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Effect of Methylmercury on Midbrain Cell Proliferation during Organogenesis: Potential Cross-Species Differences and Implications for Risk Assessment

T. A. Lewandowski,*,† R. A. Ponce,*,‡ J. S. Charleston,§ S. Hong,* and E. M. Faustman*,¶,[1]

*Department of Environmental Health, University of Washington, Seattle, Washington 98105; †Gradient Corporation, Mercer Island, Washington 98040; ‡Zymogenetics Inc., Seattle, Washington 98102; §ICOS Corporation, Bothell, Washington 98021; and ¶Center for Child Environmental Health Risks Research, Seattle, Washington 98105

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5′-bromodeoxyuridine (BrdU) labeling was employed to explore the effects of methylmercury (MeHg) on cell cycle kinetics in the developing rat midbrain during gestational days (GDs) 11 to 14. Contrary to what has been previously reported in mice, no effects of MeHg on cell cycle kinetics were observed up to embryonic brain concentrations of 3–4 μg/g. The absence of an effect was confirmed using stereology and counts of midbrain cell number. Treatment with colchicine, the positive control, resulted in significant effects on cell cycle kinetics in the developing rat midbrain. The parallelogram method, borrowed from genetic toxicology, was subsequently used to place the data obtained in the present study in the context of previously collected *in vitro* and *in vivo* data on MeHg developmental neurotoxicity. This required developing a common dose metric (μg Hg/g cellular material) to allow *in vitro* and *in vivo* study comparisons. Evaluation suggested that MeHg's effects on neuronal cell proliferation show a reasonable degree of concordance across mice, rats, and humans, spanning approximately an order of magnitude. Comparisons among the *in vivo* data suggest that humans are at least or more sensitive than the rodent and that mice may be a slightly better model for MeHg human developmental neurotoxicity than the rat. Such comparisons can provide both a quantitative and a qualitative framework for utilizing both *in vivo* and *in vitro* data in human health risk assessment.

Key Words: **methylmercury; cell cycle; parallelogram; interspecies; stereology; midbrain.**

Cell production in the developing embryo and fetus is a highly regulated and coordinated activity, with waves of cell division, migration, and differentiation occurring in different brain regions at different times (Rodier, 1995). Disruption of these processes, particularly cell proliferation, is a common feature of many neurodevelopmental toxicants. Environmental agents that alter cell proliferation include rubella, ionizing radiation, cancer chemotherapeutic or antiviral therapy, and exposure to agricultural fungicides or metals (Scott, 1977). Experimental evidence supports the theory that cell cycle inhibition caused by environmental agents can produce regionally defined alterations in nervous system morphology, and the resultant neuropathological effects can be linked with specific neurobehavioral deficits (Rodier, 1983; Rodier *et al*., 1984). For example, *in utero* exposure to valproic acid and thalidomide at clearly defined time points has been linked with autism (Rodier *et al*., 1996). In these studies, neuropathological examination revealed a loss of specific nuclei in the hindbrain, specifically the superior olive and facial nuclei (Rodier *et al*., 1996). However, in most cases, it is not possible to correlate altered neurobehavior with central nervous system (CNS) pathology, particularly if exposure causes subtle effects on cell number, migration, orientation, or synaptogenesis. In cases where subtle changes in cell-specific population abundance and distributions are suspected, quantitative stereology can be applied to examine changes in cell number and distribution.

The developing nervous system is highly susceptible to the toxic effects of methylmercury (MeHg). The effects of *in utero* exposure include both sensory and motor disturbances, with more severe exposures producing mental retardation (Burbacher *et al*., 1990). Pathology studies of human infants and animals exposed to MeHg during gestation demonstrate alterations in cell number, brain size, cell orientation, and distribution (Chen *et al*., 1979; Choi *et al*., 1978; Eto *et al*., 1992; Rodier *et al*., 1984). Burbacher *et al*. (1990) noted that cell loss and reduced brain size are consistently observed effects of MeHg exposure across species, from high-dose examples in humans (i.e., Minimata, Iraq) to low-dose examples in rodents (primarily mice). Several studies have indicated that the decreased number of brain cells is a result of the inhibition of proliferation rather than cell death, because decreased proliferation is observed at doses below those that result in cytolethality (Ponce *et al*., 1994; Sager *et al*., 1984). MeHg-mediated mitotic arrest has been observed *in vivo* (Choi, 1991; Choi *et al*., 1978; Rodier *et al*., 1984) and *in vitro* (Miura *et al*., 1978;

[1] To whom correspondence should be addressed at 4225 Roosevelt Way NE, Suite 100, Seattle WA 98105. Fax: 206-685-4696. E-mail: faustman@u.washington.edu.

Ponce *et al*., 1994; Sager, 1988; Vogel *et al.*, 1986). Disruption of microtubule formation, oxidative stress, altered cellular signaling, gene expression, and protein phosphorlylation have been reported at low MeHg exposure concentrations, i.e., 1 μM or lower (Minnema *et al*., 1989; Miura *et al*., 1984; Rajanna *et al*., 1995; Sarafian and Verity, 1991; Vogel *et al*., 1985; Zucker *et al*., 1990). Such effects may contribute independently or jointly to the observed effects of MeHg on mitotic progression.

Ponce *et al*. (1994) studied the effects of MeHg exposure on micromass cells, that is, primary rat midbrain cells dissociated and cultured at a high density. Via flow cytometry, they observed a clear G2/M phase arrest in cells exposed to 2 to 4 μM of MeHg, which is similar to the type of cell cycle arrest that is observed when cells are exposed to the mitotic spindle–disrupting drug colchicine. These results, in a primary, nonimmortalized CNS culture, are consistent with similar effects observed in other cell culture systems (Miura *et al*., 1984; Vogel *et al*., 1986; Wasteneys *et al*., 1988; Zucker *et al*., 1990) as well as *in vivo* in mice (Rodier *et al*., 1984; Sager *et al*., 1984).

The aim of the investigation reported here was to observe the effects of MeHg on cell proliferation in rat midbrain cells *in vivo* using flow cytometry and quantitative stereology. These observations would provide an *in vivo* extension of our previous rat *in vitro* studies (Ponce *et al*., 1984). A second goal was to develop a quantitative framework that would allow for consideration and comparison of *in vitro* and *in vivo* midbrain data. In particular, we were interested not only in the differences in mouse versus rat data, but also in the differences in each species between *in vitro* and *in vivo* assessments. These comparisons would provide some indication of the similarity in quantitative response between the *in vivo* and *in vitro* systems. Our hypothesis was that, at doses similar to those used in the *in vivo* mouse studies (i.e., 1–2 μg/g in the embryonic brain), cell cycle disruption would be observed in the rat.

MATERIALS AND METHODS

Experiment 1: Cell Cycle Kinetic Studies

The design of the *in vivo* studies used to examine the cell cycle effects of MeHg are summarized in Table 1.

Animals. Time-mated female Sprague-Dawley rats were obtained from Charles River Laboratories (Hollister, CA). The morning after mating was designated GD 0. The animals were shipped from the breeding facility to the University of Washington Health Sciences Complex on GD 6. They were kept on a 12-h light/dark cycle, fed with standard rat chow, and watered *ad libitum*. Animal care and treatment followed a university-approved animal use protocol developed in accordance with NIH guidelines.

Treatment. The animals were dosed subcutaneously (sc) with MeHgOH (Alpha Aesar, Ward Hill, MA) in the afternoon of GD 11. The control animals received sc doses of sterile saline. Total maternal MeHg doses ranged from 5 to 20 mg/kg. Toxicokinetic data (Lewandowski *et al*., 2002) indicated that mercury (Hg) concentration in the embryos approached maximum levels 24 h after dosing. Twenty-four or 48 h after MeHg dosing (GD 12, Witschi stage 21; GD 13, Witschi stage 27), short-term (1.5-h) bromodeoxyuridine (BrdU) exposures were carried out. The animals received a single intraperitoneal (ip) injection of BrdU (18 mg/ml dissolved in phosphate-buffered saline (PBS), pH 7.7, total dose = 25 mg/kg) and were sacrificed 1.5 h later. After sacrifice, tissues were collected and processed as described below. A second set of experiments was carried out using 6-h BrdU exposures because previous studies (Miller and Nowakowski, 1991; Miller *et al*., 1995) indicate that changes in the fraction of BrdU-labeled cells may be easier to detect with longer labeling times. In these experiments, the animals were dosed with MeHg on GD 11, received an injection of BrdU at 8:00 A.M. on GD 12, and received a second injection 5 h later (1:00 P.M.). The animals were sacrificed approximately 1 h later (total labeling time, 6 h). Previous work indicated that the average duration of the S-phase in these cells was approximately 8 h (Lewandowski *et al*., 2003). Thus an interval of 5 h between BrdU injections was sufficient to label all cycling cells (i.e., with BrdU administration every 5 h, no cells would pass completely through the S-phase without being exposed to BrdU). To provide a positive control for MeHg treatment, several animals were also treated with colchicine, a known mitotic spindle inhibitor. Dams were dosed intraperitoneally (ip) with 0.7 or 1.0 mg/kg colchicine (three dams per treatment group) on the morning of GD 12, approximately 30 min prior to the start of BrdU administration. Dosing with colchicine (Sigma-Aldrich, St. Louis, MO) was conducted on GD 12 due to the short half-life of colchicine, reported to be 16 min in the rat (Leighton *et al*., 1990). Dosing one day prior

TABLE 1
Summary of Approach for *in Vivo* Experiment 1: Cell Cycle Kinetic Studies

		Treatment				BrdU labeling	
Group	No. of dams/litters[c]	Treatment	Maternal dose (mg/kg)	Route	Treatment day	BrdU injection	Labeling duration
1[a]	3	saline	0	sc	GD11	GD12	1.5h
2[a]	3	saline	0	sc	GD11	GD13	1.5h
3[a]	6	MeHg	5–20	sc	GD11	GD12	1.5h
4[a]	6	MeHg	5–20	sc	GD11	GD13	1.5h
5[b]	4	saline	0	sc	GD11	GD12	6 h
6[b]	3	colchicine	0.7, 1	ip	GD12	GD12	6 h
7[b]	4	MeHg	18	sc	GD11	GD12	6 h

[a]Animals in groups 1–4 were used to evaluate the effects of MeHg on the S-phase length and cell proliferation using BrdU pulse labeling. Results are presented in Figures 2 and 3.

[b]Animals in groups 5–7 were used to evaluate the fraction of cycling cells using 6-h BrdU labeling. Results are presented in Figures 4 and 5.

[c]Cell cycle kinetics evaluations were performed on a litter-by-litter basis using pooled embryos.

to BrdU treatment, as was the case with MeHg, would have resulted in the colchicine being eliminated by the time BrdU labeling occurred.

Tissue collection. At the appropriate time interval after BrdU injection, the animals were killed using CO_2. The uteri were removed, placed on ice, and maintained cold while the embryos were dissected out of the uterine tissues. Dissection on ice has been used to halt BrdU incorporation in embryonic tissues (Miller and Kuhn, 1995) and allows for control of the time of BrdU exposure. After the embryos were obtained from the uteri, the developmental stage was confirmed by measuring the crown rump length, counting somites, and evaluating the overall morphology and comparing these to a standard reference (Altman and Bayer, 1995). The embryos were examined for gross pathologies (i.e., smaller size or abnormal morphology relative to controls) when removed from the uterine tissue. The embryonic midbrains were then sectioned from the body of the embryo. All of the midbrains obtained from each litter were pooled and dissagregated using 0.25% trypsin/2.5% collagenase and trituration (25°C). The midbrain cell suspensions were then washed with PBS/5% normal goat serum (NGS), and the cells were fixed in cold 70% ethanol (4°C). All work was conducted using dark room lights to minimize BrdU photoactivation.

The embryonic bodies were frozen at −80°C and later analyzed for total Hg content according to the methods described in Lewandowski *et al*. (2002). The toxicokinetic data (Lewandowski *et al*., 2002) indicated that, at this gestational period, embryonic body Hg concentration serves as a reasonable indicator of Hg content in the embryonic brain.

Cell staining. The cells were centrifuged (10 min, 700 r.p.m., 25°C for all spins) in 70% ethanol and then resuspended in 1 ml PBS/5% NGS. The cells were allowed to rehydrate at room temperature overnight, as this was found to provide better antibody access to DNA. After the overnight rehydration, the cells were centrifuged and resuspended in 150 μl of PBS/NGS and 450 μl of membrane-shredding solution [100 ml calcium magnesium free (CMF)-PBS, 500 μl NP-40, 20 mg ethylenediamine tetracetic acid (EDTA)]. The samples were placed on ice and vortexed vigorously every 3 min for 20 min to disrupt the cells and gain access to the ethanol-fixed nuclei. The samples were then acidified with 800 μl of 4N HCl and agitated for 45 min at room temperature to denature the double-stranded DNA and allow antibody access. The nuclei were then washed twice with 3 ml 1-M Tris (pH 8.5) and counted using a hemocytometer. The samples were then completely decanted and resuspended with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Pharmingen, San Diego, CA) at a ratio of 150 μl antibody to 1E6 cells. The antibody was previously diluted to 1:20 with PBS/NGS/0.5% Tween 20. After 1 h of incubation at 25°C, the samples were washed once with PBS/NGS and resuspended in PBS for flow analysis. Immediately prior to flow analysis, propidium iodide (PI), a DNA staining dye, was added to achieve a final PI concentration of 5 μg/ml in the sample.

Flow cytometry. Flow cytometry was used to determine the fraction of cells positively labeled with BrdU and the cell cycle profile (i.e., %G1, %G2, and %S). Dual-parameter flow cytometry utilized a Coulter EPICS Elite equipped with a coherent Innova 90 air-cooled argon laser. Excitation occurred at 488 nm for both the FITC and PI. Fluorescence measurements were obtained from approximately 20,000 cells. The data were analyzed using the software package *Mplus* (Phoenix Flow Systems, San Diego, CA).

Cell cycle kinetic parameter estimation. The S-phase length (Ts) was determined from the pulse-labeling study by the shift in mean PI fluorescence of the BrdU-labeled cells after 1.5 h of BrdU exposure using the method of Begg *et al*. (1985) as modified by Terry *et al*. (1991). The fractions of labeled cells at 1.5 and 6 h of BrdU exposure were determined with adjustments for cells that divided during the labeling period (Nowakowski *et al*., 1989).

The effect of embryonic Hg concentration on the cell cycle parameters was evaluated using linear regression and a significance criterion of 0.05. In the case of the 6 h BrdU exposures, the data were also grouped by concentration and analyzed using a *t*-test or ANOVA.

Experiment 2: Stereology Studies

The design of *in vivo* stereology studies used to confirm the cell cycle results is summarized in Table 2.

Dosing and embryo isolation. To obtain confirmatory data for the cell cycle kinetic studies, four embryos each from two MeHg-treated dams were also obtained for stereological evaluation of the midbrain cell number. The dams were dosed sc with 18 mg/kg MeHg on GD 11 (i.e., the same time as dosing in the cell cycle studies). Embryos were obtained on GD 14 using the procedures described above (i.e., one day later than in the cell cycle studies). One-half of the embryos (one side of the horn) were used for counting while the other half were used for mercury analysis. Mercury analysis was performed on the whole embryonic body, which we have previously shown is a reasonable surrogate for midbrain mercury concentration at this gestational age (Lewandowski *et al*., 2002). Each embryo used for counting was assigned a specific code number so that counting was performed with the observer blind to uterine position and treatment status.

Tissue preparation. Following fixation by whole-body immersion in 4% paraformaldehyde (carried in 0.1-N dibasic sodium phosphate buffer, pH 7.2), whole bodies or heads of embryos (depending on age/size) were specifically positioned by embedment in a 3% agar matrix to facilitate handling during subsequent tissue processing and increase histology efficiency by allowing entire sets of embryos to be processed together as a single tissue set. The agar blocks containing the tissue were dehydrated in a graded ethanol series and embedded in glycomethacrylate (Historesin, Cambridge Instruments, Deerfield, IL). The blocks were exhaustively sectioned in the sagittal plane at a thickness of 20 or 30 μm on a JB-4 microtome (Sorvall) using glass knives, and all sections were collected in the order generated. The sections were stained with Giemsa (stock solution, J. T. Baker, Phillipsburg, NJ) using the procedure of Iñiguez *et al*. (1985) and following the suggestions of Brændgaard *et al*. (1986, 1990).

Stereology. Estimates of the number of neurons in the developing rat midbrain were determined by use of the Optical Volume Fractionator (OVF) procedure of Bolender and Charleston (1993) as previously described in Charleston (2000). A detailed explanation of this methodology is provided in Lewandowski *et al*. (2003).

Counting was performed on every twelfth section in the set of sections, and 100 to 300 neuroepithelial cells were counted per individual. The boundaries of the midbrain were determined by use of a developmental rat atlas (Altman and Bayer, 1995). The areas considered for cell enumeration included the developing region of the thalamus, thus enabling use of the unambiguous boundary formed by the prominent fissure separating the thalamus from the

TABLE 2
Summary of Approach for *in Vivo* Experiment 2: Stereology Studies

Group	No. of dams/litters[a]	Treatment	Maternal dose (mg/kg)	Route	Treatment day	Embryo collection day
		Treatment				
1[b]	1(4)	none	NA	NA	NA	GD11
2[b]	2(8)	none	NA	NA	NA	GD12
3[b]	2(8)	none	NA	NA	NA	GD13
4[b]	2(8)	none	NA	NA	NA	GD14
5[b]	2(8)	none	NA	NA	NA	GD16
6	2(8)	MeHg	18	sc	GD11	GD14

Note. NA, not applicable.

[a]Cell cycle kinetics evaluations were performed on a litter-by-litter basis using pooled embryos.

[b]Previously reported in Lewandowski *et al*. (2003).

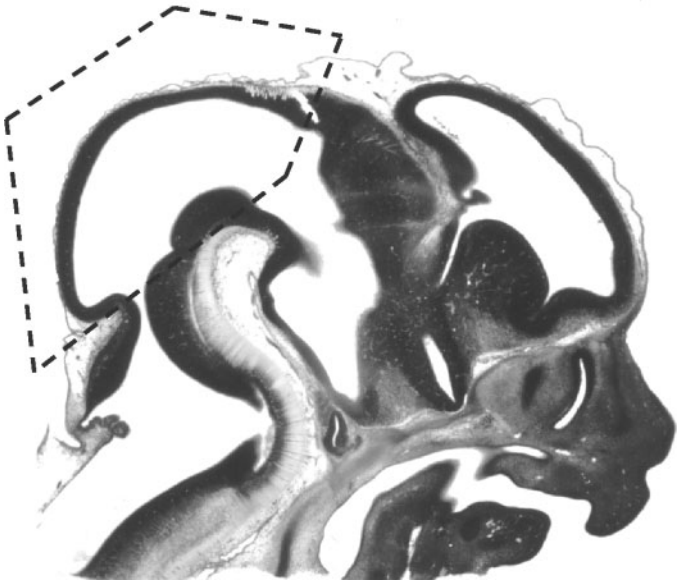

FIG. 1. Rat embryo (GD 14) indicating the brain region sectioned for both stereology and cell cycling studies. The area studied was bounded by the cerebellomesencephalic flexure and a similar flexure associated with the posterior commissure.

cerebral cortex. Otherwise, determination of the boundary between the midbrain and the thalamus would have required the use of arbitrary boundaries, resulting in equally arbitrary estimates of the total number of neuroepithelial cells. This area of interest was identified by extending a line between the base of the cerebellar sulcus and the base of the thalamus, thereby transecting the ventricular space (Fig. 1). This approach was consistent with the cell cycling studies in that the same brain region was evaluated in both sets of experiments.

The treated embryo results were compared to the embryonic midbrain counts determined from GD 11 to GD 16, which we have published previously (Lewandowski *et al*., 2003).

Experiment 3: Evaluation of Hg Concentrations in Vitro

To permit comparison of previous *in vitro* data with results of our *in vivo* study described above, a common dose metric was required. *In vitro* studies generally report chemical exposures as media concentrations (e.g., μM), whereas *in vivo* studies typically report tissue concentrations (e.g., μg/g). To establish a means of comparison, micromass (primary embryonic rat midbrain) and P19 cells (murine embryonic carcinoma cells) were used to measure the mercury uptake from the culture media. For micromass cultures, the culture medium was Hams's F12 supplemented with 10% fetal bovine serum, penicillin (50 IU/ml), streptomycin (5 μg/ml), and L-glutamine (5.8 mg/ml). For P19 cultures, the medium was the same, except that F12 was replaced with Dulbecco's Modified Eagle Medium (DMEM). The cultures were grown on Falcon Primaria dishes (Beckton-Dickinson Labware, Lincoln Park, NJ) possessing a hydrophillic polystyrene culture surface. The cells were incubated at 37°C in a 5% CO_2/95% air atmosphere with 100% relative humidity for 2 h to allow for attachment prior to treatment. The media was then removed and replaced with an MeHg-containing media (0.5, 1.0, and 2.0 μM), and the cells were further incubated for 6 h. This was believed to be sufficient time for the MeHg to equilibrate between the cells and the media (Furukawa *et al*., 1982). After treatment, the culture medium was removed and the cells were rinsed twice with CMF-PBS. The cells were then incubated for 5 min at 37°C in 500-μl CMF-PBS containing 0.05% trypsin and 0.02% ETDA. Following incubation, 500 μl of mercury-free culture medium was added to each dish, and the cells were collected into 15-ml polystyrene centrifuge tubes. The cells were centrifuged at 800 r.p.m. for 20 min at 4°C, the supernatant was decanted, and the cells were stored at -80°C until analyzed for mercury. Approximately 0.15 g of the cellular material was collected per experiment. One to two experiments were conducted at each dose level/cell type. Analysis for total cellular Hg was conducted using an atomic fluorescence spectrometer (PSA Ltd., Kent, UK), as described in Lewandowski *et al*. (2002). The media concentrations were identical to those used by Ponce *et al*. (1994), and the cell culture methods were similar to those employed in that study. During collection, the cell suspensions were washed several times to remove any extracellular Hg that might have been present in the media or loosely associated with the cell membranes. A linear regression was used to determine the relationship between the intracellular Hg content and the Hg in the culture media.

RESULTS

No overt signs of toxicity were observed in the maternal animals treated with MeHg or colchicine. The MeHg- or colchicine-treated embryos were observed morphologically under $\times$10 magnification, and no differences in viability, size, or gross morphology were noted relative to the controls. The mercury tissue concentrations for all MeHg-treated animals are provided in Table 3.

Experiment 1: Cell Cycle Kinetics Studies

Single-pulse labeling results. The results of the 1.5 h BrdU labeling experiments are presented in Figures 2 and 3. Up to embryonic concentrations of 4 μg/g, no effects were observed

TABLE 3
Mercury Concentrations in the Tissues of MeHg-Treated Animals.

Experiment	Litter	Maternal MeHg dose	Embryo	Maternal liver
Experiment 1				
Group 3[a]	1	5	1.5	3.5
(24-h MeHg exposure,	2	5	1.3	6.2
GDs 11–12)	3	8	2.4	5.6
	4	9	3.3	8.1
	5	12	2.4	8.2
	6	12	3.2	5.4
Group 4	1	5	1.6	3.1
(48-h MeHg exposure,	2	5	2.3	3.8
GDs 11–13)	3	5	1.4	3.7
	4	8	2.2	5.5
	5	9	2.9	11.3
	6	12	3.4	6.7
Group 7	1	18	2.4	15.3
(48-h MeHg exposure,	2	18	2.5	15.3
GDs 11–13)	3	18	2.6	12.9
	4	18	2.6	11.7
Experiment 2				
Group 6	1	18	2.7	16.2
(72-h MeHg exposure,	2	18	2.9	11.8
GDs 11–14)				

Note. MeHg dose measured in mg/kg; embryo measured in μg/g; maternal liver measured in μg/g.

[a]Refers to the group number indicated in Table 1.

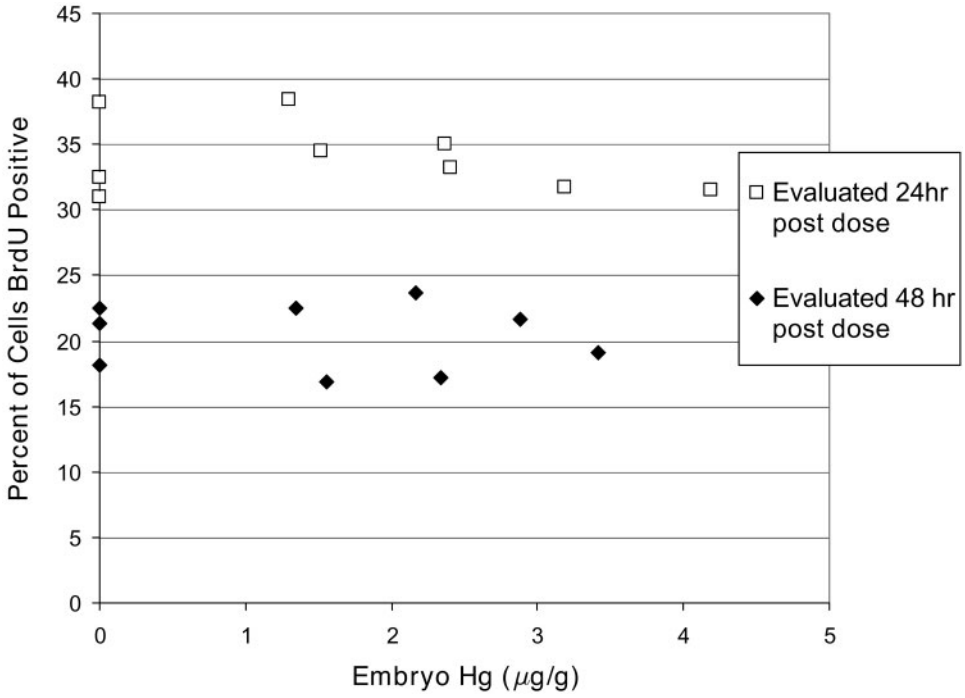

FIG. 2. Effect of methylmercury treatment on BrdU incorporation in midbrain cells. The embryos were exposed to BrdU through maternal ip dosing and sacrificed 90 min later. The percentage of BrdU-positive cells was determined by fluorescence of anti-BrdU antibody. Data were analyzed using linear regression. Neither the 24- nor 48-h exposures resulted in statistically significant effects on BrdU labeling. Each point shown represents a result for an individual pooled litter.

on the S-phase length at either 24 or 48 h after MeHg dosing (linear regression, $p_{slope} = 0.6$ [24 h], $p_{slope} = 0.8$ [48 h]). Similarly, no effects were observed on the fraction of cells labeled with BrdU (linear regression, $p_{slope} = 0.4$ [24 h], $p_{slope} = 0.7$ [48 h]). The cell cycle distribution (percent G1, S, and G2/M) was also not significantly different for MeHg-treated animals (data not shown).

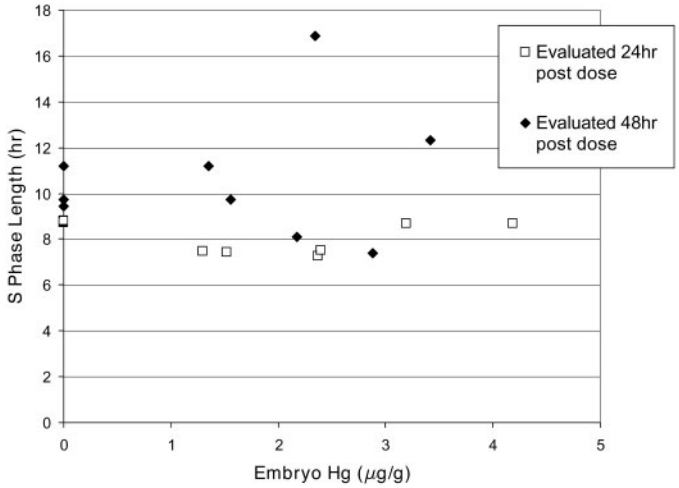

FIG. 3. Effect of methylmercury treatment on the S-phase length in midbrain cells. The embryos were exposed to BrdU through maternal ip dosing and sacrificed 90 min later. The S-phase length was estimated using the relative movement approach of Begg *et al*. (1985). The data were analyzed using linear regression. Neither the 24- nor 48-h exposures resulted in statistically significant effects on the S-phase duration. Each point shown represents a result for an individual pooled litter.

Six-h BrdU labeling. The results for the 6-h BrdU exposures subsequent to MeHg treatment are shown in Figure 4. No significant differences were observed between the MeHg-treated animals and the controls in terms of the percent of BrdU-positive cells or the cell cycle distribution. The relationship between MeHg dose and the fraction of BrdU-positive cells was examined using both linear regression ($p_{slope} = 0.2$) and by a *t*-test, grouping the MeHg-treated embryos with Hg concentrations of approximately 2.5 μg/g (SD = 0.1). The percentage of BrdU-positive cells in the MeHg-treated em-

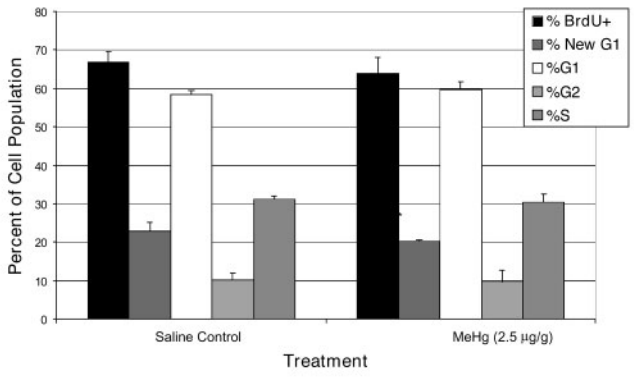

FIG. 4. Effect of methylmercury treatment on cell cycling in rat midbrain cells after 6 h of BrdU exposure. The embryos were exposed to BrdU through maternal ip dosing and sacrificed 6 h later. The MeHg data shown are those for four litters with a mean Hg concentration of 2.5 μg/g (SD = 0.1 μg/g). There were also four litters in the control group. The data were analyzed by *t*-test. Aside from a marginal significance for new G1 cells ($p = 0.05$), no other parameters were significantly altered by MeHg treatment.

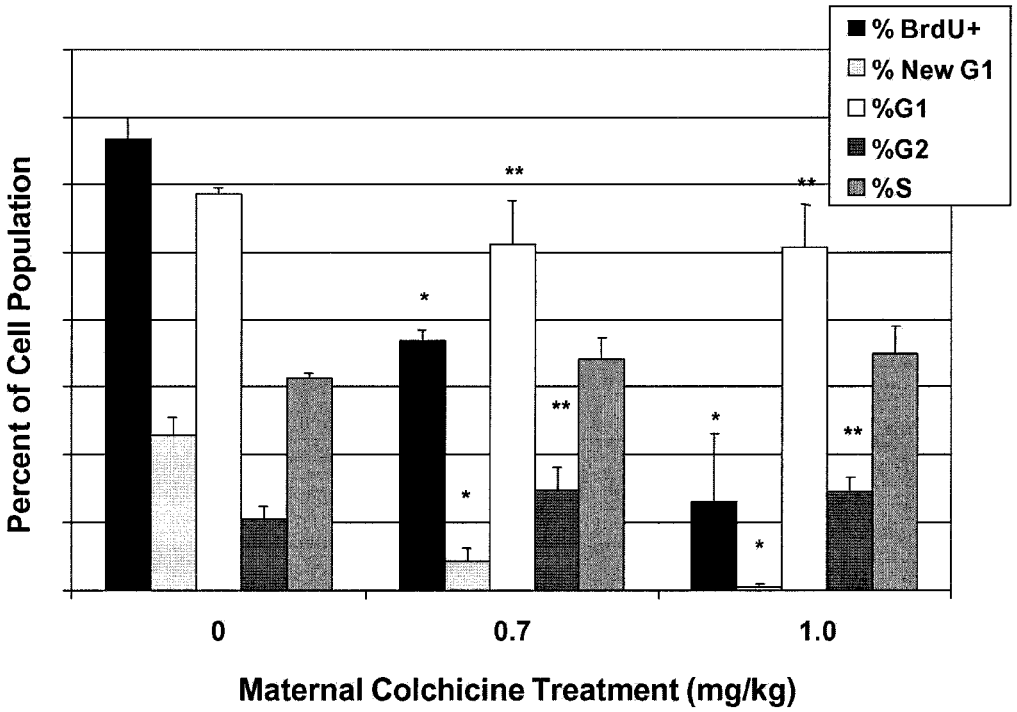

FIG. 5. Effect of *in vivo* colchicine treatment of dams on cell cycling in rat midbrain cells. Colchicine treatment (ip) occurred 30 min prior to BrdU exposure. The dams were then treated with BrdU (ip dosing, 25 mg/kg) and sacrificed 6 h later. Each treatment group shown represents three litters. The data were analyzed using ANOVA. The effect of colchicine on the fraction of BrdU-positive and new G1 cells was statistically significant in a dose-dependent manner (**$p <$ 0.01). When the two treatment groups were combined, there was also a significant effect on the cell cycle distribution (G2/M and G1, *$p < 0.05$; S **$p <$ 0.10). There were three dams/litters per colchicine treatment group and four dams/litters in the control group.

bryos (64% ± 4%) was not statistically different from the controls (66.9% ± 5%) (*t*-test, $p = 0.29$). There was a marginally significant decrease in the new G1 cell population in the MeHg-treated animals (*t*-test, $p = 0.05$, regression $p = 0.12$), but this was likely due to the small variance in the treated animals (coefficient of variation = 1.4%) and not to substantial differences in the means. MeHg treatment did not have any significant effects on cell cycle distribution (*t*-test and linear regression, $p > 0.1$). MeHg therefore did not appear to have any effects on cell cycling *in vivo* up to an embryonic Hg dose of 2.5 μg/g.

The results for *in vivo* treatment with colchicine, the positive control, are shown in Figure 5. Colchicine treatment had a clear and highly significant impact on cell cycling. For example, the fraction of BrdU-positive new G1 cells decreased in a dose-dependent manner from 23% in the controls to 4% at 0.7 mg/kg and 0.3% at 1 mg/kg (ANOVA, $p < 0.01$). A similar effect was seen in the overall fraction of BrdU-positive cells (ANOVA, $p < 0.01$). Regarding cell cycle distribution, the percentage of cells in the G2/M and S-phases was increased at the expense of the G1 population, which is consistent with the idea that colchicine causes cell cycle arrest at the point of cell division.

Experiment 2: Stereology Studies

The results of the stereological evaluation are shown in Figure 6. MeHg treatment of dams, resulting in an embryonic brain mercury level of 2.8 μg/g did not have any apparent effects on the cell number 72 h after dose administration. As is shown in Figure 6, the midbrain cell numbers in MeHg-treated embryos are consistent with the overall developmental growth patterns observed in untreated embryos from GD 11 to GD 16. We also conducted an analysis using a random effects model to determine how large a change in cell number in the MeHg-

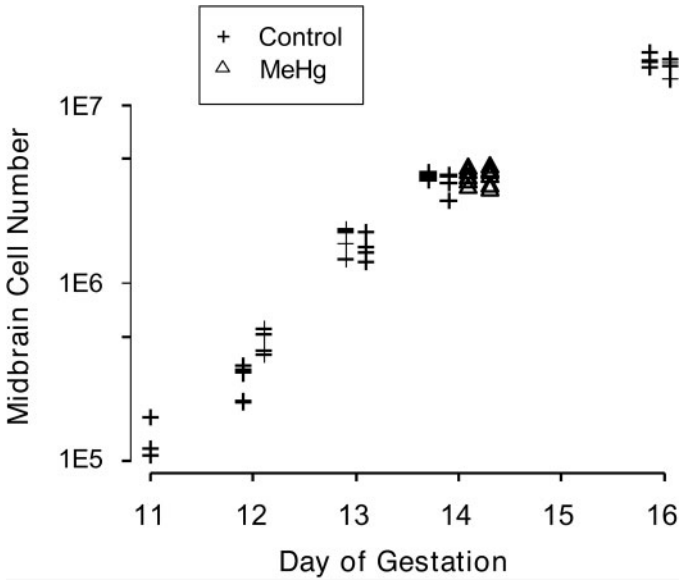

FIG. 6. Count data for midbrain neuroepithelial cells in control and MeHg-treated embryos. Each vertical column of data points represents data from an individual litter. The control data were obtained from untreated animals on GD 11 through GD 16 (Lewandowski *et al.*, 2003). For MeHg treatments (data shown as open triangle; four embryos per litter), the dams were given subcutaneous injections of 18-mg/kg MeHg on GD 11 with embryos obtained and fixed on GD 14. The mean embryonic Hg concentration on GD 14 was 2.7 μg/g (litter 1) and 2.9 μg/g (litter 2).

exposed litters would have been needed to detect a statistically significant difference. We found that an approximately 20% decrease would have been needed to detect a significant effect using our study design.

The data shown in Figure 6 are consistent with the results of the cell cycle kinetic studies described above. An evaluation of the cell number in the treated animals by position on the uterine horn did not indicate selective toxicity to individual embryos, which might have been missed when the animals were considered as a group (data not shown).

Experiment 3: Evaluation of Hg Concentrations in Vitro

The results for the analysis of Hg content in cells exposed *in vitro* to MeHg are shown in Figure 7. The increase in cellular Hg concentration was linear across the range tested (i.e., 0.5–2 μM). The relationship between the intracellular Hg (μg/g) and the culture media Hg (μM) was approximately 3 to 1 (r^2 = 0.99), indicating that a media concentration of 1 μM of MeHg results in a cellular concentration of 3 μg Hg/g tissue. A very similar relationship was observed using P19 cells.

DISCUSSION

In this paper we explored the effects of MeHg on cell proliferation in the developing rat midbrain. Our *in vivo* data on cell cycle kinetics and the stereological estimates of midbrain cell numbers both indicated no effect of MeHg on midbrain cell cycle kinetics or cell numbers at embryonic Hg concentrations up to 2.5 μg/g. Previous studies in mice reported that this embryonic brain concentration did lead to mitotic arrest. For example, Sager *et al*. (1984) observed significant increases in mitotic arrests in the cerebellum of mouse pups exposed to Hg on postnatal day 2. The pups were evaluated 24 h after dosing, and concentrations in the cerebella were 1.8 μg/g. Decreases in cell numbers were also observed at both 24 h and 19 days after treatment, indicating that the mitotic inhibition by MeHg has long-lasting consequences. Rodier *et al*. (1984) looked at the effects of MeHg administration to pregnant mice earlier in gestation, on GD 12, with evaluation on GD 13 and GD 14. Embryonic brain Hg levels were 2.3 μg/g. They observed significant decreases in the number of late mitotic figures in a unit length of the midbrain ventricular zone 48 h after exposure (decreases at 24 h were noted but were not statistically significant). The mitotic index in midbrains of MeHg-treated animals appeared to be less than those in the controls, although the results were not statistically significant. Surprisingly, the cell number actually increased in the MeHg-treated embryos, with midbrain cell numbers being significantly greater than controls at 48-h postexposure (Rodier *et al*., 1984). The authors speculated that this may have represented compensatory growth. While interpretation of the results is complicated, the authors noted that the results support the notion that *in utero* MeHg exposure arrests cells in mitosis. Other authors have observed gross malformations (Curle *et al*., 1987) and behavioral effects (Su and Okita, 1976) in mice at embryonic Hg concentrations similar to those observed in our embryonic rats.

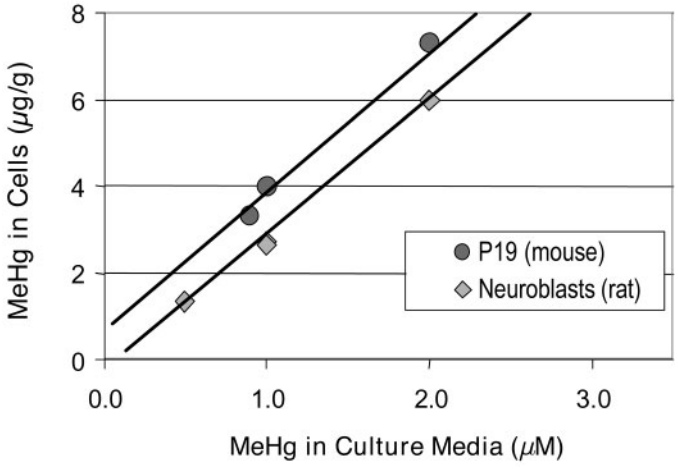

FIG. 7. The relationship between *in vitro* media Hg concentration and intracellular Hg. The cells were grown in Hg-containing media for 6 h and then harvested and analyzed for intracellular Hg content. Intracellular Hg (in μg/g) was approximately three times the concentration of Hg in the media (in μM). The experiments were performed using P19 cells (circles) and primary rat midbrain cells (micromass cells, diamonds). The lines shown were determined by linear regression.

A lesser sensitivity of the rat to MeHg developmental effects, as compared to the mouse, has been documented (e.g., Fuyuta *et al*., 1978). To a large degree, this differential sensitivity has been attributed to the different toxicokinetics of MeHg in these two species, with the rat sequestering MeHg more avidly in the blood than any other species, thereby lessening the levels distributed to other organs, such as the brain. According to this rationale, it should be possible to produce the same effects in rats and mice if the maternal loading of MeHg was adjusted to produce equivalent organ concentrations. Toxicodynamic differences between the two species, although postulated, have not been demonstrated. The work of Rodier (1995) and Sager (1988) has shown that mice exposed *in vivo* to MeHg demonstrated cell cycle effects in the developing brain at MeHg levels of 2–3 μg/g. Data in the rat are more limited but suggest a higher threshold of sensitivity. For example, Chen *et al.* (1979) observed that the rate of DNA synthesis in the brains of rat embryos exposed to MeHg *in utero* were only 36% of the control rat values. The fetal Hg levels in the Chen *et al*. study were 10 μg/g, five times higher than those reported in the mouse studies. Although cell proliferation data in the embryonic rat have not been reported by other authors, other studies with the rat do suggest a lesser sensitivity of the developing rat brain relative to the mouse (e.g., Fredriksson *et al*., 1993).

It is unfortunate that we were unable to achieve higher brain concentrations using the acute dosing procedure, because this prevented us from defining the threshold for MeHg's cell

cycling effects. Higher concentrations may have been achievable using a multiday dosing regimen (Chen, 1979; Lee and Han, 1995). The basis for our choice of acute dosing was twofold: Our interest was in performing an *in vivo* study that would parallel the *in vitro* study of Ponce *et al*. (1994), allowing us to draw a potential *in vitro–in vivo* comparison. In addition, we were interested in quantifying the effects of MeHg on cell cycle kinetics. Chronic dosing would have complicated the interpretation of our results; if we observed effects on GD 13, it would have been difficult to determine if these were attributable to the tissue burden on GD 13 or to the initial exposures on GD 7. A more invasive approach, such as delivering MeHg directly to the embryos via injection to the uteroplacental circulation, might allow for acute delivery of higher MeHg doses to the embryo, but the effects of the surgery on cell cycle patterns would likely be considerable. Alternatively, whole embryo cultures would allow for more direct control of the dosing, although this would require additional assumptions when drawing conclusions regarding the effects in intact animals.

Our data using colchicine demonstrated that colchicine clearly had a negative impact on the cell cycle. The effects of the colchicine on brain development have been well studied (Kalter, 1968). The drug blocks dividing cells at metaphase, causing a cessation of cell proliferation (Dalu *et al*., 1998). Colchicine has also been shown to decrease BrdU labeling in the brain of developing rats when administered postnatally (Carbajo-Perez and Watanbe, 1990). The positive results that we obtained using colchicine demonstrate that our analytical method is capable of detecting changes in cell cycling such as might be expected from MeHg treatment.

Using the data in Figure 7 relating *in vitro* media concentration and intracellular Hg content, we were able to compare *in vivo* and *in vitro* studies using a common dose metric of tissue Hg concentration (i.e., μg Hg/g cellular material). Figure 8 presents *in vivo* and *in vitro* data on the effects of MeHg on cell proliferation. Where applicable, the No Observed Adverse Effect Level (NOAEL), Lowest Observed Effect Level (LOAEL), and highest dose from each study are indicated. The *y*-axes are mercury concentration in μg/g or μM in culture media that are aligned and equated using the relationship shown in Figure 7. As shown in Figure 8, intraspecies differences in MeHg effects on proliferation in the developing brain are not as noticeable using *in vitro* systems as they are *in vivo*. For example, Wasteneys *et al*. (1988), using a murine carcinoma cell line, noted effects on microtubule stability at 0.33 μM (1 μg/g) and decreases in cell growth at 1 μM (3 μg/g). Similarly, Ponce *et al*. (1994), studying rat midbrain cells *in vitro*, observed decreases in cell cycle progression at 1 μM (3 μg/g). Similar data may be considered from Miura *et al*. (1978; mouse glioma cells), Zucker *et al*. (1990; mouse leukemia cells), and Vogel (1985; human fibroblasts). These *in vitro* data suggest a quantitative similarity in response to MeHg between species. However, in our *in vivo* study we observed that, at

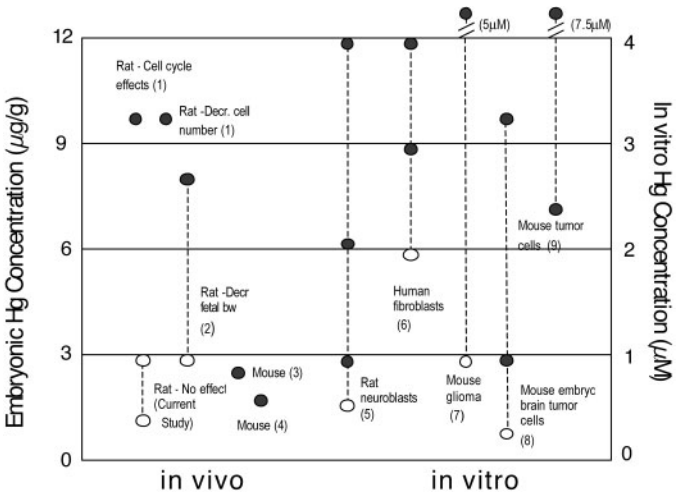

FIG. 8. Comparison of *in vivo* and *in vitro* effects of MeHg cell cycling and proliferation. The relationship shown in Figure 7 was used to relate *in vitro* media doses to tissue Hg content and provide a basis for comparison with *in vivo* studies. In this figure open circles represent the no-effects level, while filled circles represent adverse effects. For perspective, brain mercury levels determined at autopsy in "normal" or unsymptomatic human neonates were below 0.3 μg/g in a Seychelles population (Lapham *et al*., 1995) and 0.16–0.23 μg/g in a population in Japan (Nishimura *et al*., 1974). Brain mercury levels in two neonates involved in the Minimata poisoning incident ranged from 1 to 13 μg/g (Choi *et al*., 1978). References: (1) Chen *et al*. (1979); (2) Fredriksson *et al*. (1993); (3) Rodier *et al*. (1984); (4) Sager *et al*. (1984); (5) Ponce *et al*. (1994); (6) Vogel *et al*. (1985); (7) Miura *et al*. (1978); (8) Wasteneys *et al*. (1988); and (9) Zucker *et al*. (1990). Note that, where studies involved multiple doses, the NOAEL, LOAEL, and highest dose from the original studies are shown.

brain concentrations of approximately 3 μg/g, no effects on cell cycling occurred in the rat midbrain. Thus, *in vitro* systems may not be able to fully quantitatively replicate interspecies differences in isolation and without detailed analyses.

The parallelogram approach has been used to evaluate species differences in chemical sensitivity (Sobels, 1977). Although used primarily for genotoxicants, the parallelogram may also be useful for evaluating developmental toxicants. Figure 9 illustrates how *in vivo* and *in vitro* data for mice, rats, and humans can be used to evaluate cross-species differences in response. The LOAEL values for cell cycle effects *in vitro* in mice and rats were taken from Figure 8. For the mouse *in vitro* LOAEL, we chose the value of 3 μg/g from the study of Wasteneys *et al.* (1988), while the corresponding rat value of 3 μg/g was obtained from the study of Ponce *et al. (*1994). The *in vivo* LOAEL for the mouse was obtained from the study of Sager *et al.* (1984). For rats, an LOAEL was interpolated from the study of Chen *et al.* (1979), which noted substantial decreases in cell number at 10 μg/g; our own data noting an absence of effect at approximately 3 μg/g, and the study of Fredrickson *et al*. (1993) which saw substantial decreases in body weights (presumably a higher dose effect than cell proliferation) at 8 μg/g. We therefore estimated that the LOAEL for cell proliferation in the rat would be approximately 4–6

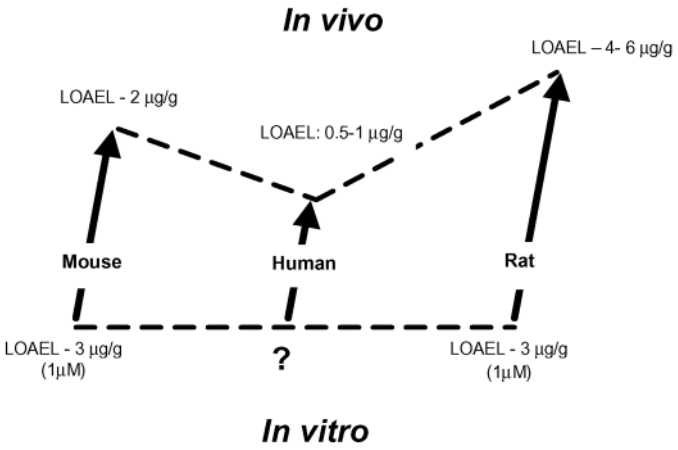

FIG. 9. Use of the parallelogram approach to compare *in vitro* and *in vivo* data across species. The LOAEL values for cell cycle effects *in vitro* in mice and rats were taken from Wasteneys *et al*. (1988) and Ponce *et al*. (1994), respectively. For the *in vitro* data, both the culture medium MeHg concentration in μM and the predicted tissue concentration in μg/g are indicated. The *in vivo* LOAEL for mice were obtained from Sager *et al*. (1984). For rats, an LOAEL was interpolated from Chen *et al*. (1979), Fredriksson *et al*. (1993), and the current study data (see discussion in text). *In vitro* data were not located for human neuronal cells. However, human *in vivo* data collected from neonates in the Minimata episode (Choi *et al.*, 1978) and data from nonsymptomatic neonates (Lapham *et al.*, 1995) suggest a human *in vivo* LOAEL in the range of 0.5 to 1.0 μg/g. Note that the LOAEL values show a reasonable degree of concordance across species, spanning approximately an order of magnitude.

μg/g. *In vitro* data were not located for human cells. However, human *in vivo* data collected from neonates in the Minimata episode (Choi *et al.*, 1978) and data from nonsymptomatic neonates (Lapham *et al.*, 1995) suggest a human *in vivo* LOAEL in the range of 0.5–1.0 μg/g. Quantitatively, all of the LOAEL values show a reasonable degree of concordance, spanning approximately an order of magnitude. Comparisons among the *in vivo* data suggest that humans are at least or more sensitive than the rodent and that mice may be a slightly better model for human developmental neurotoxicity than the rat. The current analysis is based on several assumptions, however, and is limited by differences in study design and comparisons being made across slightly different endpoints (e.g., mitotic index, labeling index, changes in cell number). Additional studies in this narrow dose-response range using consistent designs would be valuable for improving our understanding of species differences in this range of concern. Nonetheless, the parallelogram approach is a useful tool for identifying where detailed assessments are needed to refine our ability to conduct cross-species comparisons.

Our data provide further information on the effects of MeHg on neurdevelopment and provide cross-species and *in vitro* to *in vivo* comparisons. Such comparisons can provide both a quantitative and a qualitative framework for utilizing both *in vivo* and *in vitro* data in human health risk assessment. Studies such as this will help to define low-level response to chemical agents and will help to determine how we can use *in vitro* data to supplement *in vivo* observations.

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