversed in 42-day-old pups. The magnitudes of decreases in dopamine were similar in the pups between the ages of 3 to 15 days. Although, dams of the experimental groups continued to receive cocaine and continued to nurture their own pups, there was no further decrease in the levels of striatal dopamine during the postnatal development in the striatum of the offspring born to cocaine exposed mothers. Thus, the cocaine-induced insult to the striatal dopaminergic system appears to be restricted during the prenatal period rather than the postnatal stage.

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Effect of Gestational Cocaine Exposure on Cortical NMDA Receptor Concentrations in Rat Brains.

We further determine the basis for the diminution of striatal dopamine in the gestational cocaine-exposed pups since cocaine may inhibit dopamine re-uptake in the fetal brains to increase the levels of this neurotransmitter in the synaptic cleft. This extracellular increase in dopamine concentration may decrease the rate of dopamine synthesis by a feedback mechanism resulting in diminution of striatal dopamine after gestational cocaine exposure. Prenatal cocaine administration has been shown to reduce cortical GABA cell count (Yablonsky-Alter et al., 1993); a decrease in striatal dopamine may represent reduction in dopaminergic terminals induced by the excitotoxicity of glutamate caused by gestational cocaine exposure. To test this possibility, we measured the density of NMDA receptors in the cortical membranes obtained from control and experimental groups of female rats by a ^{3}H-MK-801 binding assay. Results shown in Fig. 2 indicates that the densities of NMDA receptors significantly increase in the frontal cortex of 15-day-old pups that were exposed to gestational cocaine as compared to control animals both in the presence of glutamate as well as glycine. These observations suggest that gestational cocaine exposure may enhance glutamatergic transmission by increasing ionophore opening to facilitate the occurrence of excitotoxicity.

Prevention of I*n Utero* Cocaine Exposure-Induced Diminution of Striatal Dopamine by Clozapine in Mouse Brains.

In these experiments, the levels of dopamine in striata following *in utero* cocaine exposure were assessed. A decrease in the striatal levels of dopamine produced by gestational exposure to cocaine was completely abolished by clozapine in 15 days old pups (Table 1). At the dose given, clozapine by itself did not significantly alter the levels of striatal dopamine (Table 1). Again, there was no discernible difference between the four groups in the levels of striatal dopamine in 42 days old pups (Table 1).

Prevention of an Increase in Striatal NMDA Receptor Levels Following Gestational Cocaine Exposure by Clozapine in Rat Brains.

To further confirm that clozapine protects against gestational cocaine-induced neurochemical effects, we also investigated if clozapine prevents augmentation of striatal NMDA receptors after gestational cocaine exposure. The results again indicate that there was a significant increase in the number of NMDA receptors in the striatal membranes of pups born to dams exposed to cocaine as compared to control group (Fig. 3). This cocaine-mediated effect was prevented by co-treatment with clozapine (Fig. 3). The increase in NMDA receptor density in prenatal cocaine-exposed rats may result from decrease NMDA neurotransmission.

Discussion

The present data demonstrate a diminution of striatal dopamine levels in pups born to cocaine-exposed mice as compared to control group between the ages of 3 to 15 days. In contrast, the 42 day-old pups showed no significant difference in the levels of striatal dopamine between control and cocaine-treated groups (Fig. 1). This suggests that despite initial loss of striatal dopamine at early ages, there is a recovery in the concentration of this neurotransmitter in the striatum during the course of brain maturation after gestational exposure to cocaine. These data are in accord with previously reported developmental recovery in terms of extracellular striatal dopamine levels in rats (Keller et al., 1994), auditory brain stem transmission in human newborns (Salamy et al., 1990) and postnatal rats (Church et al., 1990), and neurobehavioral indices in human infants (Chasnoff et al., 1992) following prenatal cocaine exposure.

Our results showing that gestational cocaine lowered striatal dopamine level in early postnatal ages are also consistent with another study with human neonates where prenatal cocaine exposure showed significantly lower levels of cerebrospinal fluid (CSF) homovanillic acid, the principal metabolite of dopamine (Needlman et al., 1993). The diminution of striatal dopamine may be due to excessive release of this monoamine and/or a reduction of striatal dopamine transporter protein following gestational cocaine-exposure. Consistent with this notion, an increased potassium-stimulated rat striatal dopamine release concurrent with a decrease in dopamine transporter proteins were observed in brains from the offspring of gestational cocaine-exposed rats (Salvatore et al., 2004). Moreover, the release of striatal dopamine after an acute cocaine injection was found to be greater and more prolonged in the prenatal cocaine-exposed rat pups as compared to the vehicle control group (Keller et al., 1994).

Interestingly, Keller and his associates (1994) observed, basal extracellular dopamine levels, as well as its metabolites, were increased in the prenatal cocaine-exposed pups as compared to control pups. Therefore, a diminution of striatal dopamine transporter protein after prenatal cocaine-exposure (Salvatore et al., 2004) may decrease the endogenous levels of dopamine (Fig. 1) and increase its concentration in the extracellular fluid (Keller et al., 1994). A reduction of endogenous dopamine concentration in the striatum may result in an elevated dopamine synthesis rate as a feedback mechanism in the gestational cocaine-exposed pups that in turn, accounts for the recovery of dopamine concentrations observed at the later postnatal age. This assumption is consistent with the demonstrations that prenatal cocaine administration increased fetal brain or cortical tyrosine hydroxylase activity or immunoreactivity (Akbari and Azmitia, 1992; Meyer and Dupont, 1993). In contrast to the recovery of striatal dopamine levels at 42 days of postnatal age in prenatal cocaine-exposed pups reported here, the forebrains of the mature offspring following prenatal cocaine exposure showed a reduction of dopamine concentration in the male mice (Miller et al., 1995). Such discrepancy may result from differences in doses of cocaine used (5 mg/kg daily vs 30 mg/kg twice daily), route of administration (i.p. injection vs intragastric intubation) and/or treatment paradigms (E0 to E21 vs E8 toE19) between these two studies.

In addition to dopaminergic neurotransmission, glutamatergic systems, especially the NMDA receptor, are likely involved in cocaine-mediated neurochemical and/or neurobehavioral effects. Previous investigators have shown that MK-801 prevents the development of sensitization to the convulsive response in rats and mice (Itzhak and Stein, 1992) and the augmentation of lethality rate in mice (Itzhak and Stein, 1992) following repeated administration of cocaine in adult animals. Moreover, up-regulation of cortical NMDA receptors following repeated administration of cocaine to mice appears to parallel the sensitization phenomenon

(Itzhak and Stein, 1992). These observations suggest that some of the cocaine-induced toxicity may be mediated through glutamatergic neurons. To test the possibility that gestational cocaine exposure affects the levels of cortical NMDA receptors, we measured (^{3}H)-MK-801 binding to rat cortical membranes either in the presence of glutamate or glycine obtained from 15-day-old pups of either control or experimental groups. This experiment was based on a previous finding that in the presence of either glycine or glutamate, an increase in ionophore opening that result in several fold enhancement of ^{3}H-MK-801 specific binding (Reynolds et al., 1987). There was a significant increase in the density of the NMDA receptors either in the presence of glycine or glutamate in the cortical membranes obtained from pups exposed to gestational cocaine as compared to the control group (Fig. 2). While the precise underlying mechanism for the increased NMDA receptors is currently not clear, it is possible that this may be a compensatory response to either diminution of extracellular glutamate levels or down-regulation of postsynaptic NMDA receptor activity in the experimental group. Although, there is no information on the effects of gestational cocaine exposure on the cortical extracellular glutamate levels or postsynaptic NMDA receptor sensitivity, a decrease in the levels of extracellular glutamate in the nucleus accumbens following repeated cocaine administration has been reported (Bell et al., 2000). Alternatively, disruption of the NMDA receptor subunit composition and/or their interaction with signal regulators that are critical to the pharmacological and electrophysiological activities in the cerebral cortex may have occurred during cocaine exposure to developing brains.

The above results raise the possibility that attenuation of glutamatergic transmission may protect against adverse neurochemical changes mediated by gestational cocaine. Olney and his associates have shown that noncompetitive NMDA receptor antagonists such as MK-801 are neurotoxins and of limited value as therapeutic agents (Olney et al., 1991). This MK-801

mediated neurotoxic effects may derived from blocking glutamatergic transmission mediated via NMDA receptor system located on GABA neurons leading to a tonic inhibitory influence over excitatory tone in the cortex (Olney and Farber, 1994). Reduction or blockade of this tonic inhibition by NMDA antagonist may result in imbalanced excitation and cell death. Additionally, MK-801 may also block the presynaptic NMDA receptors to increase release of glutamate that would eventually cause excitotoxicity. Despite such difficulties with non-competitive NMDA antagonists, drugs useful to tone down glutamatergic transmission are available. For example, GM_1 ganglioside has been shown to prevent glutamate-induced excitotoxicity without affecting normal synaptic transmission (Favaron et al., 1990).

Reduction in glutamate-induced excitotoxicity may also be achieved using partial agonists of the NMDA receptors such as clozapine (Banerjee et al., 1995). This hypothesis is supported by the results shown on Table 1 and Fig. 3 indicating that clozapine protect against gestational cocaine-induced decrease in the striatal dopamine concentration and an increase striatal NMDA receptor density in 15-day-old mouse or rat pups, respectively. These data suggest that the mechanism for the protection against cocaine-induced toxicity may be related to its ability to reduce glutamatergic transmission. Alternatively, clozapine may reduce cocaine-mediated vasoconstriction and hypoxia by acting as an antihypertensive by blocking D_1 receptor subtype (Witkin et al., 1993). Together with the reports demonstrating clozapine effectively block another NMDA noncompetitive antagonist, phencyclidine-induced hyper-locomotion in rats (Phillips et al., 2001), these data support the notion that clozapine could prevent prenatal cocaine-induced neurochemical changes by modulating glutamatergic activity. The pivotal role of NMDA receptor in mediating cocaine- or phencyclidine-induced neurochemical and behavioral changes is further strengthen by demonstration that despite clozapine blocks D_1, $5HT_2$, and muscarinic cholinergic receptors, more specific agents against these receptors do not

prevent increased locomotion caused by cocaine or phencyclidine unless very high dosages are used (Phillips et al., 2001; Moy and Breese, 2002).

The clinical relevance of clozapine in preventing neurochemical and neurobehavioral changes elicited by gestational cocaine exposure is supported by reports demonstrating clozapine is effective in the treatment of cocaine abuse with or without schizophrenia (Farren et al., 2000; Zimmet et al., 2000). Similarly, another atypical antipsychotic, olanzapine has also been shown to prevent locomotor sensitization induced by chronic phencyclidine administration in rats and cocaine abusers with schizophrenia (Moy and Breese, 2002; Tsuang et al., 2002). Since modafinil which increases brain glutamate and inhibits GABA release, attenuated some aspects of cocaine withdrawal including blunting of cocaine-induced euphoria (Dackis et al., 2003), the possibility that the clozapine protect against gestational cocaine-induced neurotoxicity may be expressed by enhancement of glutamatergic transmission can not be ignored. In addition, the attenuated glutamatergic neurotransmission may also be improved by directly stimulating the postsynaptic NMDA receptors because clozapine is also a partial agonist at the NMDA receptors. Alternatively, the ability of clozapine in blocking cocaine-induced neurotransmission changes mediated by other systems, especially the dopaminergic receptors may also involve. Although future research is required to elucidate the precise mechanism of action for clozapine in its protection against gestational cocaine-induced neurotoxicity, our data presented here directly demonstrating that co-administration of clozapine with cocaine during gestational period prevents cocaine-induced neurochemical changes support the notion that clozapine and other agents that modulate the NMDA neurotransmission may be used in the management of neurochemical effects induced by prenatal cocaine exposure.

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FIGURE LEGENDS

Figure 1. Striatal dopamine levels in pg/mg of wet tissue measured by HPLC in 3-, 7-, 11-, 15-, and 42-day old pups obtained from control and 10 mg/kg/day gestational cocaine-exposed female mice. There was a significant decrease in the striatal levels of dopamine at all ages except in the 42-days old pups. The data are expressed as mean ± S.E.M. derived from 8 to 10 individual determinations at each age. Statistical differences were evaluated using two-factor ANOVA followed by Newman-Keul's test for multiple comparisons.

$^{*p} < 0.05$ when compared to respective value in control group.

Figure 2. The density of NMDA receptor in the cortical membranes of 15-days old pups born to either saline (control) or pre- and post-natal 5 mg/kg/day cocaine-exposed female rats. The number (B_{MAX}) of NMDA receptors was estimated by generating displacement curve of ^{3}H-MK-801 binding in the presence of eight different concentrations (0.1 nM- 0.1 mM) of unlabeled MK-801. The binding experiments were conducted either in the presence of 100 μM glutamate or 30 μM glycine. The data are expressed as mol/mg wet weight x 10^{-14}. The results are expressed as mean ± S.E.M. derived from 6 determinations at each point and show significant differences between control and the experimental groups either in the presence of glutamate or glycine. Apparent dissociation constants (K_d) were comparable among groups. Statistical significances between groups were evaluated by two-tailed student's t test.

$^{*p} < 0.05$ when compared to respective value in control group.

Figure 3. The number of NMDA receptor binding sites in the striatal membranes of 15-day-old pups born to saline (control) or 5 mg/kg/day cocaine- or 5mg/kg/kg cocaine- plus 3 mg/kg/day clozapine-exposed female rats. The procedure to measure NMDA receptor binding was similar to that described in the Methods or the legend to figure 2. All binding assays were conducted in the presence of 100 μM glutamate. The results are expressed as mol/mg wet weight x 10^{-14} (mean ±S.E.M.) derived from 4 independent determinations. The data show a significant increase in the density of NMDA binding sites in the cocaine-exposed group as compared to control and cocaine-plus clozapine-exposed group. The control and cocaine- plus clozapine exposed groups showed no significant difference. Also, there were no significant differences in the apparent dissociation constants between the three groups. Statistical differences were evaluated using one-factor ANOVA followed by Newman-Keul's test for multiple comparisons.

$^{*}p < 0.01$ when compared to control group.

$^{+}p < 0.01$ when compared to cocaine-treated group.

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Table 1.

Clozapine co-treatment prevents cocaine-induced changes in striatal dopamine levels. Striatal dopamine in pg/mg wet tissue weight in 15- or 42-days old pups born to control, 5 mg/kg/day cocaine, 3 mg/kg/day clozapine, or 5 mg/kg/day cocaine plus 3 mg/kg/day clozapine- exposed female mice. The results are averages (± S.E.M.) of 4 determinations in each group. Although, clozapine prevented decline of striatal dopamine levels caused by gestational cocaine in 15- day-old pups, there was no significant differences between the four groups in 42-days old pups. Statistical differences were evaluated using one-factor ANOVA followed by Newman-Keul's test for multiple comparisons.

[a] $p < 0.01$ when compared to controls in each age group.

[b] $p < 0.01$ when compared to cocaine-treated animals in each age group.

Striatal Dopamine Level

(pg/mg wet tissue weight)

	Control	**Cocaine**	**Clozapine**	**Cocaine + Clozapine**
15-day-old	**219.5 ± 2.2**	**154.5 ± 3.1**	**238.2± 1.8**	**230.1 ± 3.2**
42-day-old	**200.4 ± 5.4**	**169.4 ± 12.3**	**186.5 ± 10.0**	**198.1 ± 11.1**

Fig. 1

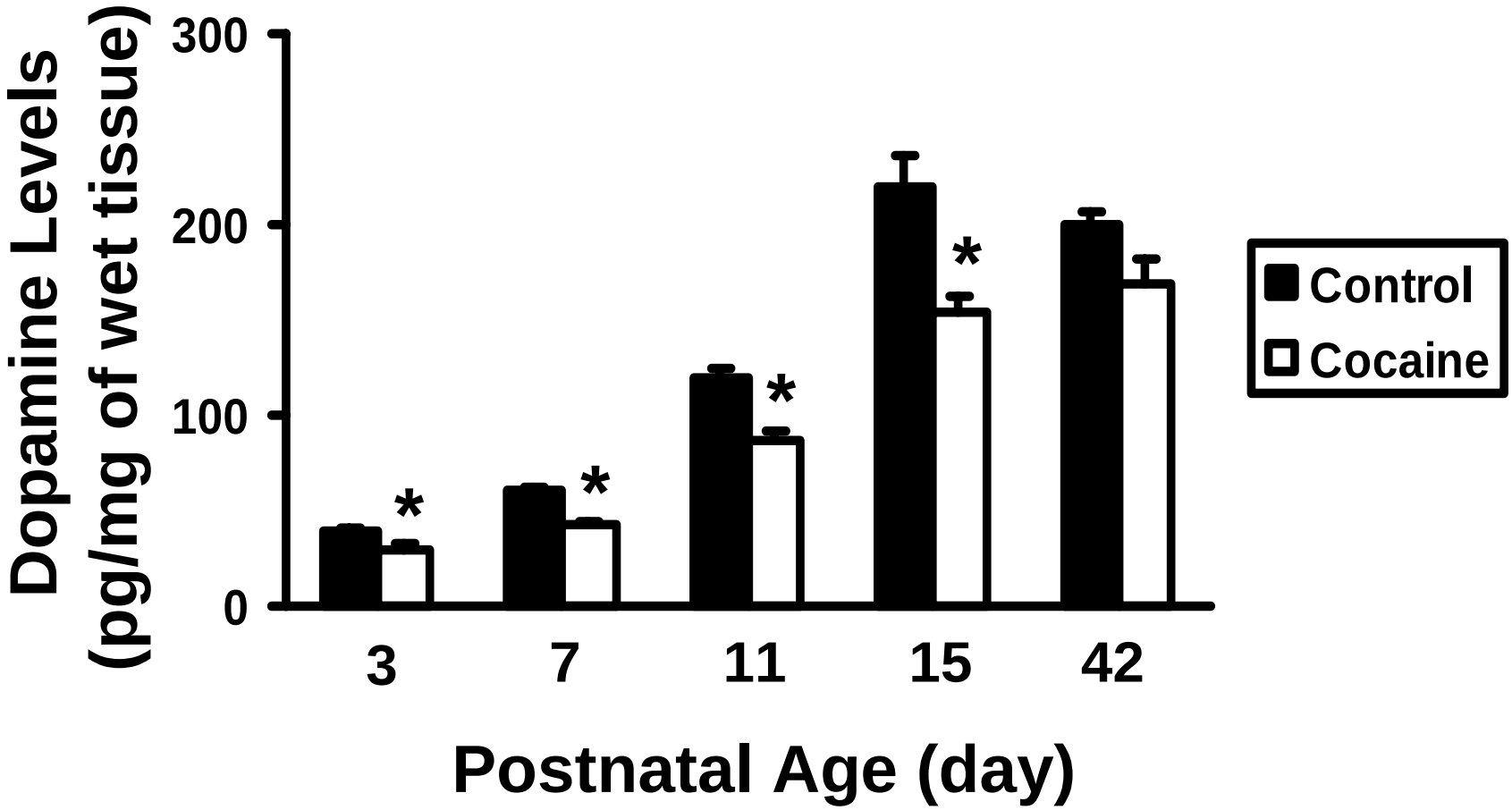

Fig. 2

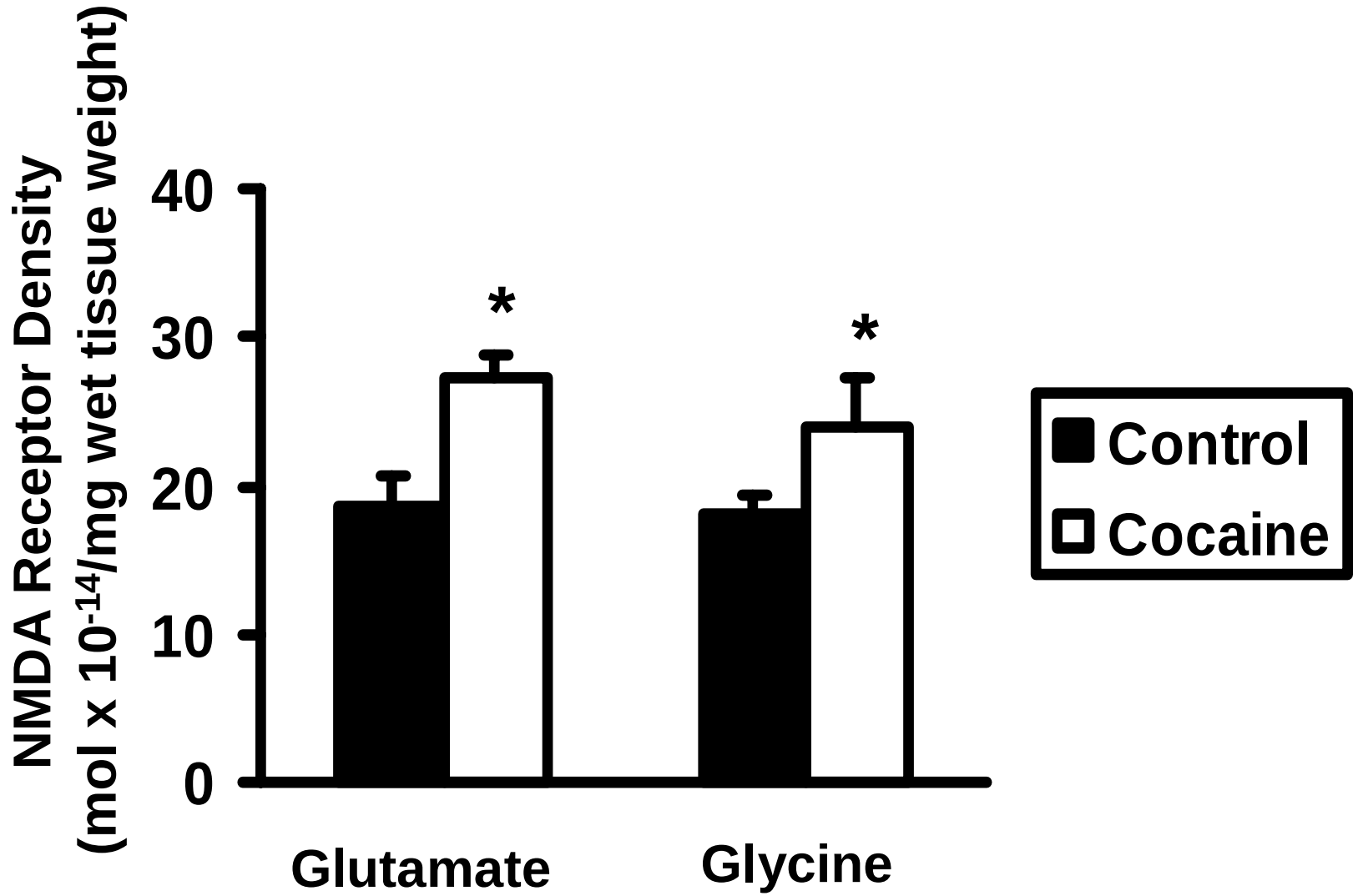

Fig. 3