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Prenatal Benzodiazepine Administration. II. Lorazepam Exposure Is Associated With Decreases in [^{35}S]TBPS Binding But Not Benzodiazepine Binding[1]

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MILLER, L. G., S. CHESLEY, W. R. GALPERN, D. J. GREENBLATT AND R. I. SHADER. *Prenatal benzodiazepine administration. II. Lorazepam exposure is associated with decreases in [^{35}S]TBPS binding but not benzodiazepine binding.* PHARMACOL BIOCHEM BEHAV **40**(2) 429–432, 1991.—Prenatal benzodiazepine exposure has been associated with neurobehavioral alterations in humans and animals. To determine effects of prenatal benzodiazepine exposure on binding at the benzodiazepine and t-butylbicyclophosphorothionate (TBPS) sites on the $GABA_A$ receptor in mature offspring, we treated mice with lorazepam, 2 mg/kg/day, during days 13–20 of gestation. Binding was assessed at 6 weeks of age. There were no differences among controls, vehicle- or lorazepam-exposed mice in benzodiazepine receptor binding determined in vivo or in vitro. However, receptor density for [^{35}S]TBPS binding sites was decreased in lorazepam-exposed offspring compared to the other groups. These data are consistent with prior neurochemical results indicating decreased TBPS binding and $GABA_A$ receptor function in several systems.

Lorazepam Prenatal exposure Benzodiazepine GABA

SEVERAL clinical studies indicate that prenatal benzodiazepine exposure is associated with neurobehavioral alterations in exposed infants (5–7). In animals, a considerable literature supports these observations with regard to alterations in behavior [for reviews, see (4,22)]. Such alterations include motor activity, active and passive avoidance, operant response, and startle response. Indeed, a number of studies indicate that behavioral alterations in exposed offspring can persist into adulthood.

A more modest literature concerns effects of prenatal benzodiazepine exposure on neurochemical parameters [e.g., (18–20)], including alterations at the benzodiazepine binding site located on the $GABA_A$ receptor complex in brain [discussed in (1,17)]. Studies have indicated decreases or no change in binding in young and adult offspring after prenatal exposure. These conflicting results may be due in part to differences in drug choice, dosage, and receptor assays, all of which can influence results of binding studies (14).

Recently, we demonstrated that prenatal exposure to lorazepam was associated with decreases in $GABA_A$ receptor function and binding of the putative chloride channel ligand, t-butylbicyclophosphorothionate (TBPS) in chicks (17), and decreases in $GABA_A$ receptor function in mice (1). To determine if the benzodiazepine binding site or the putative chloride channel site labeled by TBPS was associated with changes in receptor function, we studied benzodiazepine and TBPS binding in mice exposed to lorazepam during the last week of gestation.

METHOD

Materials

Female CD1 mice, 6–8 weeks of age, were purchased from Charles River (Wilmington, MA), maintained on a 12-hour light/dark cycle, and given food and water ad lib. Osmotic pumps were obtained from Alza (Palo Alto, CA). [^{3}H]Flunitrazepam (spec.act. 70 Ci/mmol), [^{35}S]TBPS (spec.act. 90 Ci/mmol), and [^{3}H]Ro15-1788 (spec.act. 81 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Muscimol was obtained from Sigma (St. Louis, MO). Unlabeled TBPS was purchased from Research Biochemicals (Natick, MA).

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Flunitrazepam was a gift from Hoffmann-La Roche (Nutley, NJ). Lorazepam was a gift from Wyeth (Radnor, PA). All other reagents were obtained from standard commercial sources.

Drug Administration and Rearing

Lorazepam (2 mg/kg/day) and vehicle (PEG 400) were administered by subcutaneously implanted osmotic pumps during days 13–20 of gestation as previously reported (1). After birth, litters were culled to 8 mice, with 3–5 males and 3–5 females in all cases. All mice were transferred to untreated control dams for fostering. Mice were weaned at 3 weeks and transferred to single-sex cages, with litter distinctions maintained. All assays were performed at 6 weeks of age. Alterations were observed in $GABA_A$ receptor function at this time point in a prior study (1).

Benzodiazepine Binding

Benzodiazepine binding in vivo was performed as previously described (13). Briefly, mice were injected IV with 3 μCi [^{3}H]Ro15-1788. After 20 min, animals were sacrificed and brains rapidly removed and dissected on ice. After weighing, brain regions were dissolved in Protosol (40°C for 24 hours) and then counted by scintillation spectrometry. Synaptosomal membranes for benzodiazepine binding in vitro and binding studies in cortex were prepared as previously described (15). To duplicate or triplicate samples was added [^{3}H]FNTZ, 0.1–20 nM. To determine nonspecific binding, to an identical set of samples was added flunitrazepam, 10^{-5} M. After incubation at 40°C for 45 min, samples were filtered using a Brandel M48R (Gaithersburg, MD) onto Whatman GF/B filters. Filters were washed twice with cold buffer and counted by scintillation spectrometry.

TBPS Binding

TBPS binding was performed as previously described (15). Briefly, to samples of synaptosomal membranes, [^{3}H]TBPS, 2 nM was added, along with unlabeled TBPS (0–500 nM). Nonspecific binding was determined using picrotoxin, 10^{-5} M. After incubation for 90 min at 25°C, samples were filtered using a Brandel M48R (Gaithersburg, MD) onto Whatman GF/B filters. Filters were washed twice with cold buffer and counted by scintillation spectrometry.

Data Analysis

Binding data were analyzed using the EBDA programs (11). Data are analyzed by litter rather than by individual offspring, to control for possible litter effects. Litter data was derived from means of individual data. Data were compared using analysis of variance with Dunnett's test.

RESULTS

Benzodiazepine Receptor Binding In Vivo

Benzodiazepine binding in all five brain regions evaluated (cortex, hippocampus, hypothalamus, cerebellum, and pons-medulla) was similar in 4 to 5 litters among the three exposure groups (Fig. 1). Binding was similar in male and female offspring, so data reflect mice of both sexes.

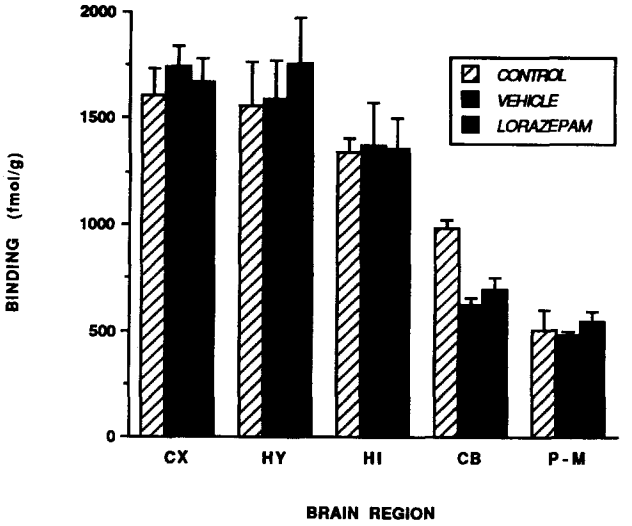

FIG. 1. Effects of prenatal benzodiazepine exposure on benzodiazepine receptor binding in vivo. Mice were exposed to lorazepam, 2 mg/kg/day, during days 13–20 of gestation. Assays were performed at 6 weeks of age using uptake of [^{3}H]Ro15-1788. Results are mean ± SEM of data including both male and female offspring from 4–5 litters for each exposure group, including 5–8 determinations per litter. There are no significant differences.

Benzodiazepine Receptor Binding In Vitro

Benzodiazepine binding in cortex was similar in lorazepam-exposed mice compared to vehicle-exposed and control offspring (Table 1). No significant changes were observed in apparent affinity or receptor density among the three exposure groups. Binding was similar in male and female offspring, so data reflect mice of both sexes.

TBPS Binding

Binding of the putative chloride channel ligand TBPS was decreased in lorazepam-exposed mice compared to vehicle-exposed and control offspring (Table 2). Receptor density was significantly reduced in lorazepam-exposed mice, with no change in apparent affinity. Vehicle-exposed mice were similar to control mice in both affinity and receptor density. Binding was

TABLE 1

EFFECTS OF PRENATAL BENZODIAZEPINE EXPOSURE ON BENZODIAZEPINE RECEPTOR BINDING

Exposure	Apparent Affinity (nM)	Receptor Density (pmol/mg protein)
Control	1.31 ± 0.19	1.34 ± 0.11
Vehicle	1.16 ± 0.14	1.20 ± 0.17
Lorazepam	1.45 ± 0.18	1.26 ± 0.10

Mice were exposed to lorazepam, 2 mg/kg/day, during days 13–20 of gestation. Assays were performed at 6 weeks of age using [^{3}H]flunitrazepam. Results are mean ± SEM of data including both male and female offspring from 3 litters for each exposure group, including 3–4 determinations per litter. There are no significant differences.

TABLE 2

EFFECTS OF PRENATAL BENZODIAZEPINE EXPOSURE ON [^{35}S]TBPS BINDING

Exposure	Apparent Affinity (nM)	Receptor Density (pmol/mg protein)
Control	42 ± 4	2.30 ± 0.11
Vehicle	48 ± 10	2.54 ± 0.16
Lorazepam	50 ± 15	1.81 ± 0.21*

Mice were exposed to lorazepam, 2 mg/kg/day, during days 13–20 of gestation. Assays were performed at 6 weeks of age using [^{35}S]TBPS. Results are mean ± SEM of data including both male and female offspring from 3 litters for each exposure group, including 3–4 determinations per litter. There are no significant differences. *$p<0.05$ vs. control and vehicle groups.

similar in male and female offspring, so data reflect mice of both sexes.

Offspring Survival and Development

As observed in prior studies (1), litter size and sex composition were similar in all three exposure groups. Lorazepam-exposed mice had decreased weight compared to the other two groups at 1 and 2 weeks, but no differences in weight were observed at 3 weeks and subsequently (data not shown).

DISCUSSION

Our data suggest that mice exposed prenatally to benzodiazepines do not develop persistent alterations in benzodiazepine binding, but that alterations in TBPS binding occur in mature animals. Similar binding to both sites was observed in male and female mice. These results are similar to those observed by several previous investigators using the rat as a model (8,12). The results are also consistent with our prior findings indicating no change in benzodiazepine binding but decreased TBPS binding and $GABA_A$ receptor function in chicks exposed to lorazepam in ovo (16), and decreased $GABA_A$ receptor function in mice exposed to lorazepam in utero (1). Although the benzodiazepine site is located on the $GABA_A$ receptor complex, alterations in receptor function may not be reflected in benzodiazepine binding. In contrast, alterations in TBPS binding largely occur in parallel with changes in receptor function (21).

Several investigators reported decreased benzodiazepine receptor binding in animals exposed prenatally to benzodiazepines. Gavish et al. (2) reported decreased benzodiazepine receptor density in cortex and cerebellum, while Livezey et al. (9,10) found decreased benzodiazepine binding in thalamus in the rat, and cortex and hypothalamus in the cat. The discrepancies among these studies, the studies cited above, and the present study may be in part due to species differences, choice of benzodiazepine and dose administered, interval of administration, and rearing of offspring. All prior studies used intermittent oral or parenteral administration, in contrast to constant administration used in the present study. Constant infusion was used in the present study to simulate the relatively constant plasma concentrations which occur in most patients taking benzodiazepines therapeutically (3). In addition, only one prior study determined benzodiazepine concentrations in plasma or cortex (4). In the model used in the present study, lorazepam was determined in dam, fetus, and offspring to ensure drug delivery (1).

With regard to drug choice and dose, Gavish et al. (2) used chlordiazepoxide, a precursor for diazepem, while all other studies used diazepem itself. Diazepam is metabolized in rodents and in humans to desmethyldiazepam and oxazepam, both of which are active. Doses used by Massotti et al. (12) are relatively large compared to other studies (100 mg/kg/day vs. 5–10 mg/kg/day). To avoid problems associated with multiple active metabolites and high doses, we used lorazepam, which has no active metabolites in the mouse or in humans. The dose chosen, 2 mg/kg/day, was effective in long-term studies in mice, and produced plasma and brain concentrations analogous to human therapeutic levels (15). Prior studies also varied in the interval of benzodiazepine administration during gestation. Massotti et al. (12) and Gavish et al. (2) treated dams during most of gestation, while Livezey et al. (9,10), Lauer et al. (8) and the present study treated dams during late gestation only.

Most prior studies did not control for rearing effects, and analyzed results by individual rather than litter. However, substantial evidence indicates that benzodiazepine treatment of dams may lead to alterations in rearing (4), which in turn might confound results. In addition, litter effects are well described in rodents, so that the unit of analysis should be the litter rather than the individual (22).

It is also possible that timing of assays might account for differences in results. Since behavioral alterations due to prenatal benzodiazepine exposure have been shown to be specific for developmental stages, it is possible that similar specificity occurs for neurochemical changes (4). Thus alterations in benzodiazepine binding might occur at a different time point than was evaluated in the present study. Similarly, the radioligands used in in vivo and in vitro benzodiazepine binding studies, [^{3}H]Ro15-1788 and [^{3}H]flunitrazepam, respectively, do not differentiate between benzodiazepine receptor subtype binding (21). It is possible that prenatal exposure might affect subtypes differentially but that this might not be reflected in total binding determinations.

Finally, differences in benzodiazepine receptor binding determinations may affect results (14). Differences in tissue preparation, buffers, temperature, and radioligand can affect binding results. A number of these variables can be avoided using in vivo binding as in the present study. The lack of change in both in vivo and in vitro binding noted here argues against assay procedures as an explanation for these results.

In adult mice, chronic lorazepam exposure is associated with decreased benzodiazepine binding in several brain regions (15). Binding returns to control levels rapidly after drug discontinuation (16), but then "overshoots" several days after discontinuation before again returning to control levels at 1 week after discontinuation (16). Since we did not determine receptor binding in the present study immediately after drug administration, results in adult mice cannot be directly compared to young animals. However, the reduction in TBPS binding in mice exposed in utero is in contrast to the lack of change in TBPS binding in adult mice exposed to lorazepam (16).

In summary, the present study used a treatment regimen known to provide adequate drug concentrations without active metabolites, and controlled for rearing and litter effects. Our results are consistent with prior studies of $GABA_A$ receptor binding and function in chicks and mice, and with considerable behavioral data indicating behavioral alterations in mature animals exposed to benzodiazepines prenatally. Further studies may identify the mechanism whereby prenatally administered benzodiazepines lead to persistent behavioral and neurochemical alterations.

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REFERENCES

1. Chesley, S.; Lumpkin, M.; Schatzki, A.; Greenblatt, D. J.; Shader, R. I.; Miller, L. G. Prenatal exposure to benzodiazepine. I. Prenatal exposure to lorazepam in mice alters open-field activity and $GABA_A$ receptor function. Neuropharmacology 30:53–58; 1991.
2. Gavish, M.; Avnimelech-Gigus, N.; Feldon, J.; Myslobodsky, M. Prenatal chlordiazepoxide effects on metrazol seizures and benzodiazepine receptor density in adult albino rats. Life Sci. 36:1693–1698; 1985.
3. Greenblatt, D. J.; Shader, R. I. Dependence, tolerance and addiction to benzodiazepines: Clinical and pharmacokinetic considerations. Drug Metab. Rev. 8:13–28; 1978.
4. Kellogg, C. K. Benzodiazepines: influence on the developing brain. Prog. Brain Res. 73:203–228; 1988.
5. Laegreid, L.; Olegard, R.; Wahlström, J.; Conradi, N. Abnormalities in children exposed to benzodiazepines in utero. Lancet 1:108–109; 1987.
6. Laegreid, L.; Olegard, R.; Wahlström, J.; Conradi, N. Teratogenic effects of benzodiazepine use during pregnancy. J. Pediatr. 114: 126–131; 1989.
7. Laegreid, L.; Olegard, R.; Conradi, N.; Hagberg, G.; Wahlström, J.; Abrahamsson, L. Congenital malformations and maternal consumption of benzodiazepines: a case-control study. Dev. Med. Child Neurol. 32:432–441; 1990.
8. Lauer, J. A.; Adams, P. M.; Johnson, K. M. Perinatal diazepam exposure: Behavioral and neurochemical consequences. Neurotoxicol. Teratol. 9:213–219; 1987.
9. Livezey, G. T.; Radulovacki, M.; Isaac, L.; Marczynski, T. J. Prenatal exposure to diazepam results in enduring reductions in brain receptors and deep slow wave sleep. Brain Res. 334:361–365; 1985.
10. Livezey, G. T.; Marczynski, T. J.; Isaac, L. Prenatal diazepam: Chronic anxiety and deficits in brain receptors in mature rat progeny. Neurobehav. Toxicol. Teratol. 8:425–432; 1986.
11. McPherson, G. A. A practical computer based approach to the analysis of radioligand binding experiments. Comput. Prog. Biomed. 17:107–114; 1983.
12. Massotti, M.; Alleva, F. R.; Baalazs, T.; Guidotti, A. GABA and the benzodiazepine receptors in the offspring of dams receiving diazepam: ontogenetic studies. Neuropharmacology 19:951–956; 1980.
13. Miller, L. G.; Greenblatt, D. J.; Paul, S. M.; Shader, R. I. Benzodiazepine receptor occupancy in vivo: correlation with brain concentrations and pharmacodynamic actions. J. Pharmacol. Exp. Ther. 240:516–522; 1987.
14. Miller, L. G.; Greenblatt, D. J.; Shader, R. I. Benzodiazepine receptor binding: influence of physiologic and pharmacologic factors. Biopharm. Drug Dispos. 8:103–114; 1987.
15. Miller, L. G.; Greenblatt, D. J.; Barnhill, J. G.; Shader, R. I. Chronic benzodiazepine administration: I. Tolerance is associated with downregulation of benzodiazepine receptor binding and γ-aminobutyric $acid_A$ receptor function. J. Pharmacol. Exp. Ther. 246: 170–176; 1988.
16. Miller, L. G.; Greenblatt, D. J.; Summer, W. R.; Shader, R. I. Chronic benzodiazepine administration: II. Discontinuation syndrome is associated with upregulation of γ-aminobutyric $acid_A$ receptor binding and function. J. Pharmacol. Exp. Ther. 246:177–182; 1988.
17. Miller, L. G.; Roy, R. B.; Weill, C. L.; Lopez, F. Persistent alterations in $GABA_A$ receptor binding and function after prenatal lorazepam administration in the chick. Brain Res. Bull. 23:171–174; 1989.
18. Miranda, R. C.; Wagner, J. P.; Kellogg, C. K. Early developmental exposure to benzodiazepine ligands alters brain levels of thiobarbituric acid-reactive products in young adult rats. Neurochem. Res. 14:1119–1127; 1989.
19. Miranda, R.; Cekcler, T.; Guillet, R.; Kellogg, C. K. Early developmental exposure to benzodiazepine ligands alters brain spectra in young adult rats. Brain Res. 506:85–92; 1990.
20. Miranda, R.; Ceckler, T.; Guillet, R.; Kellogg, C. K. Aging-related changes in brain metabolism are altered by early developmental exposure to diazepam. Neurobiol. Aging 11:117–122; 1990.
21. Schwartz, R. D. The $GABA_A$ receptor-gated ion channel: biochemical and pharmacological studies of structure and function. Biochem. Pharmacol. 37:3369–3375; 1988.
22. Tucker, J. C. Benzodiazepines and the developing rat: A critical review. Neurosci. Biobehav. Rev. 9:101–111; 1985.