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Consequences of Oral Gavage during Gestation and Lactation on Rat Dams and the Neurodevelopment and Behavior of Their Offspring

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Oral gavage is a popular route of drug administration during preclinical testing. Despite the growing body of information regarding the effects of oral gavage and the stress associated with this technique, the consequences of such exposure during pregnancy or lactation have rarely been investigated. Therefore, we sought to determine the consequences of oral gavage exposure during pregnancy and lactation on the neurodevelopment and behavior of rat offspring. Pregnant Sprague–Dawley dams underwent either no treatment or oral gavage of distilled water once daily from gestational day 7 until postnatal day 21. Oral gavage treatment had no significant effect on maternal parameters, including bodyweight gain, duration of gestation, litter size, and incidence of neonatal death. Compared with their counterparts from untreated dams, male and female progeny of gavaged dams had longer body lengths on PND 7 and 14 but reduced forelimb grip performance on PND 14 and 17. Therefore, the use of oral gavage during pregnancy and lactation in rats can have opposite effects on the somatic and behavioral development of the offspring. These factors should be considered when using oral gavage as a route of administration during pregnancy. In addition, the inclusion of no-treatment controls is important because they may reveal various restraint-associated effects.

Abbreviations: GD, gestational day; PND, postnatal day

Preclinical research, particularly in the last decade, has witnessed a growing emphasis on translation to and close modeling of the clinical situation. In addition, preclinical research has aimed to incorporate validity, particularly face, construct, and predictive validity. These criteria are essential when developing an animal model, and to predict the clinical outcome, the preclinical model should be robust, with findings that are consistent and reliable. Therefore, research groups have increasingly been incorporating clinically relevant parameters into their study designs. One such aspect is the oral administration of drugs. Since the late 1970s, researchers have been interested in the molecular and behavioral consequences associated with various routes of drug administration.

For example, in studies assessing behavior in the context of intraperitoneal, intravenous, or subcutaneous administration of methamphetamine, locomotor activity is affected most by intraperitoneal dosing, whereas stereotypy ratings are most influenced by subcutaneous administration[4]. More recently, our own studies found that neonatal outcome after prenatal methamphetamine exposure is route-dependent, with subcutaneous administration having a greater effect than oral gavage[10]. Therefore, it is unsurprising that Figure 1 highlights an increasing trend for oral administration since the early 1980s. The term 'oral administration' refers to the administration of an item in a food or drink or by gavage. Among these methods, gavage is the most common technique for dosing in pharmacokinetic and toxicokinetic studies,[6] with increasing numbers of researchers using this method of administration (Figure 2).

However, the effects associated with the gavage route itself remain unclear. The stress associated with the restraint that is necessary when using the oral gavage procedure has been of concern, but to date few studies have attempted to determine the level of stress induced by oral gavage in a controlled manner. In one study,[2] blood pressure, heart rate, and body temperature were all significantly elevated in rats for as long as 60 min after oral gavage administration of barium sulfate. Given that stress is defined as any external stimulus that challenges homeostasis,[11] it is reasonable to conclude that rats are acutely stressed by either the gavage administration or the restraint used to accomplish this procedure.

Little is known about the consequences of oral gavage during pregnancy, and this route of administration has become quite popular in recent years for preclinical drug evaluations in pregnancy, given the common clinical use of the oral route.[3,7-9,13,14] Therefore, the aim of the current study was to determine whether oral gavage, as a route of administration, during pregnancy and lactation affects neurodevelopmental and behavioral outcomes in the offspring of rat dams. We hypothesized that the stress associated with the oral gavage technique would adversely affect rat offspring, as measured by delays in neurodevelopmental parameters and deficits in behavior.

Materials and Methods

Animal housing. Adult male (weight, 275 to 325 g; age, approximately 4 mo) and naïve female (weight, 275 to 325 g; age, approximately 4 mo) Sprague–Dawley rats were used for this

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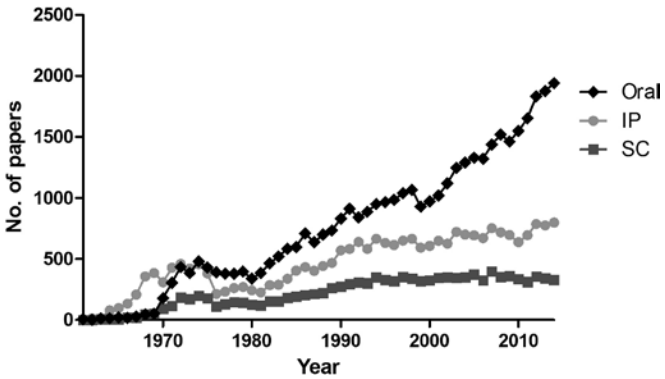

Figure 1. Number of articles published annually between 1961 and 2014. Total number of articles returned when 'oral administration and rats', 'ip administration and rats', or 'sc administration and rats' was entered into PubMed search engine.

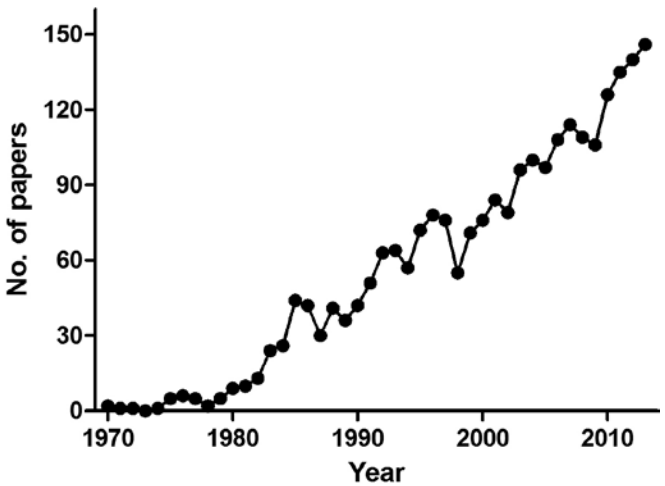

Figure 2. Number of studies involving oral gavage published annually between 1970 and 2014. Total number of articles returned when 'gavage administration and rats' was entered into PubMed search engine.

study. All female rats were bred inhouse; all male rats were obtained from Charles River (Kent, United Kingdom), and all animals were habituated for 1 wk after arrival. Our sentinel surveillance indicated that all rats were free of known bacterial, viral, and parasitic pathogens. After mating, all female rats were housed singly in plastic-bottom cages with appropriate bedding material (Pellets, 3Rs, United Kingdom) and additional nesting materials (unbleached cotton and Nesteldown bedding, Petworld, Galway). All rats were maintained under standard laboratory conditions under artificial 12:12-h light:dark cycle (lights on, 0800), and temperature was maintained at 20 to 24 °C with relative humidity at 35% to 60%. Food and water were provided without restriction. After parturition, pups remained with their biologic dams until postnatal day (PND) 21, at which point the pups were weaned. All experiments were approved by the Animal Care and Research Ethics Committee of the National University of Ireland (Galway; 12/NOV/07) and in compliance with the European directive (2010/63/UE).

Mating, gestational period and delivery. Three female rats were housed overnight with each sexually mature male rat (3:1). At the beginning of the light phase the following morning, vaginal smears were obtained from all female rats to check for the presence of sperm by light microscopy. Gestational day (GD) 0 was deemed the day that sperm was present in the smear. The expected day of delivery (birth) in rats is GD 21 to 22, and pregnant dams were checked daily. The pups were randomly culled (using a random number generator) to 10 per litter on PND 1, with equal numbers of male and female offspring whenever possible. One male and one female pup were selected for testing from each litter to avoid litter effects, and these same pups continued through all neonatal testing stages. Pups were selected based on weight; selected pups weighed the closest to the sex-specific average for the litter at PND 1. Each of the selected pups was injected intradermally with black India ink in the footpad for unique identification purposes within the litter.

Treatment. Rats were assigned randomly to no-treatment (NT) or gavage-treated groups according to body weight (n = 10 for NT, n = 12 for gavage). All female rats were habituated to handling and weighing for 1 wk before treatment began. Rats in the gavage group received distilled water (1 mL/kg) by oral gavage once daily at 1400 from GD 7 until PND 21 (time of weaning). Rats in the NT group were handled and weighed at the same time and frequency and differed only in not having to undergo oral gavage.

Daily maternal measurements and litter characteristics. Maternal body weight, food intake, and water consumption were recorded daily from GD 0 to PND 21 prior to dosing or handling of each rat (between 1400 and 1600). The duration of gestation was recorded for all mothers. At birth, pups were counted, sexed, and weighed. Any stillbirths were recorded. Offspring in each litter were checked and counted daily during the week after delivery to monitor for pup mortalities (dead or cannibalized). On PND 21 (day of weaning), all dams were euthanized by decapitation. Gross dissections were performed, and organs were removed and weighed.

Development of offspring. The development of the offspring involved examination of somatic development and behavioral testing. The day on which each test was performed related to the time at which the development milestone normally occurs in rats, and each test was performed on a specific PND. Both dam and pups remained in the home-cage room during testing. At the time of testing, the dams were removed from the home cage and placed in a separate cage. The pups were taken directly from the home cage for testing and were replaced into the home cage after testing was completed. The amount of time that pups spent outside the home cage was minimized and did not exceed 30 s.

Somatic parameters included pinna (ear) unfolding, fur appearance, eye opening, anogenital distance, body length, and body weight. Pinna unfolding was recorded from PND 3, eye opening was recorded from PND 14, and fur appearance was recorded from PND 3 for both male and female pups. The time of first appearance of fur was considered the first day of occurrence, whereas both pinna had to unfold or both eyes open to denote the first day of appearance. Recording of these parameters continued until all pinna had unfolded, eyes had opened, and fur was present in all rat pups.

Anogenital distance in pups was measured (for comparison) to assess possible masculinizing or feminizing effects of gavage treatment of dams. To this end, a digital calipers was used to measure the distance between the base of the genitals and the top of the anus was measured on PND 3.

To compare growth between groups, body length was measured on PND 7 and 14 by using a digital calipers between the tip of the nose and the base of the tail. In addition, each pup was weighed on PND 1, 2, 4, 8, 11, 15, 18, and 21 prior to behavioral testing.

To measure surface righting, pups were placed in the supine position on a flat surface and the time taken to turn over and restore its normal prone position (on all fours) was recorded.

The maximum time allowed was 30s. A time of 30s was recorded if the pup did not right itself within this period and the test was terminated. This test was performed on PND 2, 3, 4 and 5.

To measure forelimb grip in pups, the testing apparatus consisted of a thin steel bar supported by 2 adjustable poles. The bar was approximately 20 cm in length and 0.2 cm in diameter and lies 25 cm above the base of the platform. The handler grasped the pup at the base of the tail and lowered it to the bar. The length of time the pup held onto the bar before falling was recorded and the maximum time allowed was 30 s. A time of 30 s was given if the pup did not fall during this period, and the test was terminated. This test was performed on PND 14 and 17.

Statistical analysis. All figures representing the data from the testing period were constructed by using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). The data were then analyzed by using the statistical package SPSS 21. (SSPS, IBM, Armonk, NY). First, data were evaluated to assess normality of distribution and homogeneity of variance (Shapiro–Wilks and Levene tests, respectively; $P > 0.05$). This process determines whether the data are parametric or nonparametric. In addition, the data were assessed to determine whether they displayed sphericity (Mauchly test, $P > 0.05$); when this test failed, Greenhouse–Geisser correction was applied (that is, degrees of freedom corrected). For the parametric data, tests included repeated-measures ANOVA to compare overall effects for related data; two-way ANOVA to compare the effect of treatment groups and sex; and one-way ANOVA and Student–Newman–Keuls posthoc tests. Nonparametric data were evaluated by using Friedman ANOVA by ranks to compare the overall effect for related data; Wilcoxon matched-pairs test to compare the effect of time; Kruskal–Wallis test to compare the effect of treatment groups; and Mann–Whitney *U* tests to define where the significance lay. For pinna unfolding, eye opening, and fur appearance, the data were recorded as present or absent, whereas for surface righting and forelimb grip, the data were recorded as the ability to perform the test or not; therefore chi-squared testing was performed for these parameters. All results reported refer to the gavage groups compared with the NT groups. The level of significance was set at a *P* value of less than 0.05 for all parameters except the chi-squared test, for which the threshold was a *P* value of less than 0.02 (that is, Bonferroni correction used because of multiple comparisons).

Results

Prenatal maternal measurements. Gestation day (time) had a significant effect ($F_{1.95, 39.03} = 502.17$, $P < 0.001$) on maternal body weight in rats, with all groups gaining weight as gestation progressed. Treatment had no significant effect on maternal body weight (Figure 3), nor did gestation day and treatment show significant interaction. Treatment had no effect on maternal body weight gain in the first, second, or third week of gestation nor on total body weight gain through gestation (data not shown).

Gestation day (time) had a significant effect ($F_{5.43, 86.90} = 15.17$, $P < 0.001$) on maternal food consumption, which increased slightly throughout the gestation period and then decreased during the last few days before birth (GD 18 to 21). Neither treatment nor the interaction of gestational day and treatment significantly affected food consumption by dams (data not shown). Treatment had no effect on total food consumption during the first, second, or third gestational week.

Maternal water consumption showed a significant effect of gestational day ($F_{1.81, 30.68} = 3.53$, $P < 0.05$), with water consumption increasing slightly throughout the gestation period and then decreasing for the last few days preceding birth (GD 18 to 21).

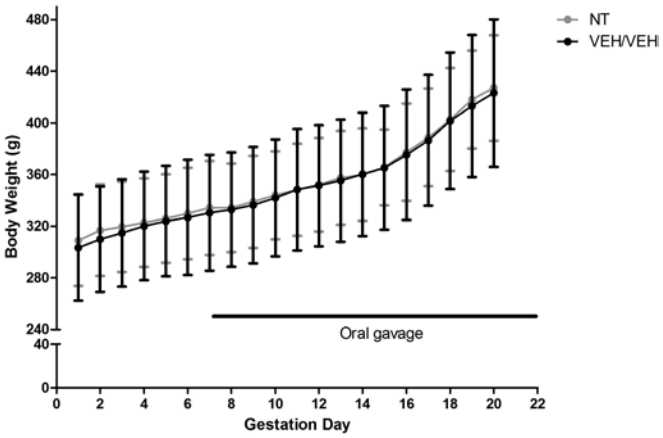

Figure 3. Maternal weight gain during gestation in rats. Weight gain for each day of gestation for untreated control (NT; $n = 10$) and gavage ($n = 12$) groups. Line indicates dosing period. Data are expressed as mean ± 1 SD.

No significant effect of treatment was found (data not shown), nor was there a significant interaction effect of gestation day and treatment. Treatment did not influence total water consumption during the first, second or third gestation week.

Postnatal maternal measurements. Postnatal maternal body weight gain showed a significant effect of PND ($F_{2.54, 40.69} = 10.45$, $P < 0.001$), with both groups gaining weight as lactation progressed. Treatment had no effect on the weight gain of dams (NT, 27 ± 16 g; gavage, 28 ± 17 g). No significant interaction effect of PND and treatment was present, and treatment did not affect body weight gain during the first, second, or third week of lactation (data not shown).

Dams in the gavage group consumed more food on PND 3 ($U = 12.50$, $P < 0.01$) than did the NT group; food consumption did not differ between groups on any other PND (data not shown). Treatment did not affect total food consumption during the first or second lactation week, but the gavage group ingested more food than did the NT dams during the third week of lactation ($t_{18} = 2.75$, $P < 0.05$).

PND (time) had a significant effect on maternal water consumption after delivery ($F_{2.84, 31.19} = 29.14$, $P < 0.001$). No significant interaction effect of PND and treatment was found. Treatment did not affect overall water consumption during the first, second, or third lactation week (data not shown). Furthermore, oral gavage had no effect on the relative weight of the liver, kidneys, thymus, adrenals, lungs, heart, pancreas, or spleen (data not shown).

During the study, all dams were monitored daily, and all remained in good health, with no signs or symptoms of distress throughout the study. Gavage of dams had no effect on day of delivery (22 ± 0 d for both NT and gavage groups), total number of pups in a litter (NT, 15 ± 2 pups; gavage, 12 ± 3 pups), number of male pups per litter (NT, 7 ± 2 pups; gavage, 5 ± 2 pups), number of female pups per litter (NT, 8 ± 2 pups; gavage, 7 ± 2 pups), percentages of pups that died (NT, 0%; gavage, 4.2%) or were cannibalized (NT, 0%; gavage, 2.4%) in the postnatal period, number of stillborn pups (NT, 0.7%; gavage, 0%), or the total percentage of pup deaths (NT, 0.7%; gavage, 6.5%).

Development of offspring. No significant effect of treatment or sex or an interaction effect of treatment and sex was demonstrated for birth weight of pups; unfolding of pinna on PND 3, 4, or 5; or appearance of fur on PND 3, 4, or 5 (data not shown).

Pup sex influenced eye opening on PND 14 ($\chi^2 = 9.52$, $P < 0.01$) and 15 ($\chi^2 = 9.52$, $P < 0.01$), and treatment influenced this

parameter on PND 14 ($\chi^2 = 13.79$, $P < 0.01$), PND 15 ($\chi^2 = 26.67$, $P < 0.001$), and PND 16 ($\chi^2 = 21.05$, $P < 0.001$; Table 1). Posthoc testing showed that among female pups, the gavage group had more pups with eyes open on PND 15 than did the corresponding NT group. Whereas treatment had no effect, sex significantly affected anogenital difference ($F_{1, 36} = 58.43$, $P < 0.001$; data not shown), with female pups having a shorter anogenital distance than male pups, as expected. No significant interaction of treatment and sex occurred.

Neither sex nor the interaction of sex and treatment affected the body length of pups on PND 7 or 14. Pup body length differed between treatment groups on PND 7 ($F_{1, 36} = 8.34$, $P < 0.01$) and PND 14 ($F_{1, 36} = 22.64$, $P < 0.001$; Figure 4). Posthoc testing showed that female pups on PND 7 and 14 and male pups on PND 14 were longer in the gavage groups compared with the NT groups. In addition, PND significantly affected pup weight ($F_{3.24, 110.24} = 3850.44$, $P < 0.001$), with all pups gaining weight with age. Furthermore, pup weight showed a time×treatment interaction ($F_{3.24, 110.24} = 5.71$, $P < 0.01$), but neither sex, treatment, nor sex×treatment was found to influence pup weight. In addition, neither sex nor an interaction effect of sex and treatment affected total body weight gain in pups. However, although total body weight gain differed between treatment groups ($F_{1, 36} = 7.61$, $P < 0.01$), posthoc testing did not reveal any difference once pups were analyzed according to sex.

Surface righting in pups did not show a significant effect of sex on PND 2, 4, or 5. A significant effect of sex was found on PND 3 ($\chi^2 = 19.78$, $P < 0.001$), with more male pups performing this task. Treatment affected surface righting of pups on PND 2 ($\chi^2 = 28.57$, $P < 0.001$), PND 3 ($\chi^2 = 87.50$, $P < 0.001$), PND 4 ($\chi^2 = 39.64$, $P < 0.001$), and PND 5 ($\chi^2 = 30.77$, $P < 0.001$; Table 2). According to posthoc testing, among female pups on PND 2 and 3 and male pups on PND 3 and 4, more pups in the gavage groups could right themselves in less than 10 s, compared with the NT groups.

Sex did not affect forelimb grip in pups on PND 14 or 17, but treatment-associated effects occurred on PND 14 ($\chi^2 = 59.45$, $P < 0.001$) and PND 17 ($\chi^2 = 47.62$, $P < 0.001$; Figure 5). Results of posthoc analyses showed that among female offspring on PND 14 and 17 and male pups on PND 17, fewer pups could perform the task in gavage groups compared with the NT groups.

Table 1. Percentage (%) of rat pups ($n = 10$ per group) with both eyes open

	PND 14	PND 15	PND 16	PND 17
Male pups				
No treatment	20	80	100	100
Gavage	30	70	90	100
Female pups				
No treatment	40	60	100	100
Gavage	20	90[a]	90	100

[a]$P < 0.05$ compared with value for relevant no-treatment group.

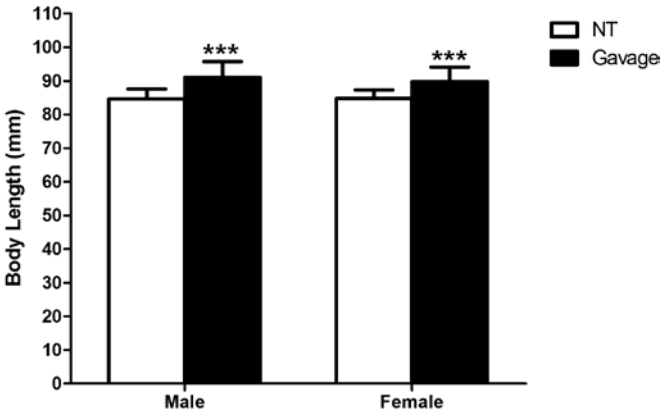

Figure 4. Pup body length on PND 14. Body length for male and female rat pups on PND 14 ($n = 10$ per group). Data are expressed as mean ± 1 SD; ***, $P < 0.001$ compared with relevant no-treatment (NT) group.

Table 2. Percentage (%) of rat pups ($n = 10$ per group) able to right themselves on a surface in less than 10 s

	PND 2	PND 3	PND 4	PND 5
Male pups				
No treatment	60	80	80	90
Gavage	70	100[a]	100[a]	100
Female pups				
No treatment	60	50	90	100
Gavage	90[a]	90[a]	100	100

[a]$P < 0.05$ compared with value for relevant no-treatment group

Discussion

Oral gavage is the most commonly used technique for dosing orally in pharmacokinetic and toxicokinetic studies.[7] Several studies[2,6,11,16] have attempted to establish the level of stress induced due to the restraint necessary to perform oral gavage. However, to our knowledge, no previous study has investigated the influence of gavage treatment during pregnancy on the offspring, and we therefore cannot directly compare our current results with other studies. The most applicable comparisons available would be with studies of maternal stress caused by various means, such as chronic restraint, maternal separation, social stress, tail shock, and endocrine activation. Our study showed that maternal parameters including body weight, food and water consumption, gestational length, number of offspring, and organ weights were all unaffected by oral gavage treatment. These findings are in contrast to a previous studies, which found that gavage treatment resulted in weight loss[12] or that pregnant females subjected to prenatal stress experienced longer pregnancies and had fewer viable young than did nonstressed rats.[5]

However, habituation to the oral gavage procedure is common, and the chronic dosing period we used in the present study likely accomplished this effect. For example, a recent study showed that chronic orogastric gavage of male rats with aqueous solutions (5 mL/kg) does not negatively affect their welfare; the same study also states that habituation of rats to handling during the week prior to dosing improved the ease of gavage administration.[16] We similarly handled the dams before beginning the dosing regimen in the present study. Mice habituated to the oral gavage procedure after only a single exposure, and this habituation continued as the study progressed.[6] In addition, whereas the relative weight of the adrenal glands increased in chronically stressed rats,[16] we found no difference in the adrenal gland weights in our current study. Therefore, we conclude that daily oral gavage during gestation and lactation has no significant effect on rat dams.

The second aim of this study was to investigate the consequences of oral gavage administration on various neonatal outcomes. The present study showed that gavage treatment of dams had no effect on developmental parameters in their offspring, including birth weight, pinna unfolding, fur appearance, anogenital distance, body weight, and neonatal death. These

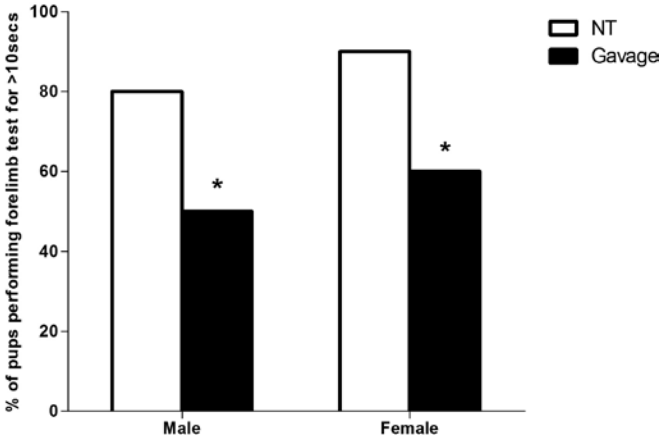

Figure 5. Forelimb grip of pups on PND 17. Forelimb grip of male and female rat pups on PND 17 (n = 10 per group). Data are expressed as the percentage of pups that could perform the task for at least 10 s. *$P < 0.05$ compared with relevant no-treatment (NT) group.

data contrast with findings from the offspring of prenatally stressed mothers, which were exposed to physical and environmental stressors such as heat, restraint, and bright light; these pups had smaller body weights and were less likely to survive in the neonatal period.[5] However, the maternal stressors in the previous study[5] were quite marked and usually were performed several times each day for an extended period. Therefore, such treatment would not unexpectedly result in a magnified response compared with that due to gavage treatment, which usually lasted less than 5 s in our current study.

Nonetheless, somatic development in the offspring, including eye opening and body length, and neuromuscular development, including surface righting and forelimb grip, highlighted significant consequences of gavage treatment in the present study. Gavage had a positive effect on all of these developmental parameters, except for forelimb grip which showed a deficit in the ability. Although our result may seem minimal relative to the number of parameters assessed, it is quite important given that the performance of gavage-group pups is more than 30% less than that of the NT offspring. This result suggests that development of the cerebellum (known to control coordination and muscular activity) has been compromised in some way. However, to date the literature contains no evidence that supports our finding. In fact, previous studies have found that the hippocampus is the brain region most affected by prenatal stress. In the offspring of rat mothers that were exposed to daily restraint stress in late pregnancy, the morphologic and functional maturation of hippocampal granule cells are impaired.[15] In another study,[1] pregnant dams were stressed (varied stressors) from GD 15 to 20, and prepubertal male and female offspring had shorter and less complex dendrites in the hippocampus, compared with unstressed controls. Such measurements were not assessed in the present study and may warrant further investigation. Another limitation of the current study was the inability to follow these offspring into adulthood to investigate whether the present findings are persistent and are still apparent in later life.

Although the findings of the present study cannot be explained mechanistically, it is clear that the gavage treatment poses less of a risk to neonatal development than do models of prenatal stress, such as restraint stress or shock. The brief restraint necessary to perform the technique, coupled with habituation (reexposure) to the technique, means that the distress caused to the mothers and therefore the stress to which their offspring are exposed are minimal. As a route of administration, oral gavage seems safe and feasible for use during pregnancy and lactation. However, because oral gavage of dams had several consequences in their pups, the inclusion of both no-treatment as well as negative-control (for example, saline) groups in experiments may be necessary.

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