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Behavioral Effects of Exposure to Halothane during Early Development in the Rat:

Sensitive Period during Pregnancy

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Pregnant female rats were anesthetized with halothane for two hours during the middle of either the first, second, or third trimester (each trimester in the rat is seven days). Adult male offspring of these exposed mothers and of unexposed controls were tested on a difficult visual discrimination task and assessed for sensitivity to electric footshock (painful stimulus). Measures of activity, water intake, and adult body weight were also taken. Although exposure to halothane produced no significant change in the latter three measures, offspring of mothers exposed in the first and second trimesters took 39 and 41 per cent more error trials, respectively, to learn the maze task. First- and second-trimester-exposed offspring also had 26 per cent lower footshock response thresholds to the highest magnitude of response. Offspring of mothers exposed in the third trimester were not significantly different from controls in any of the measures taken. The impairments seen in the first-trimester-exposed (day 3–blastula) offspring may have been due to residual halothane or halothane metabolites retained until later in pregnancy. The data indicate that the (possibly teratogenic) effects of exposure to halothane during early development in the rat are maximal during the second trimester, when organogenesis is occurring, and that exposure to halothane during this period produces learning deficits and changes in footshock sensitivity in adult offspring. (Key words: Anesthesia, volatile: halothane. Pregnancy: teratogenicity. Toxicity: fetal; neurotoxicity; teratogenicity.)

A RECENT SERIES of reports has demonstrated that chronic exposure to low (10 ppm) concentrations of halothane during early development produces in the offspring a variety of behavioral effects that can be demonstrated in adulthood. Two learning tasks, a shock-motivated visual discrimination and a food-motivated symmetrical maze, were more difficult for the offspring of exposed mothers to learn.[1,2] While it is difficult to use these tests as an indicator of specific brain damage, poor performance on both of these tasks might be indicative of a general learning deficit, and might indicate damage to either cortical areas of brain[3] or the limbic system.[4] In addition to the reported deficits on learning tasks, adult rats exposed to halothane during early development were more sensitive to footshock (painful stimulus) than unexposed controls.[2] Increased sensitivity to footshock has been related to disruption of the serotonergic neurochemical system of the brain,[5] which has been implicated in a number of other functions, including sleep.[6] All of these changes were specific to exposure during early development, since a comparable exposure period in adulthood produced no detectable change in any of these measures. Such behavioral data suggest that early exposure to halothane might have teratogen-like effects. Indeed, electron microscopic analysis of cerebral cortical sections from rats exposed during early development has demonstrated that such exposure produces central nervous system (CNS) damage such as synaptic malformation, disruption of the nuclear envelope, and cell death.[7] The behavioral methods used for assessing changes in the CNS subsequent to early exposure to halothane can thus detect functional changes at halothane levels that also produce anatomic damage at the electron microscopic level.

Chronic exposure to low concentrations of halothane cannot, however, show whether there are sensitive periods during early development. If halothane had teratogen-like effects on the CNS, it would be likely to have maximal effects during the periods of sensitivity to other known teratogens, *i.e.*, during the organogenesis stage of development in the second trimester of development in the rat.[8] In the present study, therefore, we sought to answer the question of whether a sensitive period during pregnancy exists, when brief exposure to halothane produces maximal effects on the behavior of the offspring during adulthood.

Materials and Methods

Pregnant female Sprague-Dawley rats were anesthetized in 1.5-l plexiglass chambers during the middle of the first, second, or third trimester of pregnancy, *i.e.*, on day 3, day 10, or day 17. Halothane, 2.5 per cent, was administered for 5 min, followed by 1.2 per cent for another 115 min. The halothane was

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TABLE 1. Total Numbers of Error Trials on the Maze Task According to Three Increasingly Difficult Criteria (Mean ± SEM)

	Criterion (Per Cent Correct)		
	70 Per Cent	80 Per Cent	90 Per Cent
Control	116 ± 8.8	144 ± 7.5	161 ± 10.9
Day 3	129 ± 21.9	183* ± 26.2	225* ± 26.7
Day 10	150 ± 26.0	210* ± 25.6	227* ± 25.2
Day 17	98 ± 9.9	122 ± 14.1	162 ± 17.0

* Significantly different from control, $P < .05$.

metered through a Dräger vaporizer and added to a stream of nitrogen, 65 per cent–oxygen, 35 per cent, at a total flow of 1.33 l/min. Gas samples were taken from various parts of the exposure chamber and analyzed for halothane, as previously described.[1] The combination of small chamber size, flow rate, and inspired oxygen concentration prevented hypoxia during anesthesia. Blood-gas analyses of eight adult rats similarly anesthetized indicated an absence of hypoxia or significant hypercarbia (Pa_{O_2} 89 ± 2.1 (SEM) torr, Pa_{CO_2} 45 ± 1.8 torr), compared with eight awake animals breathing ambient room air (Pa_{O_2} 75 ± 1.2 torr, Pa_{CO_2} 34 ± 1.8 torr). At all times except during the anesthetic exposure, the pregnant rats were housed in individual breeding boxes, as were control animals that received no anesthesia.

Following birth, litters were culled to eight pups by discarding females, and were reared by their natural mothers in breeding boxes. Only males were used in behavioral testing, to avoid cyclic hormonal influences on footshock sensitivity, which have been reported to occur in females.[9] These procedures produced 25 subjects from six litters in the control group, 12 subjects from five litters in the day-3 group, 14 subjects from six litters in the day-10 group, and 11 subjects from five litters in the day-17 group. There were fewer litters delivered from the day-3 group than from other groups; this difference was slight (approximately 75 per cent delivered *vs.* 80–85 per cent for all other groups), and no attempt was made to determine whether this was due to increased fetal resorption or a slightly lower impregnation rate. There was no systematic difference in litter sizes among the treatment groups, although all had somewhat smaller litters than control mothers.

Litters were weaned at 28 days, and testing began at 75 days of age, which developmentally corresponds approximately to young adulthood in man. All animals were tested on the shock-motivated Y-maze discrimination task, and one to two weeks after maze training, on footshock sensitivity, both of which have been reported to reflect effects of early exposure to halothane.[1,2] In addition, adult body weight, activity as measured in a running wheel, and water intake were also monitored for periods during adulthood. The sequence of testing was fixed and was the same for all groups.

The automated Y-maze has been described.[1] For each trial, the rat was required to choose between a steady light and a 2/sec flashing light in the alternative goal boxes. Failure to run into the flashing goal box within 5 sec of the start of the trial resulted in an intermittent 0.6-ma shock to the floor of the chamber until the correct response was made. The correct side was randomly alternated. Rats were tested for 30 trials per day until they reached a criterion of 90 per cent correct choices, or for a maximum of 28 days.

Shock sensitivity was determined in a small plexiglass cage (7 × 7 × 20 cm) with a shock grid floor, patterned with modifications from a device described by Hoffman and Fleshler[10] and used by Hoffman, Fleshler, and Abplanalp.[11] The chamber was rigidly connected to a telephone diaphragm so that any movement of the cage produced a voltage output, which was then amplified, rectified, and recorded on an Esterline-Angus recording milliammeter. The magnitude of the rat's response to the shock thus produced a proportional movement on the pen record. Shocks of 0.2-sec duration were administered by electromechanical programming from a BRS/LVE shock generator/scrambler. The rat received four series of shocks, with each series consisting of ten ascending amplitudes from 0.2 to 2.0 ma in 0.2-ma increments, followed by ten shocks is descending order of intensity. Fifteen seconds elapsed between shocks, and series were separated by 2 min.

TABLE 2. Shock Thresholds (ma) for Four Response Amplitudes (Mean ± SEM)

	Pen Deflection			
	5 mm	10 mm	20 mm	40 mm
Control	1.053 ± .118	1.183 ± .131	1.273 ± .128	1.463 ± .115
Day 3	.711* ± .167	.833* ± .166	.944* ± .169	1.077* ± .175
Day 10	.572* ± .067	.760* ± .062	.905* ± .062	1.078* ± .071
Day 17	1.103 ± .108	1.235 ± .110	1.359 ± .111	1.620 ± .101

* Significantly different from control, $P < .05$.

For the maze data, the number of error trials to each of the successively more difficult criteria of 70, 80, and 90 per cent correct trials in a given day were analyzed using a two-factor repeated-measures analysis of variance. Newman-Keuls post-hoc tests were used to determine which groups differed significantly from controls.[12] For all animals not reaching criterion within 28 days of testing, the number of error trials during the 28 days of testing were used as data, rather than discarding those subjects; this conservative estimate of their performance tended to bias the data *against* differences between control and exposed groups, since all animals failing to reach criterion were in the day-3 and day-10 groups.

For the footshock sensitivity data, thresholds to four magnitudes of response, corresponding to 5, 10, 20, and 40 mm of pen deflection, were determined for each subject. This range roughly covers the animal's response from a small flinch to a vigorous jump, and thus samples a wide range of responsiveness. Two-factor analyses of variance were used to determine whether significant treatment effects existed, with post-hoc tests as on the maze task.

In addition, since several animals from each litter were tested, we sought to confirm that any patterns of significance from the individual animal's data were not due to litter effects. Since using the mean of each litter's performance as the data to be analyzed decreased the power of the statistical tests, we used planned comparisons to maximize power.[12] Wilson[8] has suggested that teratogenic effects are maximal during organogenesis, and that in the rat these effects should be maximal during early pregnancy, while becoming less severe in later pregnancy (although some development continues for a considerable length of time postnatally). Accordingly, we hypothesized that, if halothane had teratogen-like effects, these should be maximal at the day-3 and day-10 exposures, and less pronounced on day 17. Our planned comparisons for the litter mean analyses therefore compared the combined day-3 and day-10 offspring with the control and day-17 offspring. A statistical significance of $P < .05$ was used for all tests. F and t values, and degrees of freedom (df) for each, are reported.

Results

The numbers of error trials required by each group to reach the successively more difficult criteria of 70, 80, and 90 per cent correct responses in a given day indicated that there were significant treatment effects ($F = 4.03$, $df = 3, 58$) and criterion effects ($F = 54.11$, $df = 2, 116$) (table 1). Newman-Keuls post-hoc tests indicated that the offspring of mothers exposed on day 3 or day 10 of gestation took significantly more error trials to learn the task to the two most difficult criteria, requiring 39 and 41 per cent more error trials, respectively, to reach the 90 per cent criterion. Although both of these groups tended to require more trials to reach the 70 per cent criterion, this was not statistically significant. The more difficult criteria enhanced the deficit produced by early exposure to halothane. Offspring of mothers exposed on day 17 of pregnancy had no tendency to perform differently from controls.

TABLE 3. Running-wheel Activities, Water Intakes and Body Weight (Mean ± SEM)

	Control	Day 3	Day 10	Day 17
Running-wheel activity (revolutions/day)	198 ± 39.3	203 ± 46.1	224 ± 39.3	346 ± 102.4
Water intake (ml/day)	33 ± 2.1	34 ± 3.9	33 ± 1.2	33 ± 1.9
Body weight (125 days)	375 ± 11.2	377 ± 10.4	374 ± 8.4	375 ± 9.9

A litter mean analysis of the maze data tested the hypothesis that day-3 and day-10 groups performed worse than day-17 and control groups; this t test was significant ($t = 1.77$, $df = 18$) indicating that the patterns of significance in the individual animals' analyses were due to treatment effects, rather than to litter effects.

The footshock sensitivity data (table 2) also indicated that there were significant treatment ($F = 4.97$, $df = 3, 68$) and response criterion ($F = 120.20$, $df = 3, 204$) effects. Post-hoc tests to analyze these differences indicated that the day-3 and day-10 offspring were significantly more sensitive to footshock at each response amplitude analyzed, while day-17 offspring again failed to differ from controls. For example, at the highest response amplitude we analyzed, day-3 and day-10 offspring required a 27 per cent lower shock intensity to produce a 40-mm pen deflection, while the day-17 offspring required a nonsignificantly greater shock than controls. As with the maze data, planned comparisons demonstrated that litter effects were not responsible for the observed patterns of significance; litter mean analysis indicated significant treatment effects ($F = 8.67$, $df = 1, 57$), with day-3 and day-10 litters more sensitive to footshock than control or day-17 animals.

There was no significant effect of exposure to halothane at any of the three points in development upon the adult body weight of the offspring, or on activity or water intake (table 3). This indicated that, although

effects of exposure to halothane on maze learning and footshock sensitivity were detected, effects on other measures commonly used to detect drug effects were not found.

Discussion

The procedures used here for halothane anesthesia avoided signs of systemic anoxia in blood-gas analyses; hence, the observed effect on maze learning and pain sensitivity would appear to result from some action of halothane on the central nervous system. As a mechanism of halothane toxicity at the cellular level, it is possible that halothane induces local as opposed to systemic anoxia. In support of this, Vannucci and colleagues have recently reported that halothane anesthesia at 10,000 ppm, but not at 4,000 ppm, produced neurochemical changes indicative of anoxia at the cellular level, despite normal oxygen values in blood.[13] The concentration we used, 12,000 ppm, would presumably produce similar changes.

Whatever the cellular mechanism for halothane toxicity, the present data indicate that a sensitive period for halothane toxicity exists during early pregnancy in rats. It is plausible that the learning deficits and the hyperalgesia observed in our rats are mediated by central neural damage resulting from exposure to halothane. This thesis is commensurate with the long-term residual nature of the observed effects, and with our previous observations of electron microscopic evidence of central neural damage. Our data indicate that exposure on day 17 did not significantly affect performance on any of the measures we used, although there are no data to indicate whether other measures are affected by day-17 exposure. Since the exposure on day 3 occurred before significant development of the CNS, it is likely that the effects of exposure to halothane on offspring of mothers exposed on day 3 of gestation may be due to maternal retention of sufficient halothane and/or metabolites to be toxic later in development. Although no data appear to exist for such retention in the rat, it has been reported that halothane metabolites are present in human urine for at least eight days following anesthetic exposure.[14] Our data, then, suggest that the middle trimester of pregnancy in the rat is the point during which the fetal nervous system is most susceptible to the effects of halothane.

Since previous studies have indicated that chronic exposure to low concentrations of halothane produces learning deficits in the offspring not specific to method of deprivation (food or shock),[1] it is possible that the deficit in maze learning indicated by the present data resulted from a general learning deficit in day-3 and day-10 offspring. However, it is not possible to infer specific brain damage from a learning deficit on a maze task, and an analysis of the behavioral nature or neural mechanism of this learning deficit remains for future research.

While it is not yet possible to specify the site of action for halothane toxicity in the developing fetal nervous system, some speculation can be advanced. Since serotonin has been repeatedly implicated in sensitivity to footshock,[5] and also in sleep[6] and stimulation-produced analgesia,[15] it is tempting to hypothesize that halothane might act via the serotonergic system in brain, and that such action at a sensitive period in development might interfere with normal development. Olsen and Seiger[16] have shown that the differentiation of the serotonin-containing cell bodies of the fetal nervous system occurs on days 11–15 in the rat. Hence, this differentiation occurs at such a time as to be influenced by residual halothane from either day-3 or day-10 exposures, and to be completed prior to a day-17 exposure. There is, furthermore, evidence that halothane has effects on brain serotonin. Roizen *et al.*[17] reported that halothane increased serotonin levels in several, but not all, areas of adult rat brain following anesthetic exposure. Bourgoin *et al.*[18] found no differences in whole-brain levels of serotonin following exposures to halothane and to nitrous oxide, but reported that such treatment lowered rates of synthesis of serotonin. Finally, offspring of rats chronically exposed to halothane throughout pregnancy had about 15 per cent lower levels of brain 5 hydroxyindoleacetic acid (5-HIAA), a catabolite of serotonin, than those found in unexposed offspring.[19] Thus, while the actions of halothane on the serotonergic system and the significance of these actions are not yet clear, there are some indications that such actions might occur. It thus appears possible that exposure to halothane during early fetal development may impair the development of the fetal serotonergic system, to produce increased footshock sensitivity in the adult.

The data from our study suggest that exposure to halothane prior to or during organogenesis in the rat produces behavioral effects analogous to teratogenesis. With respect to implications for man, these data should be interpreted cautiously. Day 10 of gestation in the rat, which was the point at which the present data indicated maximal effects of halothane, approximately corresponds to day 30 in man.[20] Thus, if halothane toxicity comparable to that seen in the rat is also present in man, exposure early in pregnancy could possibly be critical. No human data presently exist to support or refute this hypothesis.

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