Bull. Environ. Contam. Toxicol. (1988) 40:294–300

Bulletin of Environmental Contamination and Toxicology

Effects of Prenatal Exposure to 2,4-D/2,4,5-T Mixture on Postnatal Changes in Rat Brain Glutamate, GABA, Protein, and Nucleic Acid Levels

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The opportunity of maternal exposure to various chemicals in the workplace and the general environments have increased, and the fetus and neonate may be at greater risk than the adult (Friedman 1984). However, the embryotoxic and teratogenic effects of the chlorinated phenoxy herbicides 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), the main chemicals in Agent Orange, are well documented only in laboratory animals (Friedman 1984; Fagan and Pollak 1984). The brain of the developing fetus is vulnerable to the toxic effects of the phenoxy herbicides which readily cross the placental barrier and distribute into fetal tissues, including brain (Courtny et al. 1977; Dencker 1976; Lindquist and Ullberg 1971). Consequently 2,4,5-T is a behavioral teratogen in rats (Sjoden and Soderberg 1978; Crampton and Rogers 1983) and chickens (Sanderson and Rogers 1981). A 1:1 mixture of 2,4-D and 2,4,5-T at doses not resulting in malformations (non-teratogenic) is also behaviorally teratogenic in rats (Mohammad and St. Omer 1986; St. Omer and Mohammad 1987).

Although the neurochemical basis for the behavioral teratogenicity of the phenoxy herbicides is not known, Mohammad and St. Omer (1985) recently reported that non-teratogenic doses of a 1:1 mixture of 2,4-D and 2,4,5-T delayed the ontogeny of dopamine and serotonin in the brain of the developing rat. This communication provides further descriptive information about the ontogeny of rat brain nucleic acid, protein, glutamate and γ-aminobutyrate (GABA) following *in utero* exposure to non-teratogenic levels of a 1:1 mixture of 2,4-D/2,4,5-T.

MATERIALS AND METHODS

Female (age, 66-85 days) Sprague Dawley rats (Sasco) were bred

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in our laboratory with day of pregnancy (day 0) determined by visualization of vaginal plugs. Pregnant animals were caged individually in a room at 22-23°C, with a 12:12 hr light-dark cycle. Water and laboratory rat chow were available ad libitum.

On gestational days 6 to 15 pregnant rats were gavaged once daily with 2 ml/kg of peanut oil vehicle with 0 (control), 50 or 125 mg/kg of a 1:1 mixture of the acid form of 2,4-D and 2,4,5-T (Sigma, St. Louis, MO) containing 0.0125 ppm of 2,3,7,8 tetrachloro-p-dioxin (Verified at the Environmental Trace Substances Research Center, Columbia, MO). Each litter was reduced to 8 pups with equal number of males and females on postnatal day (PND) 1 and weaned on PND21. Offspring were killed by immersion in liquid nitrogen for 10 seconds on PND1, 15 or 22. The near-frozen brain was dissected out at 0°C and the cerebrum, cerebellum, neocortex or thalamus-hypothalamus were removed and frozen immediately in liquid nitrogen. Brain tissues were homogenized in 3 ml of 75% ethanol (U.S. Industrial Chemical Co., New York) containing 1% potassium acetate (Sigma). The homogenate was centrifuged at 2500 rpm for 10 min at 4°C. The supernatant was used for GABA and glutamate determinations using enzymic methods and fluorometric measurements of pyridine nucleotide coenzymes (Graham and Aprison 1966). The remaining pellet was used to extract DNA, RNA and protein (Munro and Fleck 1966). The RNA levels were determined by the cupric ion catalyzed orcinol reaction (Lin and Schjeide 1969). A diphenylamine colorimetric method was used to determine DNA levels (Geel and Timiras 1967). For protein analysis, the Coomassie blue dye-binding method of Bradford (1976) was used with some modifications. Aliquots of 0.1 ml of protein samples were diluted with 0.5 ml Tris-KCl buffer consisting of (Sigma) 0.15 M KCl, 0.01 M Tris HCl at pH 7.5. A 0.1 ml aliquot of this buffered protein sample was mixed with 5 ml of the diluted (1:4) and filtered Coomassie blue G-250 reagent (Bio-Rad, Richmond, CA). The protein-dye mixture was allowed to stand at room temperature for 10 min before reading the absorbance at 595 nm. A model 2600 UV/VIS colorimetric spectrophotometer (Gilford, Oberlin, OH) was used for all colorimetric procedures. The data were analyzed by one-way anaylsis of variance followed by the least significance difference test. The accepted level of statistical significance was at $p<0.05$.

RESULTS AND DISCUSSION.

DNA concentration is a chemical estimate of cell density while RNA/DNA and protein/DNA ratios are indicators of cell size (Winck and Noble, 1965). With the exception of a significant ($p<0.05$) 8% reduction in RNA content of neocortex in the 125 mg/kg group offspring on PND22, there were no other significant developmental effects of treatment on regional brain contents of protein and nucleic acids (RNA,DNA) on PND1, 15 or 22 (Table 1). The overall effects of 2,4-D/2,4,5-T treatments on the developmental pattern of the ratios of protein and RNA contents of regional brain areas to DNA were not significant (Table 1).

Table 1. Postnatal changes in regional brain protein and nucleic acid concentrations and protein/DNA and RNA/DNA ratios of rats prenatally exposed to vehicle or 2,4-D/2,4,5-T mixture on gestational days 6 to 15.

Brain	Age (day)	Treatment Groups								
		0 mg/kg	50 mg/kg	125 mg/kg	0 mg/kg	50 mg/kg	125 mg/kg	0 mg/kg	50 mg/kg	125 mg/kg
		Protein (mg/g wet tissue)			DNA (mg/g wet tissue)			RNA (mg/g wet tissue)		
Cerebrum	1	49.9±2.4	51.3±2.4	46.3±2.9	3.1±0.2	3.8±0.2	3.2±0.2	4.2±0.3	4.1±0.3	3.7±0.3
Cerebellum	1	48.8±1.7	48.1±1.6	45.0±2.1	1.9±0.1	2.0±0.1	1.8±0.3	3.1±0.2	3.2±0.2	3.1±0.2
Cerebellum	15	75.4±1.7	77.1±1.6	77.8±1.1	6.0±0.2	5.9±0.3	6.4±0.1	3.5±0.2	3.7±0.2	3.8±0.1
Thalamus-Hypothalamus	15	54.4±2.1	55.0±1.1	54.8±2.2	1.0±0.1	1.0±0	1.0±0	2.8±0.1	2.8±0.1	2.9±0.1
Neocortex	15	56.2±1.7	56.7±1.8	57.1±1.6	1.0±0	1.1±0.1	1.1±0.1	2.9±0.1	2.9±0.1	3.1±0.1
Thalamus-Hypothalamus	22	59.3±7.0	61.8±2.2	61.3±1.8	0.9±0	1.0±0	1.1±0	2.5±0.1	2.8±0.1	2.7±0.1
Neocortex	22	72.6±1.4	74.6±4.0	77.0±1.5	1.1±0	1.0±0.1	1.1±0	2.9±0.1	2.8±0	2.7±0.1*
		Protein/DNA (mg/mg)			RNA/DNA (mg/mg)					
Cerebrum	1	16.5±1.0	15.9±0.6	14.6±0.8	1.4±0.1	1.3±0.1	1.2±0.1			
Cerebellum	1	26.5±1.0	25.5±1.9	25.6±1.8	1.7±0.1	1.7±0.1	1.8±0.1			
Cerebellum	15	12.8±0.6	13.2±0.8	12.3±0.4	0.6±0	0.6±0	0.6±0			
Thalamus-Hypothalamus	15	54.9±3.3	57.8±0.7	56.5±2.8	2.8±0.2	2.9±0.1	3.0±0.1			
Neocortex	15	56.8±1.4	53.5±2.5	52.7±2.5	2.9±0.1	2.7±0.1	2.9±0.1			
Thalamus-Hypothalamus	22	71.0±2.2	64.7±2.6*	59.0±1.7*	2.7±0.1	3.0±0.2	2.6±0.1			
Neocortex	22	67.4±2.5	75.2±2.4	70.3±2.7	2.7±0.1	2.9±0.2	2.5±0.1			

Values for neurochemistry are means ± SEM.
Number of rats (N)/group = 9 to 12, except in neocortex (day 22) N = 5.
*Significantly different from the appropriate control values, $P<0.05$. control

However, on PND22 the protein/DNA ratios in the thalamus-hypothalamus of the 50 and 125 mg/kg groups were significantly reduced below control values by 9% and 17% respectively. These results did not indicate any generalized gross deficiencies in the treatment groups prior to weaning, but a postweaning effect was suggested. It is of interest that Duffard et al (1982) reported that 2,4-D, when painted on preincubated eggs at fetotoxic doses, decreased protein and lipid contents in the brains of chicks that were examined 24 hr after hatching. The subcutaneous administration of 50 mg/kg of 2,4,5-T to pregnant rats on gestational day 14 resulted in the inhibition of 16 day old fetal cerebral ribonucleotide reductase (a rate-limiting enzyme in DNA synthesis) assayed in vitro. (Millard et al. 1973). Thus the possibility of an ontogenetic lag in the expression of postnatal effects of 2,4-D/2,4,5-T on brain maturation and growth should be further examined.

Glutamate and GABA are important putative excitatory and inhibitory neurotransmitters respectively. Glutamate is the precursor of GABA. Both have an intimate biochemical relationship to the tricarboxylic acid cycle in the brain as well as synaptic connections to the catecholaminergic system (Donzanti and Uretsky 1983). From an ontogenetic point of view, it is of interest that in one day old pups exposed prenatally to 50 and 125 mg/kg of 2,4-D/2,4,5-T, the glutamate levels in the cerebrum were significantly lower than control values by 9% and 13% respectively, and in the cerebellum, by 23% and 11% respectively (Table 2). No significant changes occurred in glutamate levels in any of the regional brain areas examined on PND 15 and 22. The levels of GABA in the cerebrum, cerebellum, neocortex or thalamus-hypothalamus were not significantly affected by the 2,4-D/2,4,5-T treatments (Table 2). GABA is formed by decarboxylation of glutamate, but there is a significant lag in the development of the biosynthetic enzyme glutamic acid decarboxylase (Coyle and Enna 1976). At birth, for example, the specific enzymic activity is 12% of that of the adult whereas the relative concentration of GABA is 5 fold higher. It is thus possible that a neonatal decrease in glutamate levels, as was observed in this study, would not be reflected as a change in neonatal GABA levels.

The reduction in the brain glutamate levels seen on PND 1 in the present study, together with previously reported reductions in brain dopamine levels and dopamine turnover (Mohammad and St. Omer 1985; St. Omer and Mohammad 1987) during the preweaning period, suggest that prenatal exposure of rats to non-teratogenic levels of 2,4-D/2,4,5-T results in specific rather than generalized changes in neurotransmitter systems in the brains of the preweaned offspring. If so, then the relationship between the catecholaminergic and glutaminergic and/or GABAergic systems deserves further study. This is important in order to understand the nature of the neurological consequences associated with in utero exposure to the phenoxy herbicides 2,4-D/2,4,5-T.

Table 2 Regional concentrations of glutamate and GABA in the brains of rats at various ages following prenatal exposure to vehicle or 2,4-D/2,4,5-T mixture on gestational days 6 to 15.

Brain	Age (day)	Treatment Groups					
		0 mg/kg	50 mg/kg	125 mg/kg	0 mg/kg	50 mg/kg	125 mg/kg
		Glutamate (μ mol/g wet tissue)			GABA (μ mol/g wet tissue)		
Cerebrum	1	4.17 ± 0.12	3.81 ± 0.13*	3.61 ± 0.15*	0.69 ± 0.02	0.70 ± 0.02	0.74 ± 0.03
Cerebellum	1	2.88 ± 0.11	2.21 ± 0.15*	2.56 ± 0.09*	1.03 ± 0.04	0.92 ± 0.06	0.98 ± 0.04
Cerebellum	15	3.96 ± 0.21	4.09 ± 0.21	4.36 ± 0.27	0.73 ± 0.06	0.69 ± 0.04	0.70 ± 0.04
Thalamus-Hypothalamus	15	5.21 ± 0.35	5.49 ± 0.35	5.31 ± 0.54	2.32 ± 0.17	2.49 ± 0.18	2.31 ± 0.24
Neocortex	15	8.85 ± 0.84	8.28 ± 0.85	7.86 ± 0.62	1.68 ± 0.14	1.53 ± 0.17	1.42 ± 0.15
Thalamus-Hypothalamus	22	5.83 ± 0.52	4.89 ± 0.96	5.56 ± 0.40	1.94 ± 0.09	1.89 ± 0.30	2.34 ± 0.22
Neocortex	22	8.35 ± 0.81	7.09 ± 0.85	6.94 ± 0.60	1.51 ± 0.12	1.37 ± 0.06	1.39 ± 0.11

Values are means + SEM.
Number of rats ($\bar{N}$)/group = 8 to 12, except at age 22 day, N = 5.
*Significantly different from the appropriate control values, $P<0.05$.

Acknowledgements. Supported by grants from the Veterinary Medical Research Council, University of Missouri-Columbia and Biomedical Research Grant RR07053 (NIH).

REFERENCES

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Courtney KD, Ebron MT, Tucker AW (1977) Distribution of 2,4,5-trichlorophenoxyacetic acid in the mouse fetus. Toxicol Lett 1:103-108.

Coyle JT, Enna SJ (1976) Neurochemical aspects of the ontogenesis in the GABAnergic neurons in the rat brain. Brain Res 111:119-133.

Crampton MA, Rogers LJ (1983) Low doses of 2,4,5-trichlorophenoxyacetic acid are behaviorally teratogenic to rats. Experientia 39:891-892.

Dencker L (1976) The herbicide 2,4,5-T: Early placental barrier and accelerated fetal uptake with advancing gestation. Acta Pharmacol Toxicol 39:59-72.

Donzanti BA, Uretsky NJ (1983) Effects of excitatory amino acids on locomotor activity after bilateral microinjection into the rat nucleus accumbense: possible dependence on dopaminergic mechanisms. Neuropharmacology 22:971-981.

Duffard R, De Moro GM, De Duffard AME (1982) Hatching and lipid composition of chicks brain from eggs treated with 2,4-dichlorophenoxyacetic butylester. Toxicology 24:305-311.

Fagan K, Pollak JK (1984) The effect of the phenoxyacetic acid herbicides 2,4,5-trichlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid as ascertained by direct experimentation, Res Rev 92:29-58.

Friedmann, JM (1984) Does Agent Orange cause birth defects? Teratology 29:193-221.

Geel SE, Timiras PS (1967) The influence of neonatal hypothyroidism and of thyroxine on the ribonucleic acid and deoxyribonucleic acid concentrations of rat cerebral cortex. Brain Res 4:135-142.

Graham Jr LT, Aprison MH (1966) Fluorometric determination of asparate, glutamate, and γ-aminobutyrate in nerve tissue using enzymic methods. Anal Biochem 15:487-497.

Lin RI, Schjeide DA (1969) Micro estimation of RNA by the cupric ion catalyzed orcinol reaction. Anal Biochem 27:473-483.

Lindquist NG, Ullberg S (1971) Distribution of the herbicides 2,4,5-T and 2,4-D in pregnant mice. Accumulation in the yolk sac. Experientia 27:1439-1441.

Millard SA, Hart MB, Shimek JE (1973) Enzymes controlling cerebral DNA synthesis: Response to 2,4,5-trichlorophenoxyacetic and acid and hexachlorophene. Biochem Biophys Acta 308:230-233.

Mohammad FK, St. Omer VEV (1985) Developing rat brain monoamine levels following in utero exposure to a mixture of 2,4-dichlorophenoxyacetic acids. Toxicol Lett 29:215-223.

Mohammad FK, St. Omer VEV (1986) Behavioral and developmental effects in rats following in utero exposure to 2,4-D/2,4,5-T mixture. Neurobehav Toxicol Teratol 8:551-560.
Munro HN, Fleck A, (1966) The determination of nucleic acids. Methods Biochem Anal 14:113-176.
Sanderson CA, Rogers LJ (1981) 2,4,5-trichlorophenoxyacetic acid causes behavioral effects in chickens at environmentally relevant doses. Science 211:593-595.
Sjoden PO, Soderberg U. (1978) Phenoxyacetic acids: Sublethal effects. in: Ecological Bulletins 27:149.
St. Omer VEV, Mohammad FK (1987) Ontogeny of swimming behavior and brain catecholamine turnover in rats prenatally exposed to a mixture of 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acids. Neuropharmacology (in press).
Winick M, Noble A (1965) Quantitative changes in DNA, RNA and protein during prenatal and postnatal growth in the rat. Dev Biol 12:451-466.

Received June 22, 1987; accepted October 3, 1987.